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The Italian Journal of Food Science is an international journal publishing original, basic and applied papers, reviews, short communications, surveys and opinions on food science and technology with specific reference to the Mediterranean Region. Its expanded scope includes food production, food engineering, food management, food quality, shelf-life, consumer acceptance of foodstuffs. Food safety and nutrition, and environmental aspects of food processing.

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LETTER FROM THE ITALIAN JOURNAL OF FOOD SCIENCE EDITOR-IN-CHIEF

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Dear Readers and Authors,

We are starting our thirty-second year of IJFS, which will be an important anniversary for all of us.
The Journal has substantially improved his IF and Citation index (0.736 published in 2018 Journal of Citation Reports, Scopus CiteScore 2019: 1.11, CiteScore Tracker2019: 1.00)
I consider these values very important and rewarding the journal inside the list of peer reviewed international Journals.
Everyone of us may locate easily our Journal in the 2018 official JCR list for Food Science and Technology.

During the last five years (2015-2019) we published 316 papers of 1736 received, with an acceptation rate of 18,2%.
Accepted Paper from abroad raised steadily during the years, reaching 79,4 % during 2019. Their acceptation rate (20,1%) is also consistent with the general value.

Since 1989, when the University of Perugia founded the Journal, I was appointed as the Editor-in-Chief (EIC). Starting 2009 I took over the Journal, still leaving, as before, the Editorial responsibility to Chiriotti Editori.

In October 16, 2019, in consideration of the importance of the requesting Company and of the official scientific guaranties to the preservation of the existing evaluation system, I decided to transfer the Journal Headline to Codon Publications, Brisbane, Australia, but maintaining for myself the permanent appointment as EIC.

Codon Publication agreed also to continue the Journal publication 1) under the seal of the University of Perugia , appointing the Journal "under the Aeges of the University of Perugia" and 2) under the seal of the Italian Society of Food Science (SISTAl), enforcing, as before, the existing papers evaluation system managed by the Co-Editors of the SISTAl.

I would like in this occasion express my gratitude to the following Professors who acted in the past as unique Journal SISTAl Co.Editors and accepted to continue their work even in the future:

Chiavaro Emma - Università degli Studi di Parma
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Practically, all future papers submitted to IJFS until 30th of June 2020 will be processed, as before, inside the existing web platform of Chiriotti Editori.

Starting 1st of July 2020, this Open Access platform will not accept anymore papers.

All submitting Authors will be asked to redirect and submit their papers to the new Codon Publication wOpen Access web platform.

You will find proper and sound information during the next months in the section "Announcement" of the IJFS Chiriotti platform.

In looking forward to continue our important collaboration, I wish all of you a fruitful 2020.

Paolo Fantozzi

OPINION PAPER

PREBIOTIC EFFECTS OF XYLANASE MODIFICATION OF β-GLUCAN FROM OAT BRAN ON *BIFIDOBACTERIUM BIFIDUM*

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ABSTRACT

Oat β-glucan (BG) was isolated from oat bran, and the xylanase treatment was conducted to obtain modification of β-glucan (MBG). The relative molecular weight (M_w) of BG and MBG was determined by gel permeation chromatography (GPC). Results demonstrated that the M_w of BG was reduced from 1.66×10^4 to 5.43×10^3. We assessed the prebiotic effect of BG and MBG on human colon *Bifidobacterium bifidum* (*B. bifidum*). Our findings suggest that the addition of BG and MBG resulted in a lower pH of the fermentation broths. Both lactic and acetic acid production increased in the fermentation broths. While BG was found to significantly promote the proliferation of *B. bifidum* , MBG had a greater effect on *B. bifidum*.

Keywords: β-glucan, *Bifidobacterium bifidum*, modification, prebiotic, xylanase

1. INTRODUCTION

Oat bran is a by-product of the oatmeal production, produced during the milling process. It is a mixture that is mainly composed of seed coat, the aleurone layer, and oat germ, which accounts for 8%~12% of the total mass of oat seeds. It contains a variety of nutrients, such as dietary fibre, fat, protein, and minerals, and a large amount of dietary fibre represents a valuable renewable resource (ZHENG *et al*., 2017). Oat BG is a soluble dietary fibre (SDF) present in the oatmeal grain endosperm and aleurone cell wall. Its main components are (1-3) and (1-4)-β-D-glucan. After oatmeal processing, the BG is enriched in wheat bran. Following claims made by the US Food and Drug Administration (FDA), many researchers have demonstrated an association between oat BG and a reduction in the risk factors of cardio-vascular disease, in particular by lowering the blood cholesterol and glucose levels (VITAGLIONE *et al*., 2008; YAN *et al*., 2017), and at the same time regulating the immune system and strengthening resistance (LI *et al*., 2018). Oat BG has also been found to relieve the immunosuppression of tumour cells and to have a good therapeutic effect on patients with early-stage cancer (MEI *et al*., 2018).

The enzymatic method involves the use of enzymes to enzymatically decompose a raw material and to remove surface impurities to obtain insoluble dietary fibre, which is then further enzymatically modified to obtain a water-soluble dietary fibre. Studies have shown that the enzyme treatment of bran can effectively change the functional properties of dietary fibre by changing the structure or molecular rearrangement of polysaccharides (SANTALA *et al*., 2014). The modification of bran by xylanase and cellulase can increase the soluble dietary fibre content of oat bran and reduce its water binding ability (LEBESI *et al*., 2012). Laccase assisted by high hydrostatic pressure and cellulose bran can increase the content of soluble dietary fibre, alter the honeycomb structure of dietary fibre, and produce new polysaccharides (MA *et al*., 2016). After the treatment of rice bran with xylanases, including amylase, glucoamylase, protease, and cellulase, the total phenolic, flavonoid, iron reducing antioxidant capacity, and oxygen free radical absorption capacity of modified dietary fibre has been found to significantly improve (LIU *et al*., 2017). However, the precise role of BG in the enzymatic treatment of the human requires further elucidation.

The definition of prebiotics has been suggested by GIBSON *et al*. (2017) as "a substrate that is selectively utilized by host microorganisms conferring a health benefit". The catabolism of prebiotic carbohydrates by metabolic activity of the gut microbiota primarily produces short chain fatty acids (SCFA). The most abundant SCFA in the colon is acetate, which in general represents more than half of the SCFA content detected in feces (LOUIS *et al*., 2007). Prebiotic substrates can selectively promote the growth of beneficial microorganisms and induce changes in the levels of these SCFA in healthy individuals (LECERF *et al*., 2012). Thus, SCFA levels represent an indirect measure of the level of beneficial microorganisms in the gut and their impact on human health. In this study, Inner Mongolia oat bran was used as raw material to extract oat BG by xylanase enzymatic hydrolysis to explore the prebiotic effects on *Bifidobacterium bifidum* before and after enzymatic hydrolysis.

2. MATERIALS AND METHODS

2.1. Chemicals and media

The xylanases (1.67 millikatal (mkat)/g) used in this study were purchased from Yuanye Biotechnology Co., Ltd. (Shanghai, China). All other chemicals and media were of analytical grade and obtained from Haibo Biotechnology Co., Ltd. (Qingdao, China). *B. bifidum* (strain number CICC 6168) was purchased from China Center of Industrial Culture Collection. HPLC-grade water was prepared using a Milli-Qplus purification system (Millipore Corp., Bedford, MA, USA). Oat bran was produced in the Inner Mongolia Autonomous Region (Hohhot, China).

2.2. Crude BG extraction

The isolation of BG was performed as follows: fat was removed from oat bran using a 60-mesh screen with a Soxhlet extractor. The defatted oat bran (50 g) was then soaked in distilled water (0.5 L), then gelatinized at 100°C for 15 min, and finally incubated with heat-resistant a-amylase at 95°C for 1.5 h to remove starch, followed by centrifugation (2000 ×g, 15 min). Insoluble dietary fibre (IDF) was the resulting precipitation. The supernatant was isoelectrically deproteinized in a NaOH solution (0.75 mol/L, 50°C, 2 h). The pH was adjusted to 6.5 to remove protein. After centrifuging at 2000 ×g for 5 min, 200 mL of 950 g/kg ethanol solution was added to the crude BG precipitate (60°C, 2 h). The precipitation (crude BG) was then dried at 60°C for 12 h in an air-drying oven.

2.3. Enzymatic hydrolysis of BG

The enzymatic treatment was conducted as follows: the precipitation (IDF) was dried at 60°C for 12 h using an air-drying oven, then grinded and sieved through a 250 mm mesh. The crude BG and IDF were combined and treated with xylanase. The mixture (30 g) was soaked in hot water (500 mL) at 50°C for 0.5 h. Then, xylanase was added at 667 nanokatal/g (mixture) and the slurry was incubated for 2 h (50°C, pH 5.0). The treated slurry was centrifuged at 2000 ×g for 5 min after the inactivation of the enzyme in a boiling water bath for 10 min. The resulting insoluble material was washed twice with hot water (pre-heated to 50°C) and centrifuged (2000 ×g, 5 min) again. Then, 150 mL of 950 g/kg ethanol solution was added to the precipitated crude modification of β-glucan (MBG), and the residue was dried at 60°C for 12 h using a hot-air oven.

2.4. BG and MBG purification

BG crude powder (0.1 g) and MBG crude powder (0.1 g) were dissolved in 5 mL of water, respectively. After full dissolution, the solution was used for anion exchange column chromatography, using water as the eluent, at a flow rate 30 mL/h. Using 10 mL per tube, the eluent was collected from each tube. The polysaccharide distribution was determined using the phenol-sulfuric acid method, and the protein content was determined by 280 nm colorimetry. A single peak of polysaccharide was combined. The BG solution and MBG solution obtained were concentrated to 5 mL under reduced pressure, respectively, and the supernatant was centrifuged for use.
The BG and MBG concentrates treated by DEAE Sepharose CL-6B anion exchange column chromatography was respectively used for gel permeation column chromatography using

water as an eluent at a flow rate of 30 mL/h. The eluate was collected from each tube. Polysaccharide distribution and protein content were detected by the phenol-sulfuric acid method and 280 nm colorimetry, respectively. A single peak of polysaccharide was combined. BG and MBG were concentrated under reduced pressure before freeze drying.

2.5. Determination of average molecular weight (M_w) of BG and MBG

The M_w of BG and MBG was determined by GPC. The purified BG and MBG (1 mg) were analysed using a PL aquagel-OH MIXED chromatographic column at 45°C. The mobile phase consisted of 0.1 mol/L $NaNO_3$ at a flow rate of 0.9 mL/min. The quantification was performed using a VEX differential refractive index detector (PL GPC-220, Agilent Technologies Inc. California, USA).

2.6. The prebiotic effect of BG and MBG on *B. bifidum*

2.6.1. Strains activation

B. bifidum freeze-dried strains were used, such that the strains were activated and cultured before carrying out the experiments. The formulation of medium was provided by the China Center of Industrial Culture Collection, and is provided in Table 1.
Here, 30 mL of BBL medium was placed in 100 mL vial, followed by vacuum pumping and sterilization at 121°C for 30 min. The inoculation operation was carried out in an anaerobic incubator. For this, *B. bifidum* lyophilized powder was fully dissolved and inoculated into 1 mL of sterile medium. The culture period of bacteria is normally 1~2 days, however the first-generation revival culture needed to be extended appropriately. As such, the duration of this experiment was 3 days. The cells were statically cultured at 37°C in an anaerobic culture incubator. The first-generation revival cultured cells that survived were sub-cultured for two generations with 10% inoculation, with a culturing time per generation of 2 days.

Table 1. *Bifidobacterium bifidum* medium (BBL) formula (per litre, /L).

Name	Dose	Name	Dose
yeast extract	3 g	beef extract	10 g
peptone	10 g	soluble starch	1 g
glucose	5 g	L-cysteine hydrochloride	0.5 g
sodium chloride	3 g	sodium acetate	3 g
resazurin	3 mg	pH	6.8

2.6.2 Effect of BG and xylanase MBG on the growth of B. bifidum

To investigate the effect of BG and MBG on the growth of *B. bifidum*, BG and MBG were used as the sole carbon source. BG and MBG were substituted for the glucose in the BBL medium. Here, 0.5 g of BG and MBG were used to replace glucose, and 100 mL of BBL medium was prepared with an inoculation amount of 5%. After inoculation, the solution was placed in a 37°C anaerobic culture incubator for 24 h. The OD value was measured using an ultraviolet-visible spectrophotometer at a wavelength of 600 nm after 24 h. The

BBL medium not inoculated with bacteria was used to adjust the reading to zero. As can be seen in Table 1, 15 g of agar was added to BBL medium to prepare the BBL agar medium. The fermentation broth was coated and inoculated into BBL medium before culturing in an anaerobic incubator at 37° C for 24 h.

2.6.3 B. bifidum fermentation broth pH determination

The initial pH of the *B. bifidum* fermentation broth was measured before fermentation, and a sample was measured once every 6 h. For the measurement, 5 mL of the fermentation broth was sampled, centrifuged at 2000 ×g for 10 min, and the resulting supernatant was measured using a pH meter.

2.6.4 Effect of BG and MBG on the concentration of SCFA

Here, 0.5 g of BG and MBG were added to 100 mL of BBL medium with glucose. BBL medium with 5 g of glucose per litre was used as a negative control. The inoculation amount was 5%. After the inoculation was completed, it was incubated at 37°C in an anaerobic culture incubator for 24 h. The bacteria solution was filtered through a 0.22 μm filter membrane before performing high performance liquid chromatography after 24 h. The three groups of *B. bifidum* solutions were analysed using a HPX-87H chromatographic column at 50°C. The mobile phase consisted of 0.005 mol/L sulphuric acid at a flow rate of 0.5 mL/min. The quantification was performed by high performance liquid chromatography (Waters 2695, Waters Technology Co., Ltd. Milford, USA). This quantification was also carried out on a bacterial solution cultured for 0 h, that is immediately after inoculation, to determine the increased in lactic acid and acetic acid production and used as the control group.

2.7. Statistical analysis

All measurements were carried out at least in triplicate. The results presented are the mean±standard deviation (SD) of each treatment (n=3). The differences were considered significant when $p<0.05$.

3. RESULTS AND DISCUSSION

3.1. Determination of M_w of BG and MBG

The M_w distribution of untreated BG and MBG is shown in Fig.1. The M_w represents the statistical average molecular weight of the polysaccharide compared to the average weight of different molecular weights, and the average molecular weight (Mn) represents the statistical average molecular weight of the molecules in the polysaccharide with different molecular weights. According to Fig. 1(a) and Table 2, the M_w of the BG which was not subjected to the enzyme treatment was 1.49×10^4. The M_w/Mn value was 1.03, which was close to 1, indicating that the M_w distribution of BG was uniform, and the distribution was concentrated around the average molecular weight. As shown in Fig. 1(b) and Table 2, the MBG exhibited four M_w segments, 5.98×10^5, 2.68×10^4, 1.66×10^4, and 5.43×10^3, respectively. The M_w/Mn values were 1.10, 1.01, 1.02, and 1.23, respectively, that is close to 1, indicating that the M_w distribution of MBG was uniform and that the distribution was concentrated

on the average of the four molecular weight segments. Among them, the MBG of two M_w fractions, 5.98×10^5 and 2.68×10^4, accounted for 2.20% and 8.78% of the total content, respectively, and the content was low. After xylanase enzymatically cleaves the bran xylan, the macromolecular BG originally linked to xylan may be isolated. Its high molecular weight (HM_w) of 1.66×10^4 is similar to that of non-enzymatically-treated BG, and should be the same type of BG. The MBG with a low molecular weight (LM_w) of 5.43×10^3 could be the result of enzymatic hydrolysis of xylanase. Xylanase destroys the β-1,4-glycosidic linkage of connecting BG, thereby decreasing the M_w of BG, with a resulting molecular weight of 1.66×10^4. Among these, MBG with molecular weight of 5.43×10^3 accounted for 86.28% of the total content. The use of xylanase can be also a smart strategy BG to convert LM_w, the M_w of MBG showed that the M_w of BG was reduced from 1.66×10^4 to 5.43×10^3. The M_w of BG plays an important role in determining the physiological efficacy of BG in terms of health benefits. Incorporating LM_w BG may influence the palatability of food and has been shown to lower cholesterol in men (PINS *et al*., 2005) and animals (WILSON *et al*., 2004).

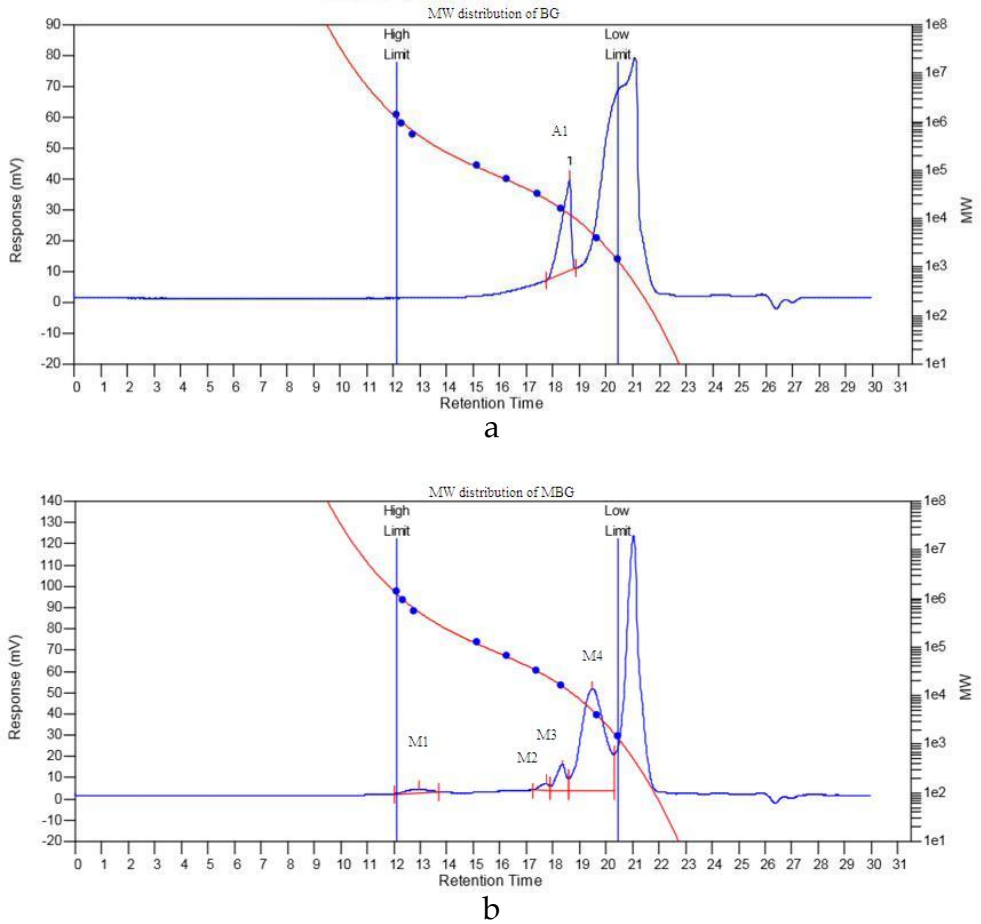

Figure 1. M_w distribution of BG and MBG.

Table 2. M_w of each component of BG and MBG.

Peak number	Mp	Mn	Mw	Mz	Area (mV.secs)	% Area
B1	12237	14501	14938	15420	777.567	100
M1	578382	544525	598568	660548	94.3102	2.75
M2	25501	26587	26868	27167	75.6232	2.20
M3	15732	16311	16600	16899	300.825	8.78
M4	5024	4415	5435	6492	2959.55	86.28

Furthermore, the action of xylanase on IDF leads to the formation of two M_w fractions, 5.98×10^5 and 2.68×10^4, which may be water soluble. As illustrated in Fig. 2, this conversion of highly polymerized IDF into SDF was achieved by performing a tailored enzymatic treatment. Polysaccharides and other polymers are cross-linked to the cell wall of the cereals with other components to form a structural network. B-(1-3,1-4)-D-glucans and arabinoxylans are the major cell wall polysaccharides in oat bran and are composed of a backbone of β-(1,4) linked D-xylopyranosyl residues. Moreover, a-L-arabinofuranoside can be present at the C (O)-3 and/or the C (O)-2 positions of the xylose moieties, and arabinoxylans can be cross-linked to ferulic acid at the C (O)-5 positions via ester linkages. Many of these polysaccharides would require enzymatic hydrolysis to be removed from the structure, formed by covalent and non-covalent cross-linking. For example, cellulose and hemicelluloses treatments can be used to improve the quality of fiber-enriched oat bran by using xylanase treatment on the fibre fraction. Enzymatic methods have been used as means for modification to improve the extractability of polysaccharides and increase yields (LAURIKAINEN *et al*., 1998). Similar studies have reported changes in the chemical bonds of the polysaccharide and bran dietary fibre molecular structures via various enzymatic treatments (SAULNIER *et al*., 2009; YA *et al*., 2017).

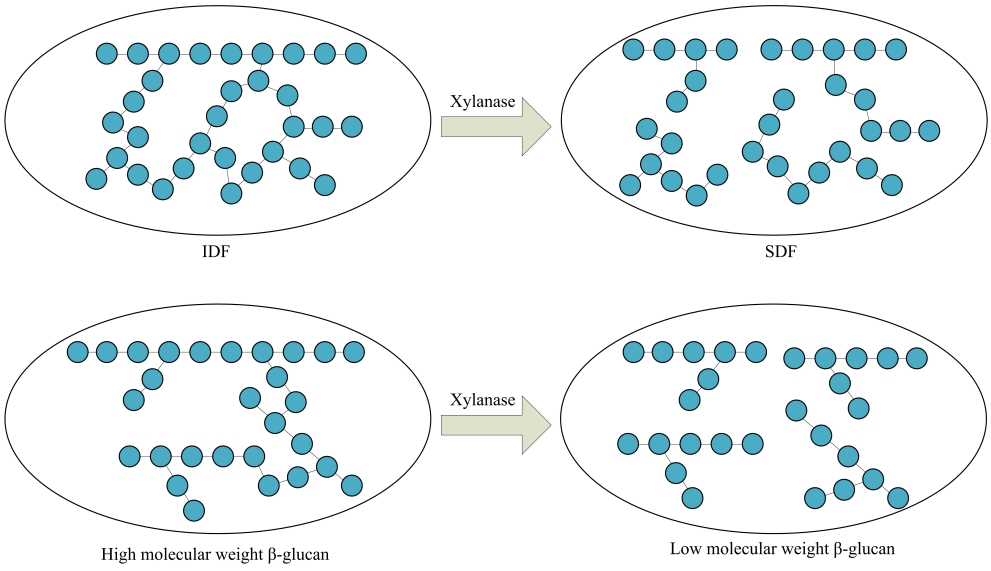

Figure 2. Schematic representation of the effects of xylanase on BG.

3.2. The prebiotic effect of BG and MBG on *B. bifidum*

3.2.1 Effect of BG and MBG on the growth of *B. bifidum*

According to the experimental methods, in which the OD_{600} of the bacterial solution of *B. bifidum* was measured *in vitro* after 24 h of anaerobic fermentation, the OD value of the bacterial solution was 0.041 cultured in BBL medium of 5 g/L glucose at 37 °C for 24 h, while the OD value of the bacterial solution were 0.089 and 0.244 cultured in BBL medium of 5 g/L BG and 5 g/L MBG, respectively. The OD value of the bacterial solution increased significantly after the addition of BG. The OD value of the BG bacterial solution then increases again after xylanase treatment. The positive correlation between the OD value of the bacterial solution and the number of bacteria suggests that BG can promote the proliferation of *B. bifidum*. However, the promotion of proliferation was greater after the addition of MBG. This may be due to *B. bifidum* being more easily oxidized in the LM_w segment than in the HM_w segment of BG during the oxidation of sugar.
Non-digestible polysaccharides cannot be degraded by mammalian enzymes. Therefore, following ingestion, these glycopolymers are delivered intact to the large intestine, where they may influence the growth or metabolic activity of members of the gut microbiota. In this context, there is growing scientific evidence of the possible prebiotic effects elicited by non-digestible polysaccharides towards various microorganisms of the mammalian gut (VITAGLIONE *et al*., 2008; TAN *et al*., 2006). After BG enters the large intestine as a soluble dietary fibre, probiotics such as *B. bifidum* pass through the extracellular glycosidase to promote BG degradation and utilization, thereby promoting the proliferative metabolism of probiotics.

3.2.3 Change in pH of B. bifidum fermentation broth

The decrease in the pH of the fermentation broth was mainly the result of organic acid production during fermentation. As can be seen in Fig. 3, compared to the negative control, the pH of the fermentation broth decreased rapidly after the addition of untreated BG and MBG. The environment changed from alkaline to acidic after 24 h of fermentation. The addition of MGB resulted in a more rapid decrease of the fermentation broth's pH compared to BG. Metabolically produced organic acid reduced the intestinal pH environment, resulting in an acidic environment for the intestines, thereby inhibiting the growth of harmful bacteria and promoting to intestinal health. There is also considerable evidence that supports the role of fibre in the promotion of health by its ability to modulate gut microbiota composition and metabolism (SLAVIN *et al*., 2013). The proposed benefits of fibre on the intestinal microbiota are associated with their uptake and utilization by putative health-promoting bacteria species and the subsequent cross-species metabolism of fermentation by-products (HOLSCHER *et al*., 2015; VERBEKE *et al*., 2015; TAP *et al*., 2015).

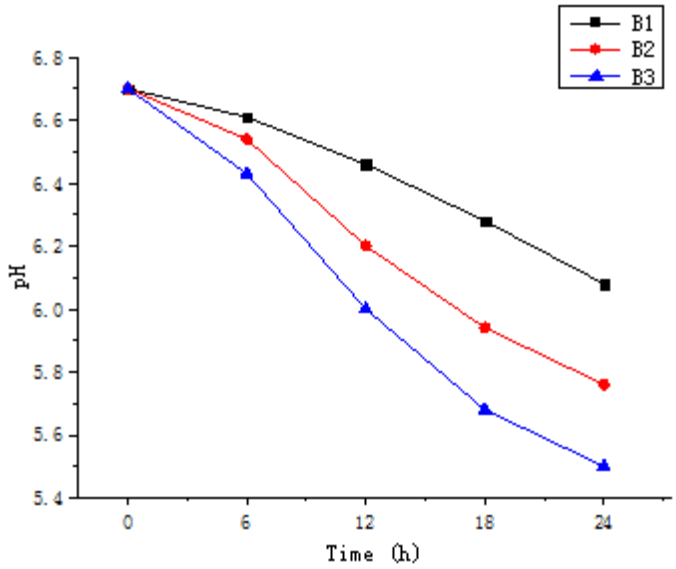

Figure 3. The effects of time on pH in fermented broth. Note: B1: BBL medium of 10 g/L glucose; B2: 5 g/L BG plus 5 g/L glucose BBL medium; B3: 5 g/L MBG plus 5 g/L glucose BBL medium.

3.2.4 Effect of BG and MBG on the concentration of SCFA

The formula of the medium (Table 1) was obtained via an optimization study based on strong metabolic and molecular studies by the China Center of Industrial Culture Collection. The production of lactic acid in the bacterial solution was measured after culturing the cells, as described in the Methods. The effect of BG and MBG on the concentration of lactic acid and acetic acid in the fermentation broth is shown in Figure 4. *B. bifidum* was cultured at 37°C for 24 h. The yields of lactic acid and acetic acid in the control (B1, 10 g/L glucose BBL medium) were 0.5316±0.0033 g/L and 0.3927±0.0043 g/L, whereas the fermentation of *B. bifidum* with 5 g/L BG plus 5 g/L glucose BBL medium and 5 g/L MBG plus 5 g/L glucose BBL medium, respectively, as a carbon source resulted in greater yields of lactic acid and acetic acid than the control. An analysis of the metabolic activity in both cultures showed that lactic acid production increased 56% during BG treatment (0.8316±0.0265 g/L) and by 184% during MBG treatment (1.5091±0.0151 g/L; $p<0.05$) compared to the control. The acetic acid production increased 12% during BG treatment (0.4388±0.0033 g/L) and by 30% during MBG treatment (0.5117±0.0046 g/L; $p<0.05$) compared to the control (Figu. 4). These results demonstrated that the addition of BG and MBG to *B. bifidum* during fermentation with glucose as a substrate can significantly promote the production of metabolic acid, since the increase in acid production of MBG was greater. The overall beneficial effects produced by MBG were higher than those induced by BG. The addition of MBG and BG to the cultures beneficially influenced the fermentation patterns of *B. bifidum*, demonstrate by the higher SCFA production and remarkably higher levels of lactic acid.

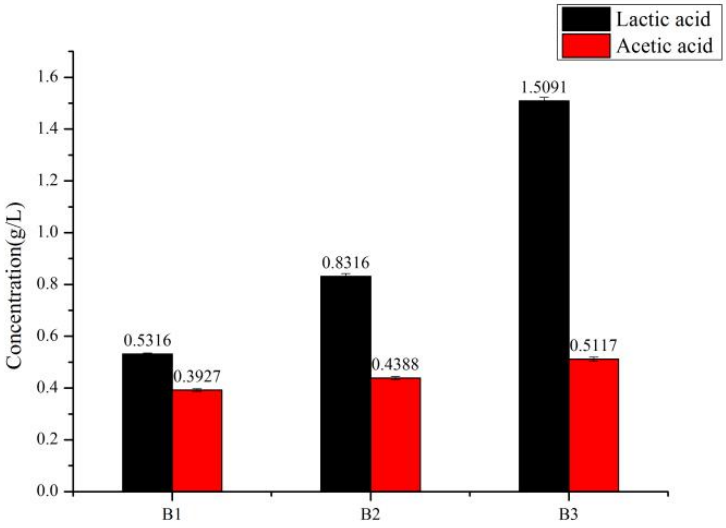

Figure 4. Effects of BG on the lactic acid and acetic acid concentration in fermentation solution (g/L). B1: BBL medium of 10 g/L glucose; B2: 5 g/L BG plus 5 g/L glucose BBL medium; B3: 5 g/L MBG plus 5 g/L glucose BBL medium.

The SCFA microbial metabolites are of particular interest, and have been suggested to promote health by regulating hormone release in the gut, as well as the cholesterol synthesis/metabolism to enhance satiety, also exerting anticancer and anti-inflammatory effects (VINOLO *et al*., 2011; WONG *et al*., 2006). The experiments showed that *B. bifidum* could produce acid by glycolysis with glucose as the carbon source. The MBG of the small molecular segment was more easily utilized by *B. bifidum* than the BG of the large molecular segment. The promotion of lactic acid production by probiotics was also more evident. The fermentation properties of *B. bifidum* are strongly influenced by the degree of polymerization of non-digestible polysaccharides. The increased SCFA production can be explained by the additional *B. bifidum* biomass resulting from the prebiotic effect of BG and MBG.

4. CONCLUSIONS

Oat bran dietary fibre was treated with xylanase to obtain MBG, which varied in its structure and properties compared to the original BG. We found that the M_w of MBG was reduced from 1.66×10^4 to 5.43×10^3, as determined by gel permeation chromatography. The addition of BG and MBG to the fermentation broth of *B. bifidum* significantly promoted the proliferation of *B. bifidum*, and the proliferation of *B. bifidum* in the LM_w segment of MBG was greater. The *B. bifidum* bacteria metabolites, lactic acid and acetic acid, were detected. Moreover, with glucose and BG as the carbon sources, the acid production of *B. bifidum* increased significantly. The production of lactic acid production in MBG increased significantly as a result.

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PAPER

BEEF TRADITIONAL FOOD: CONSUMER BEFORE PURCHASE PREFERENCES BASED ON QUALITY

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ABSTRACT

The aim of the paper is to study beef quality cues and attributes in Italy, comparing regions where beef is considered traditional food and regions where it is not. A quantitative research has been conducted; both a factor analysis and a cluster analysis were performed. Quality cues and/(or) attributes distinguish consumers when before purchase preferences are considered. Traceability and safety issues have become crucial in the before purchase phase. The paper suggests enhancing knowledge about contextual factors, besides quality cues and attributes, able to shape consumer preferencesand before purchase expectations to create new value offering to satisfy consumers' changing expectations concerning beef.

Keywords: beef quality, consumer perception, intrinsic quality cues, extrinsic quality cues, expected quality, traditional food

1. INTRODUCTION

Food quality has always beenand is still today, one of the most interesting topics not only for academics, but above all for consumers.
The meat quality issue has been in the spot light since 1995 (e.g., CARDELLO, 1995; GRUNERT, 1995; MOSKOWITZ, 1995), when beef attracted much attention just after the emergence of BSE (Bovine Spongiform Encephalopathy). However, over the last 22 years a number of quality standards, regulations and safety programs have been introduced, not only at a national level. The most recent one, at the European level, was introduced in 2014 focusing on traceability; an issue that had already emerged in literature (e.g., BANOVIĆ *et al.*, 2012) together with beef safety (e.g., DE BARCELLOS *et al.*, 2010; VAN WEZEMAEL *et al.*, 2010).
Given that beef is an experience product, consumers shape their before purchase expectations, building on extrinsic and intrinsic cues and when faced with choosing unbranded beef they almost always rely on price (BANOVIĆ *et al.*, 2012), even if the relationship between price and quality has not always been demonstrated (SOLOMON *et al.*, 2007).
Besides visual impressions understood as extrinsic and intrinsic cues (e.g., BELLO ACEBRÒN and CALVO DORPICO, 2000; GRUNERT *et al.*, 2004), also sensory impressions - e.g. quality attributes like taste – affect beef purchase choice as demonstrated through studies performed after consumption (e.g., BELLO ACEBRÒN and CALVO DORPICO, 2000).
However, to the best of the authors' knowledge, none of the papers on beef have considered that past sensory impressions could play a role together with extrinsic and intrinsic cues in consumer preferences and purchase choice, before purchase. Therefore, the paper aims at studying the impact of past sensory impressions as well as extrinsic and intrinsic quality cues in consumer before purchase preferences, paying attention to traceability and safety issues still not studied at length.
Therefore, two initial research questions emerge:

- *What is the role of extrinsic and intrinsic cues and of sensory impressions, based on past experience at the moment of purchase?*
- *In this context how do traceability and safety issues affect consumer preferences and choices?*

These two research questions could also be affected by other elements, like familiarity, which has already been studied by BANOVIĆ *et al.* (2010, 2012).
The third research question can be put forward as:

- *Do consumers in regions where beef is a traditional food consider the impact of traceability and safety issues differently at the moment of purchase?*

To perform the study the authors chose two different regions: Tuscany where beef is considered a traditional food and Latium, one of the nearest regions to Tuscany, but where beef is not a traditional food and the most famous PDO is "Abbacchio Romano", a type of lamb.
Protected Designation of Origins (PDOs) and Protected Geographical Indications (PGIs), have been defined by the European Union in the domain of geographic indications (EUROPEAN REGULATION 1151/2012). The European Union supports traditional quality products and the way they are produced, highlighting that "for a product name to

be protected as a PDO there must be an objective and exclusive link between the features of the product and its geographical origin" (LONDON ECONOMICS, 2008, p. 6).
Indeed, the paper also responds to the recent call for country-specific research into the beef domain, given that preferences for this food vary across different countries (ARDESHIRI and ROSE, 2017).
The paper is structured as follows. Firstly, there is a literature review focusing on papers that discuss various issues concerning food quality, meat quality and then beef quality. After the methodology section, results are illustrated and discussed. The paper ends with conclusions in which limitations, future steps of further research, as well as theoretical and managerial implications, are presented.

1.1. Literature review

1.1.1 An overview of quality types in food and meat studies

Although there are several definitions of food quality in literature, according to GRUNERT (2005), literature agrees that "quality has an objective and a subjective dimension", (p. 371). Objective quality refers to technical and physical characteristics necessary to have quality food, while subjective quality is about consumer perception of quality (GRUNERT, 1995; 2005). STEENKAMP (1990) elaborated this concept - perceived quality- as the match between product characteristics and consumer preferences. CARDELLO (1995) suggested that "food quality is a complex concept" (p. 163) where various factors converge and should be measured by both objective indices (e.g., nutritional or physicochemical characteristics) and subjective indices linked to person, place and time. OUDE OPHUIS and VAN TRIJP (1995) as well as MOSKOWITZ (1995) stated that "food quality is a multi-faceted concept" (p. 157) and has a very subjective nature because it changes from person to person. Following this through, food quality must be understood as a "human perceptual/evaluative construct" (MOSKOWITZ, 1995, p.167), therefore only consumer judgment can establish the quality of food. According to TOLOSA *et al.* (2005) quality is a "multidimensional phenomenon" (p. 419) and it can be described as a "set of attributes that must be perceived by the consumer" (p. 419). For these authors, subjective characteristics influence food quality more than objective features. In particular, GRUNERT (1995), proposed three distinct types of food quality: (1) product-oriented quality to be understood as all physical characteristics of food which can be objectively measured; (2) process-oriented quality, namely all characteristics of the food production process and (3) user-oriented quality, referring to consumer subjective quality perception. BRUNSØ *et al.* (2005), building on this classification, introduced a fourth quality type, namely "quality control", defined as "the standards a product has to meet in order to be approved for a specific quality class" (p. 84), e.g. Iso 9001 or specific standard quality beef.
Focusing on subjective quality, literature agrees to distinguish between multidimensional and hierarchical approaches (BRUNSØ *et al.*, 2005). According to the multidimensional approach, the combination of a number of quality dimensions or attributes determines the quality perception of a product (e.g. food) (VERDÙ JOVER *et al.*, 2004; BRUNSØ *et al.*, 2005). The two most important classifications in this approach are, on the one hand, the one regarding search, experience and credence characteristics (DARBY and KARNI, 1973;

NELSON, 1970, 1974) and on the other, the one proposing the separation of intrinsic quality cues from extrinsic quality cues (OLSON and JACOBY, 1972; OLSON, 1977).
According to the economic theory, search and experience are evaluated at a different time from the moment in which the consumer carries out his purchase - the first, before purchase, for example, refers to price or color; the second, after buying for example, refers to taste. Credence, instead, cannot be established either before or after purchase, because it is based on trust and faith in the product information provided - e.g. exclusiveness (OUDE OPHUIS and VAN TRIJP, 1995; GRUNERT *et al.*, 2004; BRUNSØ *et al.*, 2005; FANDOS and FLAVIÁN, 2006).
The second classification is part of the psychological theory and distinguishes between intrinsic and extrinsic quality cues. Intrinsic quality cues, according to GRUNERT *et al.* (2004), BRUNSØ *et al.* (2005), TOLOSANA *et al.* (2005) and ESPEJEL *et al.* (2007) can be understood as "part of the physical characteristics of the product"; they are "related to technical specifications, which also involve physiological characteristics" (BELLO ACEBRÓN and CALVO DOPICO, 2000, p. 230), while extrinsic quality cues refer to characteristics "related to the product, but are not physically part of it" (OUDE OPHUIS AND VAN TRIJP, 1995, p. 178). Quality cues can, therefore, be evaluated only prior to consumption.
Quality attributes, on the other hand, can only be ascertained through consumption, namely when the consumer eats the prepared meat (STEENKAMP, 1990; OPHUIS AND VAN TRIJP, 1995). Indeed, BELLO ACEBRÓN and CALVO DOPICO (2000) defined quality attributes as "functional and psychological benefits or consequences provided by the product and they are unobservable prior to consumption" (p. 231). Therefore, when purchasing, consumers base their choices on quality cues (STEENKAMP, 1989, 1990), while hoping quality attributes will meet their expectations.
CASWEELL (2000) maintained that quality perception depends on both intrinsic/extrinsic quality cues and "information environment", that is search, experience and credence quality, which are "vertically/horizontally differentiated" (p. 225). In his model Casweell integrates the two classifications of quality dimensions. This point of view is shared by BURNUÉS *et al.* (2003), who proposed a model integrating intrinsic and extrinsic quality cues with search, experience and credence quality, in order to analyze the extrinsic quality cues of beef perceived as indicators of quality in Europe.
The hierarchical approach focuses on the association "between product attributes and more abstract, more central cognitive categories such as values, which can motivate behavior and create interest for product attributes" (BRUNSØ *et al.*, 2005, p. 85). The frameworks on which the hierarchical approaches are based are the "means-end chain models" (OLSON AND REYNOLDS, 1983; GUTMAN, 1991), which link product characteristics to deeper purchasing motivation.
To clarify the distinction between multidimensional and hierarchical approaches, it is important to understand subjective quality perception. Indeed, these two approaches have played a key role in developing the Total Food Quality Model (TFQM) proposed by GRUNERT *et al.* (1997).
TFQM integrates several approaches to consumer quality perceptions (DARBY AND KARNI, 1973; FISHBEIN AND AJZEN, 1975; GUTMAN, 1982) and tries to explain, on the one hand, which factors are able to influence consumer purchase intentionand on the other, the concept of customer satisfaction as the gap between expected and experienced

quality (OLIVER, 1990; GRUNERT *et al.*, 2004; VIMISO *et al.*, 2012). In doing this the authors distinguished 'before' from 'after' purchase evaluation.
TFQM shows how quality expectations, in the 'before purchase' phase, come from the evaluation of available quality cues. According to STEENKAMP, (1990), consumers use 'cues' to determine the value of the product. Therefore, it is necessary to consider them together with quality attributes. For this reason, the authors proposed a more complex model than those used in the past, one where the distinction between quality cues and attributes is considered. An overview of food quality types identified by the above-mentioned studies is presented in Fig. 1.

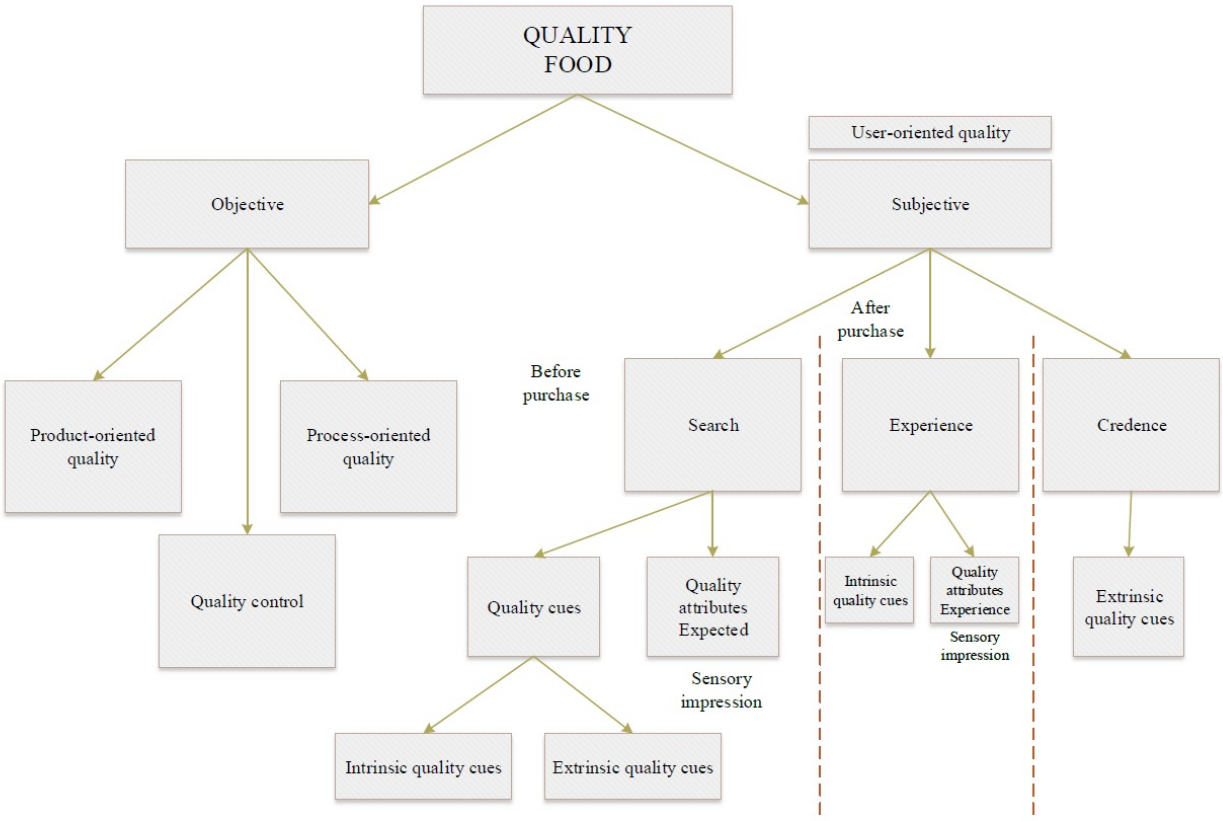


Figure 1. An overview of food quality types from the literature review.
Source: our elaboration.

1.2. Quality perception in beef consumption

Over the years, various studies have considered meat quality and especially beef quality issues. GRUNERT (1997) analyzed how consumers evaluate the quality of beef, developing research in four European countries: France, Germany, Spain and the UK. Through focus groups, the author identified the intrinsic quality cues (cut, color and fat), the extrinsic quality cues (price, origin and information on animal production) and quality

attributes (taste, tenderness, juiciness, freshness, leanness, wholesomeness, nutrition). In this study, Grunert demonstrated that some quality cues were crucial to consumer perception, even if their effect could be positive on some (e.g., on lean meat) and negative on others (e.g., price). Moreover, he observed that all quality attributes have an important impact on purchase choice and should be considered as a uni-dimensional quality concept. All the above-mentioned quality dimensions were used by the same author some years later (GRUNERT *et al.*, 2004) in order to understand how to use the feedback obtained from consumers on subjective quality perception dimensions, to develop new products in the meat sector deemed to better suit desires.

Focusing on intrinsic quality cues, color/appearance, fat and cut are the three quality dimensions most analyzed by various authors starting with GRUNERT (1997). In the same vein, MCILVEEN and BUCHANAN (2001) used these quality dimensions of intrinsic quality cues, to analyze the factors, which influence beef consumer choices. These authors demonstrated that expectations about quality play a crucial role in evaluating beef quality and that consumers combine sensory (intrinsic) properties – colour, cut and fat in this study-, with extrinsic factors like place of purchase, country of origin, price, brandand quality attributes like appearance, texture, flavour and leanness, to predict and evaluate beef quality.

BRUNSØ *et al.* (2005) also used visual stimuli - colour, fat and cut - in order to understand Danish consumer meat quality perception, demonstrating that consumers are very sensitive to visual stimuli even if this might involve dissatisfaction at the consumption moment. For this reason, BRUNSØ *et al.*, (2005) also stressed the need to educate the consumer in order to improve his consumption experience. For this sensory analysis, the following quality factors were used: cut, fat and colour (three intrinsic quality cues) and tenderness, juiciness, good taste, wholesomeness, nutritional value, freshness, leanness (the latter being quality attribute expectations).

The same quality dimensions (intrinsic quality cues and quality attributes) - with the addition of safety - were used by BANOVIĆ *et al.* (2009) in order to study how Portuguese consumers perceive beef quality. However, in their research, authors also focused on extrinsic quality cues. They also studied the relationship between intrinsic and extrinsic quality cues (price, origin and brand) and how these features were used by consumers to shape their quality perception at the moment of purchase. Results showed that brand is the predominant extrinsic quality cueand that experienced eating quality has a crucial role in future purchase intentions.

"Differences in the consumers' quality perception of national branded, national store brandedand imported store branded beef" were studied by BANOVIĆ *et al.* (2010, p. 54). They observed that consumers perceived the national branded beef as better under all quality cues and aspects in respect to all other branded beef. The same authors in 2012, published another paper focusing on how intrinsic and extrinsic cues affected beef quality consumer perception, also considering different levels of consumer familiarity with a particular beef product. Results demonstrated that color is the intrinsic quality cue most used to evaluate quality when there is high-familiarity with beef. On the contrary, for consumers not familiar with beef, brand plays a crucial role. BORGOGNO *et al.* (2015) also focused on this topic; they compared "consumer's liking and perception of meat quality attributes as a function of their familiarity and involvement with fresh meat" (p. 139) and results showed that, regardless of familiarity level, consumers assign great importance to the visual appearance of meat. Brand in the beef sector is very important because in this

domain meat is mostly sold unbranded. For this reason, according to BREDAHL (2003), analyzing "consumers' quality perception is particularly difficult" (p. 65). The author proposed further research be developed on this topic in order to improve knowledge about the formation of perceived quality and to understand how consumers use and combine quality cues, focusing on brand information. This author demonstrated that brand, as an extrinsic quality cue, is the basis for evaluating both expected eating quality and expected health quality. Intrinsic quality cues identified by BREDAHL (2003) were fat, color, meat juice and cut, while extrinsic quality cues were "brand name, price, cardboard tray, product label, package sleeve, information leaflet, recipes, promotion boards and the information scanner" (p. 69). Finally, quality attributes studied "nutritional value, healthiness, freshness, leanness, tenderness, taste and juiciness" (BREDAHL, 2003, p. 69). Research on the role of the brand in consumer quality perception, also demonstrated that consumers associate safety (quality attributes) with brand, in particular when there is no familiarity with beef. Concerning the safety topic, DE CARLOS *et al.* (2005) performed a qualitative study on the perception of beef in Spain. They observed that the most significant factors affecting quality perception were color, fat content - intrinsic quality cues - and price - extrinsic quality cue - among others (Table 1 and 2). Quite surprisingly, the study highlighted that Spanish consumers, even if aware of the controls carried out by various beef authorities, prefer not to rely on them.

According to BERNUÉNS *et al.* (2003), for some consumer groups, an indicator of safety and nutritious/healthy meat is animal feed and not origin. In their research, the authors focused on different extrinsic quality cues (origin/region of production, animal breed, environmentally friendly, processing/packaging, animal welfare storage, animal feeding) in order to study the role of this extrinsic quality cue on the willingness of consumers to pay for beef, developing their research over five European regions. They conclude identifying clusters of consumers according to the importance of extrinsic quality cues. The high level of importance given to animal welfare by consumers, as a dimension of extrinsic quality, has also been demonstrated by LAGERKVIST *et al.* (2014). The authors analyzed how food labels and packaging information on place of origin influence consumer purchasing decisions. LAGERKVIST *et al.* (2014) studied the price-quality trade-offs issue, highlighting that consumers base their decisions on price when they lack information about intrinsic quality cues. Also MERLINO *et al.* (2018) proved that price, for Italian consumers, is the most important factor in meat purchasing. However, results showed that Italian consumers are also sensitive to "animal welfare" which plays an important role in the choice of buying meat.

According to VERBEKE and WARD (2006), information cues on labels in the beef sector are very important because they help consumers orient their purchasing choices. In particular, the authors developed a study in Belgium, in order to understand which information cues on beef labels greater influenced consumers and to evaluate the impact of a campaign aimed at informing consumers about beef traceability. In this case VERBEKE and WARD (2006) focused only on extrinsic quality cues, without deepening the role of intrinsic quality cues or quality attributes on consumer purchase decisions, unlike BELLO ACEBRÓN and CALVO DOPICO (2000) who developed a study in Spain demonstrating that consumers shape their expectations about beef quality building on both intrinsic cues (e.g., color and fat) and extrinsic cues (e.g., price and origin of animal). These authors also observed that quality attributes, evaluated during consumption, are: taste, tenderness and juiciness. In particular, these authors studied the relationship

between expected quality and perceived quality at the moment of cooking. RESANO *et al.*, (2018) focused on consumer preferences of veal attributes; authors proved that regional origin and health information play a stronger role than guaranteed tenderness at the moment of purchasing.
To analyze consumer meat quality perceptions several authors used the TFQM model. In particular VIMISO *et al.* (2012) applied the TFQM model in order to compare rural consumer meat quality perceptions, measured through intrinsic and extrinsic quality cues, with meat trader quality perceptions. Quality dimensions used in this research were color and fat - intrinsic quality cues-and place of slaughter, packaging, beef class and price - extrinsic quality cues. Quality attributes considered were: juiciness, tenderness, freshness, leanness.
SAEED (2013) and SAEED and GRUNERT (2014), through the application of the TFQM model, focused on beef production processes. SAEED (2013) used the TFQM in order to analyze the change in consumer quality perception concerning four new processed beef products, both in the pre and post consumption phase. Quality cues selected for this study were: beef color, fat, appearance, cut, trim and ingredients. Taste, freshness, nutrition, juiciness, wholesomeness were considered among the quality attributes and evaluated at the point of beef consumption, in order to study consumer perceptions. SAEED and GRUNERT (2014), using TFQM, focused on four different new beef product processes and underlined that cue evaluations as well as "expected/experienced quality and purchase motive fulfillment" affect purchase intention but act differently before and after trial (p. 451). They investigated quality cues before and after trial like appearance, color, fat, etc.; expected quality and experienced quality like taste, freshness, juiciness, etc.; purchase motives before and after trial and, finally, purchase intention before and after purchase.
The studies of beef product processes are very important because according to RESURRECCION (2003) "the development of low-fat products is another strategy to increase the consumption of beef" (p. 13). Indeed, the author studied factors influencing consumer purchase behavior, suggesting that changes in consumer preferences depend on factors such as health concerns, change in demographics, need for convenience, changes in the distribution of meat, as well as price.
COLLE *et al.*, (2016) developed a technical study to determine the influence of post-fabrication ageing on beef quality characteristics and consumer sensory perceptions of biceps femoris and semi-membranous steaks. Quality attributes selected for this study were: tenderness, juiciness and flavor.
Based on previous research of consumer decision-making about red meat, from which the amount and type of visual fat emerged as a major factor in consumer choice (i.e., BANOVIĆ *et al.*, 2012, BANOVIĆ *et al.*, 2009, BANOVIĆ *et al.*, 2010, BRUNSØ *et al.*, 2005), BANOVIĆ *et al.* (2016) focused on the effect of fat content on visual attention and on the choice of red meat, as well as on gender differences, developing a study conducted on 105 Portuguese meat consumers. Results show that consumers pay more attention and more often choose meat products with lower fat content, particularly if they are female. The relationship between meat color and fat and consumer perception was also studied by RISTIĆ *et al.* (2017) who develop a sensorial analysis in order to evaluate consumer attitudes towards sensory properties of chicken, royal and beef salami, all meat products from Zlatiborac Meat Company. The authors proved that consumers pay great attention to these intrinsic quality cues; especially older consumers, perhaps because they are more aware of health aspects related to the food products they purchase. According to

SUBBARAJ *et al.* (2016), meat color is one of the cues available for consumers to gauge overall meat quality and wholesomeness; the authors, performing a technical study based on hydrophilic interaction liquid chromatography–mass spectrometry (HILIC–MS), were able to state that "colour stability of meat is determined by several factors both inherent to the animal and post-slaughter conditions, including ageing, storage/packaging and display times" (SUBBARAJ *et al.*, 2016, p. 163).
Finally, HENCHION *et al.*, (2017) developed a systematic review in order to determine the relative importance of beef quality attributes from a consumer perspective, considering search, experience and credence quality attributes. The aim of the study was to provide relevant information that may be considered in future iterations of quality assurance schemes, to increase consumer satisfaction and, potentially, to increase returns to industry. Tables 1-3 show quality dimensions studied by the above-mentioned authors in order to analyze and understand consumer perception of beef.

Table 1. Intrinsic quality cues.

Author	Type of meat analyzed	Country	Intrinsic quality cues					
			Colour/Appearance	Fat	Cut	Meat juice	Trimming	Marbling
Grunert, (1997)	Beef	France, Germany, Spain, UK	X	X	X			
Acebroen & Calvo Dopico (2000)	Beef	Spain	X	X				
McIlveen and Buchanan, (2001)	Beef	Ireland	X	X	X			
Bredahl (2003)	Beef	Denmark	X	X		X	X	
Grunert *et al.*, (2004)	Beef and pork	France, Germany, Spain, UK	X	X	X			
Resurreccion, (2004)	Beef	France, Germany, Spain, Uk and USA	X	X				
Brunsø *et al.*, (2005)	Beef	Danish	X	X	X			
de Carlos *et al.*, (2005)	Beef	Spain	X	X	X			
Banović *et al.*, (2009)	Beef	Portugal	X	X	X			
Banović *et al.*, (2010)	Beef	Portugal, Brazil	X	X	X			
Banović *et al.*, (2012)	Beef	Portugal	X	X	X			
Vimiso *et al.*, (2012)	Beef	South Africa	X	X				
Saeed *et al.*, (2013)	Beef	Denmark	X	X	X			
Borgogno *et al.*, (2014)	Beef	Italy	X	X				X
Saeed and Grunert (2014)	Beef	Denmark	X	X				
Banović *et al.*, (2016)	Beef	Portugal		X				
Colle *et al.*, (2016)	Beef	Idaho - USA	X					
Subbaraj *et al.*, (2016)	Beef	Southland, New Zealand	X					
Henchion *et al.*, (2017)	Beef		X	X				
Merlino *et al.*, (2018)	Beef	Italy	X					

Source: our elaboration.

Table 2. Extrinsic quality cues.

Author	Type of meat analyzed	Country	Extrinsic quality cues												
			Price	Origin/Quality certification	Promotion	Label Information/ Information on animal production	Place of Purchase	Brand	Butcher recom-mendation	Beef class	Store image	Storage	Package/ Presentation	Animal welfare	Recipes
Grunert, (1997)	Beef	France, Germany, Spain, UK	X	X		X									
Acebroen & Calvo Dopico (2000)	Beef	Spain	X	X	X						X		X		
McIlveen and Buchanan, (2001)	Beef	Ireland	X	X			X	X							
Bernués *et al.*, (2003)	Beef	England, Italy, France, Scotland and Spain		X				X		X		X	X		
Bredahl (2003)	Beef	Denmark	X	X	X	X		X					X*		X
Grunert *et al.*, (2004)	Beef and pork	France, Germany, Spain, UK	X	X		X									
Resurreccio n, (2004)	Beef	France, Germany, Spain, Uk and USA	X												
de Carlos *et al.*, (2005)	Beef	Spain	X	X					X		X	X			X

Verbeke and Ward (2006)	Beef	Belgium		X		X									
Banović *et al.*, (2009)	Beef	Portugal	X	X				X							
Banović *et al.*, (2010)	Beef	Portugal, Brazil	X	X		X		X					X		
Banović *et al.*, (2012)	Beef	Portugal	X	X				X							
Borgogno *et al.*, (2014)	Beef	Italy	X	X		X		X		X			X	X	X
Vimiso *et al.*, (2012)	Beef	South Africa	X			X				X			X		
Lagerkvist *et al.* (2014)	Beef	Swedish	X	X		X		X							
Henchion *et al.* (2017)	Beef		X	X		X	X	X					X		
Merlino *et al.*, (2018)	Beef	Italy	X	X		X		X		X				X	
Resano *et al.*, (2018)	Beef	Spain	X	X		X									

Source: our elaboration.
* Cardboard tray, Package sleeve.

Table 3. Quality attributes expectations/experience.

Author	Type of meat analyzed	Country	Quality attributes								
			Taste/Flavour	Tenderness	Juiciness	Wholesomeness/Healthiness	Nutrition value	Leanness	Safety	Freshness	Smell
Grunert, (1997)	Beef	France, Germany, Spain, UK	X	X	X	X	X	X		X	
Acebroen & Calvo Dopico (2000)	Beef	Spain	X	X	X						
McIlveen and Buchanan, (2001)	Beef	Ireland	X	X	X			X			

Bredahl (2003)	Beef	Denmark	X	X	X	X	X	X		X		
Grunert *et al.*, (2004)	Beef and pork	France, Germany, Spain, UK	X	X	X	X	X	X		X		
Resurreccion, (2004)	Beef	France, Germany, Spain, Uk and USA	X	X	X	X	X		X			
Brunsø *et al.*, (2005)	Beef	Danish	X	X	X	X	X	X		X		
Banović *et al.*, (2009)	Beef	Portugal	X	X	X	X	X	X	X	X		
Banović *et al.*, (2010)	Beef	Portugal, Brazil	X	X	X	X	X		X			
Banović *et al.*, (2012)	Beef	Portugal	X	X	X	X	X		X			
Vimiso *et al.*, (2012)	Beef	South Africa		X	X			X		X	X	
Saeed *et al.*, (2013)	Beef	Denmark	X		X	X	X			X		
Borgogno *et al.*, (2014)	Beef	Italy					X		X			
Saeed and Grunert (2014)	Beef	Denmark	X		X	X	X			X		
Henchion *et al.*, (2017)	Beef		X	X	X	X	X*		X*			
Merlino *et al.*, (2018)	Beef	Italy	X	X			X		X			
Resano *et al.*, (2018)	Beef	Spain		X								

Source: our elaboration.
Note: * HENCHION *et al.*, (2017) classify Nutrition value and Safety as Credence attributes together with Origin, Animal welfare, Production system/feeding, Environmental issues, Traceability, Processing technologies (ageing, irradiation, halal/kosher) and Breed.

2. MATERIAL AND METHODS

2.1. Questionnaire and data collection

Based on the study of ESPEJEL *et al.* (2007), a questionnaire was prepared to investigate the relationship between intrinsic quality cues, extrinsic quality cues, expected quality of beef and customer behavior.
The questionnaire was divided into three different areas of analysis: (i) perceived quality cues (extrinsic and intrinsic) (Table 4), (ii) evaluation of expected quality (Table 4), (iii) customer profile: containing information on socio-demographic features (Table 9). Dimensions of quality cues and expected quality attributes were drawn from the literature review.
The Likert measurement scale was used to measure consumer perception, with a score assigned to the respondents between 1 and 6, ranging from 'strongly disagree' (scoring value 1) to 'strongly agree' (scoring value 6); an even scale was chosen in order to avoid central tendency bias of the responses (LIKERT, 1932; MATELL AND JACOBY, 1971; BERNUÉS *et al.*, 2012; SILVESTRI *et al.*, 2018), To measure customer before purchase preferences and expectations, three types of questions were formulated: two single choice questions, three dichotomic questions and two questions measured on a Likert scale 1-6.
As the aim of the research was also to understand how the perception of beef quality changes from one region to another and therefore if the traditional food issue could affect consumer preferences and purchase choices, the study was performed in two Central Italian regions that have the closest percentage of beef production: Latium 35,9% and Tuscany 32,2% (ISMEA, 2016). Tuscany was selected as it is the only Italian region where beef is part of the traditional cuisine (MIELE and MURDOCH, 2002). In particular, from this region Grosseto and Orbetello were selected, both pertaining to Grosseto Province, which is the administrative center where beef livestock is the most important of all Central Italian Provinces (ISTAT, 2010; ISMEA, 2016). In Latium, Viterbo was selected as the nearest Province to Tuscany and the Province where beef livestock is less important than in other provinces in Latiumand Rome where beef livestock is the most important in the region, but the PDO is "Abbacchio Romano" (ISTAT, 2010; http://ec.europa.eu/).
The data collection was performed thus: Viterbo (Latium) June, 17-19, 2016; Grosseto (Tuscany) June, 24-26, 2016; Rome (Latium) July, 1-3, 2016; Orbetello (Tuscany) July, 8-10, 2016. To ensure both the homogeneity of data collection conditions within four hypermarkets and the possibility of contacting the most heterogeneous consumers – also working people and families - questionnaires were collected at weekends. Consumers were interviewed at the meat counter of the hypermarket once they had picked up a beef package. The difficulty in identifying the meat consumers led, as it usually does in market research activities, to the adoption of a no probabilistic model, in particular of a random sampling (BRACALENTE *ET AL.*, 2009, SAEED *et al.*, 2013). The sample analyzed was composed of 447 individuals.
The data collected was analyzed using the statistic program "STATA 12 Data Analysis and Statistical Software" (www.stata.com).

2.2. Factor analysis and cluster analysis

Data presented in Table 4 shows that all quality dimensions significantly influence preferences and beef purchase decisions. In particular, among the intrinsic quality cues, the most important attribute is color (average value of 5.40); the extrinsic quality cues are affected by price (average value of 5.85) and quality certification (average value of 5.52). Expected quality is homogeneously affected by all attributes. Safety and juiciness are the only quality attributes that present a lower average value (Safety average value of 3.99; Juiciness average value of 4.64)

Cronbach α was used to test internal consistency for all items under respective variables (NAMUKASA, 2013). Following Hair at al. (2006) who stated that Cronbach α coefficient over 0.6 is adequate for basic research, it is possible to argue that the sample of this study shows good internal consistency. Also performing the Kaiser-Meyer-Olkin (KMO) test whose result must exceed the 0.5 limit (KAISER, 1974; HAIR *et al.*, 2006; SANTOURIDIS AND TRIVELLAS, 2010), the sample was found appropriate to perform the factor analysis. Finally, the correlation test was used to verify whether or not the observed variables contain misleading redundancies or make the results insignificant.

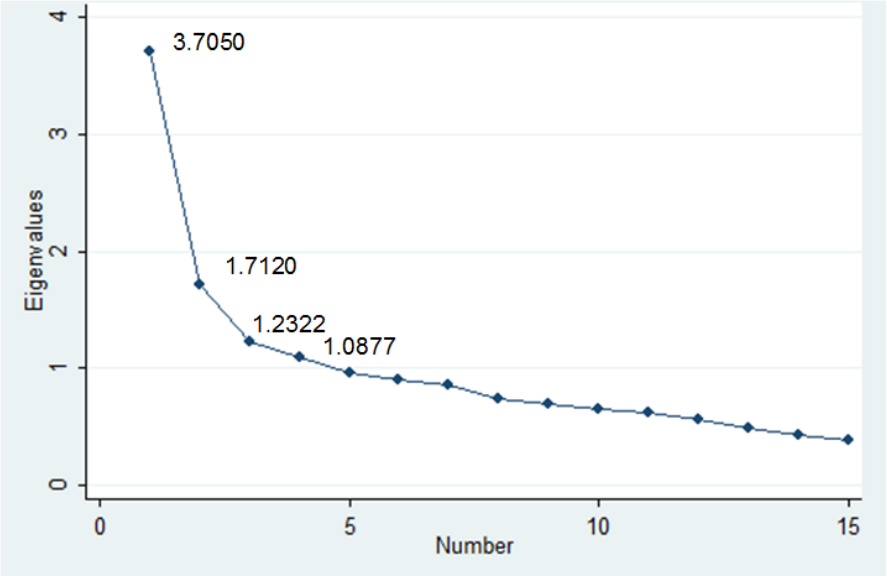
Table 4. Descriptive statistics of quality dimensions.

Measures	Items	Variable	Obs	Mean	Std. Dev.	Min	Max	Alpha	KMO
Intrinsic quality cues	Cut	IQ1	447	4.67	1.47	1	6	0.696	0.780
	Color	IQ2	447	5.40	1.06	1	6		0.811
	Fat	IQ3	447	4.79	1.33	1	6		0.823
Extrinsic quality cues	Origin	EQ1	447	4.67	1.48	1	6	0.667	0.744
	Price	EQ2	447	5.85	0.53	2	6		0.623
	Quality certification	EQ3	447	5.52	0.89	1	6		0.681
	Brand	EQ4	447	5.44	1.03	1	6		0.641
Expected quality attributes	Nutritional value	EXQ1	447	5.57	1.01	1	6	0.612	0.844
	Freshness	EXQ2	447	5.23	1.08	1	6		0.845
	Taste	EXQ3	447	5.53	0.85	1	6		0.882
	Tenderness	EXQ5	447	5.23	1.34	1	6		0.806
	Smell	EXQ6	447	5.66	0.92	1	6		0.833
	Juiciness	EXQ7	447	4.64	1.36	1	6		0.789
	Wholesomeness/Healthiness	EXQ8	447	5.57	1.01	1	6		0.782
	Safety	EXQ9	447	3.99	1.75	1	6		0.716
		Overall						0.746	0.790

Source: our elaboration on the data set.

In order to determine the number of the most important factors, the screen plots tool introduced by Cattell (1966) was used. Fig. 2 shows that the first four factors are the only ones with eigenvalues greater than 1.

Figure 2. Screen plot of eigenvalues after factor analysis.



Source: our elaboration.

Table 5 shows orthogonal Varimax rotation of the factors where the first four have eigenvalues greater than 1 and also encompass 51.58% of the information contained in the original data set.

Table 5. Rotation: orthogonal Varimax (Kaiser off).

Factor	Variance	Difference	Proportion	Cumulative
Factor 1	2.5322	0.4394	0.1688	0.1688
Factor 2	2.0928	0.2172	0.1395	0.3083
Factor 3	1.8756	0.6393	0.1250	0.4334
Factor 4	1.2363		0.0824	0.5158

Source: our elaboration on the data set; Number of obs 447; Retained factors 4.

From the results obtained from the joint use of the two above illustrated analytical tools, the first four factors were considered to identify the new variables.

Factor interpretation was achieved by considering the so-called saturation matrix (Table 6) where correlation between original variables and factors were identified.

Table 6. Saturation matrix (factor loadings).

New Variables	Measures	Items	Variable	Factor 1	Factor 2	Factor 3	Factor 4	Uniqueness
Beef quality features – FA1	Intrinsic quality cues	Color	IQ2	**0.6457**	0.2420	-0.0077	0.1189	0.5104
		Fat	IQ3	**0.5069**	0.1431	-0.0077	0.1674	0.6945
	Quality expected attributes	Freshness	EXQ2	**0.7152**	0.0449	0.1304	0.0066	0.4694
		Taste	EXQ3	**0.6020**	0.2464	0.0132	0.0346	0.5755
		Tenderness	EXQ5	**0.5764**	0.2474	0.1252	-0.0191	0.5906
		Smell	EXQ6	**0.6871**	0.1779	0.0312	0.0066	0.4952
Flavor & Healthiness – FA2	Intrinsic quality cues	Cut	IQ1	0.0792	**0.5961**	0.1815	0.2020	0.5646
	Quality expected attributes	Nutritional value	EXQ1	0.0093	**0.5929**	0.2193	0.1985	0.5609
		Juiciness	EXQ7	0.2954	**0.7237**	-0.0277	-0.1143	0.3751
		Wholesomeness/Healthiness	EXQ8	0.2429	**0.7203**	0.1169	-0.0723	0.4033
Safety & Traceability – FA3	Extrinsic quality cues	Origin	EQ1	0.0775	0.2822	**0.7074**	-0.0060	0.4140
		Quality Certification	EQ3	0.0565	0.0700	**0.8034**	0.0198	0.3460
	Quality expected attributes	Safety	EXQ9	-0.0003	-0.0473	**0.6513**	-0.0325	0.5725
Price & Brand – FA4	Extrinsic quality cues	Price	EQ2	-0.0108	0.0749	-0.1864	**0.7960**	0.3259
		Brand	EQ4	0.1347	-0.1279	0.3760	**0.6774**	0.3653

Source: our elaboration on the data set.

Table 6 shows that factor1 synthesizes the variables related to the attributes of intrinsic quality cues (like Color and Fat) and expected quality (like Freshness, Taste, Tenderness and Smell). Factor 2 synthesizes the variables related to the attributes of intrinsic quality cues (like Cut) and expected quality (like Nutritional value, Juiciness, Wholesomeness/Healthiness). Factor 3 synthesizes the variables related to the attributes of extrinsic quality cues (like Origin and Quality Certification) and expected quality (like Safety) and finally factor 4 synthesizes the variables related to the attributes of extrinsic quality cues (like Price and Brand). Through factor analysis, the number of variables was reduced from 15 to 4. This result highlights that consumers do not have a clear idea of how literature classifies the different quality dimensions of meat.
For research purposes the hierarchical method of Ward (FABBRIS, 1997; DAHL AND NÆS, 2004; ANNUNZIATA AND VECCHIO, 2013) was used and the number of groups was determined by inspecting the dendrogram.

Using the information derived by the Calinski/Harabasz indicator (Table 7) together with the dendrogram analysis, four groups were identified.

Table 7. Calinski/Harabasz indicator.

Number of clusters	Calinski/ Harabasz
2	76.06
3	86.52
4	**98.83**
5	94.23
6	91.79
7	88.22

Source: our elaboration on direct survey

Table 8 shows the four meat consumer groups related to the new variables of quality dimensions. On the basis of the correlation link intensity it is possible to define the characteristics of the four clusters.

Table 8. Cluster analysis in relation to new factors of quality – correlation link intensity.

Cluster	FA1	FA2	FA3	FA4
Cluster 1	-1.971	-0.409	-0.392	0.184
Cluster 2	**0.577**	0.038	-1.763	0.335
Cluster 3	0.189	**0.284**	0.323	-1.184
Cluster 4	0.231	-0.045	**0.390**	**0.420**
Total	-1.57E-10	5.82E-10	-1.87E-09	-1.42E-09

Source: our elaboration on direct survey.

Cluster 1 seems to be indifferent to all studied quality dimensions of beef, unlike the other three clusters. Indeed Cluster 2 is characterized by consumers focused on Beef quality features (FA1), Cut, Nutritional value, Juiciness, Wholesomeness/Healthiness (FA2) are essential for Cluster 3, while Safety and Traceability (FA3) and Price and Brand (FA4) are fundamental to Cluster 4. In order to validate the segmentation into 4 clusters, confirmatory analysis was developed.
The statistical significance of socio-demographic variables (categorical variables) was validated through the test study of Pearson Chi-square (ADANACIOGLU AND ALBAYRAM, 2012), while the statistical significance of numeric variables was validated through the study of Variance (VERMEIR AND VERBEKE, 2008; YADAVALLI AND JONES, 2014).

The largest group is Cluster 4, 51.23% followed by 23.71% of Cluster 3, while Cluster 1 and Cluster 2 are the smallest ones (Cluster 1 represents 12.08% of the sample and Cluster 2 12.98%).
Cluster 1 is mainly composed of young men, aged between 20-29 and 30-39. They are students, workers, entrepreneurs and teachers, residing mostly in Tuscany (Grosseto) and Latium (Viterbo province). They purchase beef every day or one day a weekand they do not read the traceability label because they stated they don't understand its meaning. For this reason, most Cluster 1 consumers are not willing to pay a higher price for a better beef quality system. Those who are ready to pay more declared that they would be ready to pay up to a 10% increase on the market price to have a better beef quality system. For cluster 1 consumers, quality certification is synonyms with safety (scores assigned on the Likert-scale from 3 to 5) and media information affects their perception of beef quality (scores assigned on the Likert-scale from 4 to 6).
Cluster 2 consumers were focused on Beef quality features (FA1). This represents 12.98% of the sample and it consists mainly of young men aged between 20-29 and 30-39. They are students, employees, freelancers and artisans, resident in Tuscany (Grosseto) and Latium (Viterbo). They purchase beef two or three times of week. Like cluster 1 consumers, they do not read the traceability label because they stated they don't understand it. They are not willing to pay a higher price for a better beef quality system. Those who are ready to pay more declared that they are ready to pay up to a 10% increase on the market price to have a better beef quality system. For cluster 2 consumers quality certification is not synonyms with safety (scores assigned on the Likert-scale from 1 to 2) and media information affects their perception of beef quality (scores assigned on the Likert-scale from 3 to 5).
Cluster 3 is composed of women and men aged between 20-29, 40-49 and 50-59 years, students, entrepreneurs, freelancers and unemployed resident in Tuscany (Grosseto province) and Latium (Roma). Consumers of Cluster 3 focus their attention on the traceability label and quality certification too and are willing to pay up to 10% more than the beef market price in order to have a better quality system. Some cluster 3 consumers consider beef quality certifications as synonymous with safety (scores assigned on the Likert-scale 4) while others do not (scores assigned on the Likert-scale 1). Some cluster 3 consumers claimed to be greatly influenced by media information (scores assigned on the Likert-scale 6) while others said the opposite (scores assigned on the Likert-scale). Finally, they buy beef two or three times a week, more than once a month or less than once a month.
Finally, Cluster 4 is the largest group (51.23% of simple) and it is composed of women aged between 50-59 and over 60, predominantly housewives, teachers and pensioners living in Tuscany (Grosseto province). For them the traceability label and quality certification of beef are essential factors in making their purchase decision. However, they are willing to pay up to 5% more than the current beef market price in order to have a better quality system. Finally, they consider beef quality certifications as synonymous with safety (scores assigned on the Likert-scale from 5 to 6) and they buy beef every day and once a week. Some of them are greatly influenced by media information (scores assigned on the Likert-scale 5) while others stated that they are not influenced by such information at all (scores assigned on the Likert-scale from 1 to 2) (Table 9).

Table 9. Socio-demographic characteristics and purchase intention of the meat consumers of the four clusters.

Socio-demographic behavioral variables	Sample (n)		Cluster 1 (n= 54; 12.08%)		Cluster 2 (n= 58;12.98%)		Cluster 3 (n= 106; 23.71%)		Cluster 4 (n= 229, 51.23%)	
	f	%	*f*	%	*f*	%	*f*	%	*f*	%
Gender										
Male	142	31.77	23	42.59	21	36.21	34	32.08	64	27.95
Female	305	68.23	31	57.41	37	63.79	72	67.92	165	72.05
Total	447	100	54	100	58	100	106	100	229	100
Age Group										
20-29	68	15.21	14	25.93	11	18.97	17	16.04	26	11.35
30-39	51	11.41	11	20.37	12	20.69	6	5.66	22	9.61
40-49	63	14.09	4	7.41	8	13.79	23	21.7	28	12.23
50-59	107	23.94	7	12.96	9	15.52	32	30.19	59	25.76
≥60	158	35.35	18	33.33	18	31.03	28	26.42	94	41.05
Total	447	100	54	100	58	100	106	100	229	100
Professional category										
Student	43	9.62	7	12.96	9	15.52	12	11.32	15	6.55
Employee	82	18.34	9	16.67	16	27.59	19	17.92	38	16.59
Worker	32	7.16	6	11.11	4	6.9	7	6.6	15	6.55
Housewife	69	15.44	6	11.11	7	12.07	14	13.21	42	18.34
Entrepreneur	16	3.58	4	7.41	2	3.45	6	5.66	4	1.75
Freelance	41	9.17	4	7.41	6	10.34	18	16.98	13	5.68
Teacher	23	5.15	3	5.56	2	3.45	5	4.72	13	5.68
Pensioner	104	23.27	12	22.22	8	13.79	20	18.87	64	27.95
Artisan	4	0.89	0	0	2	3.45	1	0.94	1	0.44
Unemployed	12	2.68	1	1.85	1	1.72	3	2.83	7	3.06
Other	21	4.7	2	3.7	1	1.72	1	0.94	17	7.42
Total	447	100	54	100	58	100	106	100	229	100
Residence										
Viterbo	70	15.66	7	12.96	18	31.03	14	13.21	31	13.54
Province of Viterbo	70	15.66	13	24.07	8	13.79	14	13.21	35	15.28
Civitavecchia	62	13.87	5	9.26	8	13.79	17	16.04	32	13.97
Grosseto	101	22.6	13	24.07	14	24.14	22	20.75	52	22.71
Province of Grosseto	39	8.72	4	7.41	5	8.62	14	13.21	16	6.99
Orbetello	43	9.62	5	9.26	1	1.72	6	5.66	31	13.54
Other provinces of Tuscany	62	13.87	7	12.96	4	6.9	19	17.92	32	13.97
Total	447	100	54	100	58	100	106	100	229	100

Purchase Frequency										
Everyday	22	4.93	6	11.32	0	0	4	3.81	12	5.29
2-3 times a week	220	48.43	25	45.28	32	54.39	54	50.48	109	47.14
1 time per week	163	37.22	20	37.74	20	35.09	36	34.29	87	38.33
2-3 times a month	38	8.52	3	5.66	5	8.77	10	9.52	20	8.81
Less than once a month	4	0.9	0	0	1	1.75	2	1.9	1	0.44
Total	447	100	54	100	58	100	106	100	229	100
Knowledge the traceability label										
Yes	368	82.33	42	77.78	22	55.17	96	85.85	209	88.65
No	79	17.67	12	22.22	36	44.83	10	14.15	19	11.35
Total	447	100	54	100	58	100	106	100	228	100
Read the traceability label										
Yes	370	82.74	42	77.78	22	37.93	96	90.57	210	91.67
No	77	17.26	12	22.22	36	62.07	10	9.43	19	8.33
Total	447	100	54	100	58	100	106	100	229	100
Willingness to pay a higher price for a better meat quality system										
Yes	392	87.7	42	77.78	43	74.14	102	96.23	205	89.52
No	55	12.3	12	22.22	15	25.86	4	3.77	24	10.48
Total	447	100	54	100	58	100	106	100	229	100
How much more										
5% more than the current market price	191	48.6	21	48.84	21	48.84	40	39.22	109	53.17
Up to 10% more than the current market price	130	33.08	16	37.21	15	34.88	32	31.37	67	32.68
Over 10% more than the current market price	72	18.32	6	13.95	7	16.28	30	29.41	29	14.15
Total	393	100	43	100	43	100	102	100	205	100
How much quality labels are safety synonyms for consumers										
1= In no way	52	11.63	2	3.7	10	17.24	17	16.04	23	10.04
2	23	5.15	4	7.41	7	12.07	6	5.66	6	2.62
3	48	10.74	10	18.52	8	13.79	12	11.32	18	7.86
4	105	23.49	16	29.63	13	22.41	29	27.36	47	20.52
5	146	32.66	18	33.33	15	25.86	28	26.42	85	37.12
6=Very much	73	16.33	4	7.41	5	8.62	14	13.21	50	21.83
Total	447	100	54	100	58	100	106	100	229	100

How much media information influences their meat purchasing choices										
1= In no way	227	50.78	22	40.74	26	43.86	62	58.1	121	52.42
2	32	7.16	4	7.41	4	7.02	5	4.76	19	8.37
3	44	9.84	4	7.41	8	14.04	9	8.57	22	9.69
4	57	12.75	11	20.37	11	19.3	11	10.48	22	9.69
5	52	11.63	8	14.81	7	12.28	8	7.62	28	12.33
6=Very much	35	7.83	5	9.26	2	3.51	11	10.48	17	7.49
Total	447	100	54	100	58	100	106	100	229	100

Source: our elaboration.

3. DISCUSSION

The above results help to answer the research questions on which the paper is built.
Factor analysis helps understand what the relationships are between extrinsic cues, intrinsic cues and expected quality attributes – sensory impressions based on past experience-, therefore answer the first research question:

- *What is the role of extrinsic and intrinsic cues as well as sensory impressions based on past experience at the moment of purchase?*

From Table 6 it is clear that extrinsic quality cues are linked to safety which is an expected quality attribute, while intrinsic quality cues are linked to all other expected quality attributes, namely freshness, taste, tenderness, smell.
These results are in line with previous studies. In particular, origin, safety and quality certifications – e.g. quality labels – (Cluster 4) have already been considered as quality cues important to determine consumer preferences and choices before beef is purchased (e.g. GRUNERT, 2005). BRUNSØ *et al.* (2005) also highlight the importance of quality controls, stating that this is the third dimension of quality. Instead, GRUNERT (2005) states that information available about "breed, age of animaland slaughtering date are predictive" of taste and tenderness, but "few consumers feel confident in using them" (p. 376). Cluster 4 represents 51.23% of the entire sample. It is made up of older women, aged from 50 to over 60. Consumers grouped in Cluster 4 consider beef traceability as well as quality certifications of paramount importance and predictive of beef safety. Moreover, for these consumers price and brand are the most important features to signal quality as also suggested by Grunert *et al.* (2004). Price has long been studied in beef quality literature, almost together with brand (BELLO ACEBRÓN AND CALVO DOPICO, 2000; BREDAHL, 2003; GRUNERT *et al.*, 2004; GRUNERT, 2005; TOLOSANA *et al.*, 2005; BANOVIĆ *et al L.*, 2010; BANOVIĆ *et al.*, 2012). Indeed, for GRUNERT *et al.* (2004) brand if combined with quality and reliability built over time, can be considered the most important extrinsic quality cue when purchasing beef for consumers not so aware of beef features and therefore struggling to formulate their expectations about beef quality cues. In this same vein BREDAHL (2003) and BANOVIĆ *et al.* (2010; 2012) demonstrated that consumers focus on brand when they are not so familiar with beef products, which leads to hesitation at the moment of purchase. Besides brand, price is also used by hesitant consumers as predictive of beef quality (BELLO ACEBRÓN and CALVO DOPICO, 2000; TOLOSANA *et al.*, 2005; MERLINO *et al.* 2018). Cluster 4 consumers are also ready to pay more than the

average beef market price (maximum +5%) to rely on a better quality system as already stated by BELLO ACEBRÓN and CALVO DOPICO (2000) and GRUNERT *et al.* (2004). On the contrary BREDAHL (2003), carrying out a study on the Danish beef market, found that price is not considered such an important extrinsic quality cue for Danish consumers. Our insights about Cluster 4 are in line with past studies concerning consumer behavior, stating that older women pay more attention seeking information about product safety and quality. (e.g. RICHARDSON *et al.*, 1996; ROSZKOWSKA-HOŁYSZ, 2013). From our study, media information seems decisive in determining older women's purchasing choices. In this domain, KUO *et al.* (2011) found that in general all women adopt a more "protective behavior"(p. 5) than men, in that they are more aware of food risks and the importance of safety issues. Finally, results are in line with the study conducted by BANTERLE and STRANIERI (2008), which showed that, among European consumers, Italians are more sensitive to the issue of safety and food certification. The research shows that Italians make extensive use of information reported on labels, such as information on certification and meat origins.

Intrinsic quality cues and part of the expected quality attributes, apart from safety, are of paramount importance when the consumer is aware of the product and its special quality features. In this respect BREDHAL *et al.* (1998), for example, pointed out that making the relationship clear between expected and organoleptic characteristics - e.g. intrinsic quality cues – is important to understand how consumers shape their expectations about beef. This study confirms that these characteristics are important at least for Cluster 2, which represents 12.98% of the entire sample. Cluster 2 is made up of young men who are mostly unaware of traceability labels and don't read them. They are willing to pay over 10% of the current beef market price to have better quality beef and their beef consumption is on average once a week.

These consumers seem to pay great attention to intrinsic quality cues and results are in line with several studies conducted in the literature. *In primis*, the male gender, whose result is discriminating for Cluster (2) and Cluster (1), confirms the study of several authors like e.g. SOBAL (2005), CAVAZZA *et al.* (2015), BASFIRINCI and CILINGIR UK (2017), according to whom the consumption of red meat is an expression of virility and strength and is more associated with the male identity. Indeed, the female is associated with sweet foods (LUPTON, 1996), fruit (O'DOHERTY AND HOLM, 1999) and dietetic products (MOONEY AND LORENZ, 1997; BASFIRINCI AND CILINGIR UK, 2017). Finally, LENNERNÄS *et al.* (1997), BILOUKHA and UTERMOHLEN (2001) and Piggford *et al.* (2008) showed that "sensory appeal" (PIGGFORD *et al.*, 2008, p.19) (including smell, appearance, palatability and pleasure), represent the factors that influence the male purchases. According to PENG *et al.* (2005), in fact, male consumers pay more attention to product quality and the purchasing environment, than do female consumers.

Cluster 1, representing 12.08% of the sample is made up of young men, mostly students, employees and laborers. They frequently consume beef, but seem not to be affected by any quality cue and/or attribute at the moment of purchase. These results are not surprising in that, in general, men have less shopping experience and pay less attention to information about safety and quality than women (e.g. TZIMITRA-KALOGIANNI *et al.*, 2003; KUO *et al.*, 2011).

Cluster 3 individuals (23.71% of sample) give a component as the visual aspect of the meat (cut) an attribute (succulence) that can be evaluated through taste and two other attributes (nutritional values and wholesomeness) that cannot be measured because they are part of

the beliefs, which can be found in the purchase psychological factors (FONT-I-FURNOLS AND GUERRERO, 2014). The cut is linked to these attributes, because the amount of fat in the meat varies according to the cut and, as stated by SHAN *et al.* (2016), consumers are very attentive to these aspects. In particular, young Italians, who among various purchasing factors also consider livestock feeding, since there is a relationship between this and nutritional value and healthiness (BANTERLE AND STRANIERI, 2008). Moreover, while several studies claimed that women are more attentive to factors such as nutritional value and healthiness (for both health and body care reasons) compared to men (DREWNOWSKI AND HANN, 1999; HOLM, 2003; SHAN *et al.*, 2016), this distinction does not emerge from the results of the present study. Cluster 3 is composed of both men and women. The results show that even men are becoming more sensitive to these issues nowadays.
Traceability and safety issues emerged to a certain extent in the previous discussion when we analyzed the identified Cluster characteristics, but our study also focuses specifically on this issue with the second research question.

- *How traceability and safety issues affect consumer preferences and choices?*

Traceability labels were found important for Cluster 4. In particular, consumers in this cluster are aware of traceability labels and read them. Also, it can be observed that Cluster 4 consumers are also ready to pay a higher price than the actual average beef price for a better quality system. To understand why, we considered the traditional food issueand found that consumers falling in this cluster ready to pay a higher price to have a better quality system are 205 out of 229 representing 89.52% of Cluster 4 and are mostly resident in Tuscany (43.24%) – where beef is a traditional food. The importance of safety issues as a whole has already been highlighted in literature, above all after the emergence of BSE (e.g. BRUNSØ *ET AL.* 2005; GRUNERT, 2005), but the results of this study seem to suggest that consumers today are more aware of beef quality related issues for health in general and especially when this food is known and frequently purchased, these features become of paramount importance.
The third research question introduced the traditional food issue, not yet considered in literature, which seems to play a role in beef purchase choice.

- *Do consumers in regions where beef is a traditional food, consider the impact of traceability and safety issues differently at the moment of purchase?*

Cluster 1, 2 and 3 consumers are mostly resident in Latium (45%, 58.61% and 42.46% of the sample) where beef is not a traditional food and they seem not to be affected by traceability and safety issues at the moment of purchase. On the contrary, consumers in Cluster 4 are aware of traceability and safety issues and are mostly resident in Tuscany (43.24% of the sample) where beef is a traditional food (MIELE AND MURDOCH, 2002). In this sense, it seems that residence – e.g. traditional food - could be considered a discriminating factor affecting evaluation linked to traceability and safety issues before beef purchase.

4. CONCLUSION

This paper adds some insights into beef meat consumer preferences before purchase: (a) quality cues and/or attributes diversely affect consumers with various socio-demographic characteristics; (b) being a traditional food can affect consumer choices; (c) traceability and

safety have become crucial in shaping before purchase consumer preferences, especially after the emergence of BSE some years ago. This is also because national and international bodies have focused their attention on these issues, obtaining feedback in terms of the importance of these issues recognized by some consumers.
The paper also has some limitations, which could be of help to identify future avenues of research. Principally: (a) the number of questionnaires and the limited places in which they were collected; future studies should consider other Italian regions but also other Countries, verifying the role of the traditional food issue in a more focused way; (b) the study just considers quality cues and attributes before purchasing and does not compare them with the after purchase experience; this could be another future avenue of research.
Among theoretical implications, the most important refers to the attempt to widen the perspective used to study beef quality and its cues and attributes to better understand consumer preferences and purchasing choices. Even if familiarity with beef products has been studied (BANOVIĆ *et al.*, 2010; 2012), other contextual factors could play a role and they should be understood better to paint the "full picture" in this domain.
Adopting the managerial perspective, it becomes clear that it is crucial to firms operating in this industry to know which quality cues and attributes are important in shaping different consumer cluster expectations and preferences. In particular, new value offerings could be shaped *ad hoc* for different and above all emerging clusters considering, besides beef quality cues and attributes, socio-demographic characteristics and also contextual factors like food culture and the traditional food issue. This factor, together with other contextual factors for further research, could play an important role in creating new product offerings and/or modify the present ones in the light of further enhancing consumer knowledge about beef quality and could therefore lead to somewhat modifying the "expectation side" of satisfaction, the after purchase phase of the consumer experience not investigated in this paper.

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PAPER

NUTRITIONAL AND QUALITY CHARACTERISTICS OF CHICKEN NUGGETS INCORPORATED WITH DIFFERENT LEVELS OF FROZEN WHITE CAULIFLOWER

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ABSTRACT

In the current study chicken nuggets were formulated with 5, 10, 15 and 20% of frozen white cauliflower as fat replacer instead of chicken skin. Phenolics, total flavonoids and 1,1-diphenyl-2-picrylhydrazyl (DPPH) antioxidant activity of cauliflower were determined. The proximate composition, physicochemical characteristics and sensory properties of formulated chicken nuggets were evaluated. No significant variations ($P \geq 0.05$) were recorded in the overall acceptability scores among chicken nugget batches. The incorporation of frozen cauliflower as fat replacer agent up to 20% had significant and positive effects on the acceptability and overall quality of chicken nuggets.

Phenolics content, total flavonoids of cauliflower extract were 6.76 and 1.65 mg/g,respectively. The DPPH free radicals scavenging activity of cauliflower extract was 54.9%. Chicken nuggets formulated with various levels of cauliflower had higher ash, fiber and carbohydrates contents but their fat and energy contents were lower, compared to control nuggets. No significant ($P \geq 0.05$) variations in pH values among the control samples and those samples incorporated with different levels of frozen white cauliflower. The highest values (95.60, 96.40 and 96.64%) of cooking yield were recorded for nugget

samples incorporated with 10, 15 and 20% of cauliflower, respectively. The lightness values of chicken nuggets significantly decreased with decreasing chicken skin content. Nuggets samples formulated with 20% cauliflower as fat replacer had significantly the highest value of redness (a* value). No significant differences ($P \geq 0.05$) in b* value (yellowness) were found among formulated chicken nugget samples.

Keywords: chicken, white cauliflower, nuggets, chicken skin, sensory properties

1. INTRODUCTION

Fried foods such as potato chips, tortilla chips, fried fish, fried snack products, French fries, chicken nuggets have gained worldwide popularity (OSORIO-YÁÑEZ, *et al*., 2017). Chicken nugget is one of the favorite products that consumes as fast food all over the world.

Chicken nugget products are a reformulated meat product with breaded coatings. The major composition of nugget is chicken meat, protein from vegetarian source, gum and a fair amount of chicken skin (LUKMAN *et al*., 2009; MARIKKAR *et al*., 2011). The quality and nutritional characteristics of chicken nugget significantly affected by factors such as processing treatments, raw material and additives (YOGESH *et al*., 2013). Supplementation of meat products with protein sources, legumes, and oilseed products has the ability to reduce the cost of products (ASGAR *et al*., 2010), and to improve nutritional, functional and sensory characteristics of these product. In recent times, some efforts were conducted to formulate modified meat products by changing in the contents of fat and fatty acids, and/or by inclusion of functional ingredients into meat products or by removing or reducing the substrates that are considered as a hazard to the human health (JIMENEZ-COLMENERO *et al*., 2001; FERNANDEZ-LOPEZ *et al*., 2005). Vegetables and fruit contain more than one type of bioactive compounds. Diets with high levels of vegetables and fruit have a good protection against cancers (VALCKE *et al*., 2017) and cardiovascular disease (DOS SANTOS *et al*., 2017). Cauliflower (*Brassica oleracea* var. botrytis L.) is one of the most popular vegetables grown all over the world and has a wide diversity of uses as a dish or as an ingredient in soups or salads. Cauliflower is a good source of vitamins, folic acid as, dietary fibers, proteins, mineral elements, eg, phosphorus, magnesium manganese, potassium, and iron (AHMED AND ALI, 2013; KAPUSTA-DUCH *et al*., 2017).

The main objective of the current study was to evaluate the effect of the incorporation of different levels of cauliflower on the nutritional and sensory characteristics of chicken nuggets.

2. MATERIALS AND METHODS

Fresh, boneless, skinless, chicken breast fillets and chicken skin were obtained from poultry slaughterhouse, Giza, Egypt. Cauliflower, freshly harvested, free from insect and mechanical damage was purchased from the local market in Giza Governorate, Egypt (JANUARY, 2018). Sodium tripolyphosphate was obtained from El-Gomhouria Company for Trading Chemicals and Medical Appliances (Building 23, El-Sawah Street, Al Ameria, Cairo, Egypt. Dried bread crumbs (6.8% moisture, 12.59% protein, 3.94% fat, 0.96% ash, 0.82 fiber and 81.69% carbohydrates,) were purchased from Modern Bakeries (Rich Bake) company, 6th of October City, Giza, Egypt. Refined salt, white pepper powder, fresh garlic paste and fresh onion were obtained from local market, Giza, Egypt.

2.1. Preparation of Frozen cauliflower

The cauliflower florets were cut into small bite-sized pieces (about five cm in diameter and five cm in length), blanched for three min in boiling water containing 0.5% sodium acid pyrophosphate. Blanched cauliflower samples were drained on a stainless sieve until cold, packed in polyethylene bags, and stored at -25°C for further uses.

2.2. Chicken nugget formulations

Chicken nugget samples were prepared according to the previous procedures described by ARSHAD *et al.*, 2017, using the ingredients listed in Table 1. Chicken breasts were cut into smaller chunks (2 cm height × 2 cm width × 2 cm length). Chicken breast chunks were ground twice in meat grinder (Moulinex - Model ME605131). Frozen cauliflower and seasonings (refined salt, white pepper powder, fresh garlic paste and fresh onion) were added into the formulations (Table 1). The mixture was minced twice in the above-mentioned grinder. The mixture was weighed and formed into nugget pieces (25 g weight, 1.5 cm thick, 8 cm length, and 3.5 cm width). The formulated nuggets were pre-dusted (with wheat flour), batter-coated and breaded (with dried bread crumbs). The breaded nuggets were pre-fried in sunflower oil at 175±5°C for 59 seconds. Fried nuggets were drained on absorbent paper towels, and cooled to room temperature (25°C). The samples were packed in polyethylene bags, and stored at -25°C. Hygienic practices were applied during the preparation, packaging and storage processes of the chicken nugget products.

Table 1. Chicken nugget formulations.

Ingredients (%)	Control	Formula 1	Formula 2	Formula 3	Formula 4
Chicken breast	70	70	70	70	70
Chicken skin	20	15	10	5	-
Frozen cauliflower	-	5	10	15	20
Crushed ice	5	5	5	5	5
Refined salt	1.5	1.5	1.5	1.5	1.5
Fresh garlic paste	0.5	0.5	0.5	0.5	0.5
Fresh onion	2.5	2.5	2.5	2.5	2.5
White pepper powder	0.30	0.30	0.30	0.30	0.30
Sodium tripolyphosphate	0.20	0.20	0.20	0.20	0.20

2.3. Frying (cooking) process

Two kilograms of sunflower oil were placed in frying pan, preheated and maintained at 180±5°C for 5 minutes before frying process. Frozen chicken nuggets were fried for 5 minutes. Fried nuggets were drained on absorbent paper towels, and cooled to 50°C for sensory evaluation. Other portions of samples were allowed to cool to room temperature (25°C) before further tests.

2.4. Total phenolics, total flavonoids and antioxidant activity of cauliflower extract

2.4.1 Preparation of crude extract of frozen white cauliflower

Frozen white cauliflower was subjected to water extraction.10 g of frozen white cauliflower was extracted using 100 ml deionized water for 5 h at ambient temperature (25°C). The mixture was mixed and homogenized in stainless steel blender. The resulting mixture was filtered through Whatman No 40 paper. The collected filtrates were packed in teflon tubes and kept at -25°C for further use. Total phenolic content, total flavonoids

content and antioxidant activity by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay of cauliflower extract were determined according to the procedures described by Ahmed and Ali (2013). The content of phenolics was expressed as milligram of gallic acid equivalents per g of extract. Flavonoid contents were expressed as mg of catechin equivalents per g of extract. Antioxidant activity was expressed as the inhibition (%) of the absorbance at 515nm using a UV-Vis spectrophotometer (Lambda 9; Perkin Elmer, Shelton, CT, USA).

2.5. Physicochemical properties and nutritive value of formulated nuggets

2.5.1 Determination of chemical composition

The content of moisture, ash, fiber, protein and fat were assayed according to the official procedure described by AOAC (2005). Total carbohydrate percentage was calculated by difference. Total caloric (Kcal/100g sample) were calculated according to CHOI *et al*, 2010 as follow, for fat (9 kcal g^{-1}), protein (4.02 kcal g^{-1}), and carbohydrates (3.87 kcal g^{-1}).

2.5.2 Determination of cholesterol

Cholesterol content was determined according to the previous procedures described by TURHAN *et al.* (2007). The concentration of cholesterol was expressed as mg/100 g, dry weight basis of nugget samples.

2.5.3 pH

The pH value was determined by mixing a 10 g of nugget sample with 100 ml of deionized water. The mixture was filtered through Whatman filter paper number 1, and the pH of the filtrate was measured at room temperature (YOGESH *et al*., 2013) using a pH meter (Shanghai Second Analytical Instrument Factory, Shanghai, China).

2.5.4 Colour properties

Nugget's colour was measured using a Minolta Colourimeter (Model CR-400, Minolta, Japan, calibrated using a standard white porcelain plate L*=97.75, a*=-0.48,b*=+2.31), with a measuring area of 8 mm diameter). The Nugget samples were placed in a Transparent plate and placed directly on the path of light to determine the colour parameter values of L*, a* and b*(NGADI *et al*., 2007)

2.6. Cooking properties

2.6.1 Cooking yield

The percentage of cooking yield was calculated according to the following equation (YOGESH *et al*., 2013).

Cooking yield percentage = (weight of fried (cooked)/
weight of raw (uncooked) nugget) x 100

2.7. Sensory characteristics

Sensory evaluation of fried chicken nuggets was carried out by a fifteen of trained judges from who are belonging to Food Technology Research Institute, Agriculture Research Center, Giza, Egypt). All judges are knowledgeable about the properties of chicken nuggets and familiar with chicken and meat products. The panelists were seated in individual cabins in a temperature-controlled room at 25°C lighted by daylight fluorescent lights. Rectangular strips approximately 2×2× 4 cm^3 were served to the panelists. Chicken nugget samples were evaluated for appearance, juiciness, texture, flavour and overall acceptability by using 9-point hedonic scale, 9 = like extremely, 8 = like very much, 7 = like moderately, 6 = like slightly, 5 = neither like nor dislike, 4 = dislike slightly, 3 = dislike moderately, 2 = dislike very much and 1 = dislike extremely (YOGESH *et al*., 2013). Cups of drinking water were provided for judges to clean their mouth between samples.

2.8. Statistical analysis

Results are expressed as mean±SD. Data were statistically analyzed according to the procedures described by (GOMEZ and GOMEZ, 1984). SPSS Version 18.0 (SPSS Inc., Chicago, IL, USA) was used to analyze data. Duncan's Multiple Range mean separation was performed where the ANOVA procedure showed significance.

3. RESULTS AND DISCUSSIONS

3.1. Sensory characteristics of chicken nuggets formulated with different levels of frozen cauliflower

In order to determine the acceptability of the chicken nugget formulations, sensory evaluation tests were carried out. The transformed data of appearance, juiciness, texture, flavor and overall acceptability of cooked (fried) chicken nuggets formulated with different levels of cauliflower are presented in Table 2. The appearance scores of cooked chicken nuggets ranged from 7.50 to 8.19. Nugget samples incorporated with 20% of frozen cauliflower showed the lowest ($P \leq 0.05$) scores of appearance. However, no significant differences were observed in appearance values between control samples and those nugget samples formulated with 5, 10 and 15% of frozen cauliflower. No significant ($P \geq 0.05$) variations were detected in juiciness value between control samples formulated with 20% of chicken skin and those batches formulated with 5, 10 and 15%of frozen cauliflower as fat replacer. On the contrary, the lowest juiciness value was recorded for those samples containing 20% of frozen cauliflower. This findings attributed to the effect of frying process on reduction of the moisture content of fried food and increase the crispy and hardness of fried vegetables. A critical aspect of battered and breaded chicken products is the contrast between the crispy and oily outer layer and the soft cooked interior (DOBRASZCZYK *et al*., 2006). Crispy texture of fried food products determines the consumer acceptability. The texture of cooked chicken nugget significantly affected by the addition of cauliflower as fat replacer. Chicken nuggets formulated with various levels of cauliflower had significantly ($P \leq 0.05$) higher texture scores than control samples without cauliflower addition. The highest ($P \leq 0.05$) scores of texture were recorded for chicken nuggets incorporated with 15 and 20% cauliflower as fat replacer followed by those samples containing 10 and 5% cauliflower. On the other hand, control samples

formulated with chicken skin had significantly ($P \leq 0.05$) the lowest score of texture (7.81). Additions of carrot and sweet potato were found to be effective in improving texture scores (BHOSALE *et al*., 2011). The highest flavour scores (8.22 and 8.14) were recorded for control sample and those samples incorporated with 5% frozen cauliflower. The perception of fat in food is a complex process involving many sensory modalities (texture, aroma and flavour) (FERON AND POETTE, 2013). No significant variations were detected in flavor scores among nugget batches formulated with 10, 15 and 20%of frozen cauliflower as fat replacer. The increasing of consumer awareness about healthy foods led the efforts made by the food manufacturers to develop new items of food with positive properties. Some efforts have been made to produce alternative meat products by changing in the amounts of lipid contents, by inserting of with bioactive compounds or plant-based phytonutrients into meat products (JIMENEZ-COLMENERO *et al*., 2001; FERNANDEZ-LOPEZ *et al*., 2005). No significant variations ($P \geq 0.05$) were recorded in the overall acceptability scores among chicken nugget batches. Generally speaking, the incorporation of frozen cauliflower as fat replacer had positive effects on the acceptability and overall quality of chicken nuggets.

Table 2. Sensory characteristics of cooked (fried) chicken nuggets formulated with different levels of cauliflower (n= 15).

Parameters	Control	Formula 1	Formula 2	Formula 3	Formula 4	LSD at 0.05
Appearance	8.19^{a}±0.2	8.15^{a}±0.1	8.05^{a}±0.1	8.00^{a}±0.2	7.50^{b}±0.3	0.30
Juiciness	8.17^{a}±0.14	8.09^{a}±0.18	8.04^{a}±0.24	7.90^{a}±0.10	7.45^{b}±0.15	0.28
Texture	7.81^{b}±0.18	8.10^{ab}±0.13	8.15^{ab}±0.25	8.20^{a}±0.14	8.20^{a}±0.20	0.27
Flavor	8.22^{a}±0.10	8.14^{a}±0.06	7.80^{b}±0.12	7.69^{b}±0.15	7.56^{b}±0.18	0.25
Overall acceptability	8.14^{a}±0.16	8.12^{a}±0.14	7.98^{a}±0.10	7.92^{a}±0.09	7.51^{a}±0.14	0.33

Means in a line with different letters are significantly different ($P \leq 0.05$).
LSD, Least significant difference at $p \leq .05$ according to Duncan's multiple-range test.

3.2. Total phenolics, total flavonoid and antioxidant activity by DPPH of crude extract of frozen white cauliflower

The nutraceutical quality depends on the proximate composition, particularly, the quantity of the phenols and flavonoids. Table 3 shows total phenolics, total flavonoid and antioxidant activity by DPPH of crude extract of frozen white cauliflower. The amount of total phenolics found in crude extract of frozen white cauliflower was 6.76 mg / g. Studies found that phenolics are strictly responsible for antioxidant activity of vegetarian sources. These compounds can contribute candidly to scavenge free radicals (AHMED AND ALI, 2013; ARSHAD *et al*., 2017). The total concentration of flavonoids for crude extract of cauliflower was 1.65 mg/g. It has been proven that flavonoid compounds possess antioxidant, antimicrobial, free radicals scavenging and antitumor activities (FERNANDEZ-LOPEZ *et al*., 2005). The crude extract of white cauliflower exhibited remarkable free radicals scavenging activity was 54.9%. The high level of phenolic compounds in white cauliflower makes it as a potential ingredient for meat and chicken products to improve their nutritive value and enhance their ability to scavenge free radicals.

Table 3. Total phenolics, total flavonoid and antioxidant activity by DPPH of crude extract of frozen white cauliflower.

Properties	White cauliflower extract
Total phenolics (mg/g)	6.76±0.39
Flavonoids (mg/g)	1.65±0.08
Free radical scavenging (%)	54.9±1.08

Data are expressed as the mean±standard deviation.

3.3. Chemical compositions, energy content and cholesterol content of raw and cooked chicken nuggets formulated with different levels of cauliflower

The proximate composition of raw and cooked chicken nuggets incorporated with different levels of cauliflower is shown in Tables 4. Moisture content of nugget samples was significantly affected by addition of various levels of frozen cauliflower into chicken nugget formulas. Moisture content of raw chicken nuggets ranged from 61.73 to 66.08%. Significant differences ($p \leq 0.05$) were observed in the moisture content between nugget samples formulated with cauliflower and those control samples (without cauliflower addition). Nugget samples formulated with frozen cauliflower had significantly ($P \leq 0.05$) higher moisture content than control samples without cauliflower. The highest ($P \leq 0.05$) moisture content (66.08%) was recorded for chicken nuggets incorporated with 20% cauliflower, followed by nuggets samples containing 15% cauliflower (65.76%). The lowest content of moisture was observed for control samples (61.73%). At the same time, no significant differences ($P \geq 0.05$) in moisture content were observed among chicken nuggets incorporated with 20, 15 and 10% of frozen cauliflower as fat replacer. The increases in moisture content in nuggets incorporated with cauliflower may be attributed to the ability of cauliflower fiber to hold water. Pectic-polysaccharide-rich fiber of cauliflower has been used as a water holding agent for enhancing the quality properties of model foods (MCKEE, and LATNER, 2000). Fat content of raw chicken nuggets ranged from 0.31 to 13.20%. Fat content of chicken nugget samples was markedly affected by the addition of frozen cauliflower as fat replacer. Substitution of chicken skin with different levels of frozen cauliflower caused significant decreases in fat content of nugget samples. Fat content of chicken nuggets incorporated with 20, 15, 10, and 5% of cauliflower as fat replacer were about 42.58, 3.91, 1.96 and 1.31 times as low as in control samples which containing 20% chicken skin as source of fat, respectively. The amount of fat recovered from chicken skin ranged from 22.6 to 38.9% of the initial weight of skin, according to the extraction conditions (PIETTE *et al.*, 2001). These levels of fat representing 51.5 to 89.6% of the fat initially contained in chicken skin. Blanched cauliflower contains low levels of fat ranged from 1.93 to 2.20% of the dry weight. (AHMED AND ALI, 2013), this low level of fat in cauliflower contributes well in reducing fat content of chicken nuggets formulated with different levels of cauliflower. No significant differences ($P \geq 0.05$) in protein content were observed among chicken nuggets formulated with different levels of cauliflower and control samples without cauliflower addition.

Table 4. Chemical compositions, energy content and cholesterol content of raw and cooked chicken nuggets formulated with different levels of frozen cauliflower.

Parameters	Control	Formula 1	Formula 2	Formula 3	Formula 4	LSD at 0.05
Chemical composition of raw chicken nuggets *(n= 5)*						
Moisture (%)	61.73^{c}±0.63	63.31^{b}±0.80	65.08^{a}±0.92	65.^{76a}±1.01	66.08^{a}±0.79	1.514
Fat (%)	13.20^{a}±0.72	10.01^{b}±0.11	6.71^{c}±0.31	3.37^{d}±0.29	0.31^{e}±0.08	0.688
Protein (%)	17.85^{a}±1.03	17.90^{a}±2.02	17.95^{a}±3.01	18.09^{a}±1.08	18.23^{a}±1.03	3.30
Ash (%)	1.68^{b}±0.21	1.76^{b}±0.17	1.88^{ab}±0.22	1.95^{ab}±0.09	2.17^{a}±0.13	0.307
Crude fiber (%)	0.92^{e}±0.07	1.81^{d}±0.13	2.47±^{c}0.02	3.09^{b}±0.19	3.78^{a}±0.24	0.322
Carbohydrates (%)^{b}	4.62^{e}±0.28	5.21^{d}±0.36	5.91^{c}±0.13	7.74^{b}±0.21	9.43^{a}±0.32	0.497
Energy content (Kcal per 100 g)	208.42^{a}±0.71	182.20^{b}±0.38	155.41^{c}±0.85	133.00^{d}±0.77	112.56^{e}±0.32	1.16
Cholesterol(mg/ 100g)	113.89^{a}±1.98	86.79^{b}±2.08	63.42^{c}±1.31	29.11^{d}±0.94	ND^{e}	2.65
Chemical composition of cooked (fried) chicken nuggets (*n= 5*)						
Moisture (%)	51.74^{c}±0.94	53.70^{b}±1.08	55.42^{ab}±0.87	56.24^{ab}±0.66	56.26^{a}±0.82	1.80
Fat (%)	20.31^{a}±0.17	16.53^{b}±0.31	12.81^{c}±0.33	9.97^{d}±0.69	6.85^{e}±0.43	0.76
Protein (%)	19.59^{a}±1.19	19.60^{a}±1.68	19.92 ^{a}±2.02	20.13^{a}±0.69	20.16^{a}±1.87	2.85
Ash (%)	1.79^{b}±0.16	2.15^{a}±0.23	2.28^{a}±0.08	2.46^{a}±0.17	2.51^{a}±0.12	0.292
Crude fiber (%)	1.08^{e}±0.09	1.76^{d}±0.11	2.61^{c}±0.06	3.07^{b}±0.10	3.84^{a}±0.14	0.18
Carbohydrates (%)^{b}	5.49^{e}±0.41	6.27^{d}±0.37	6.96^{c}±0.19	8.13^{b}±0.32	10.38^{a}±0.09	0.53
Energy content (Kcal per 100 g)	282.78^{a}±0.69	251.82^{b}±0.47	222.29^{c}±0.76	202.11^{d}±0.47	182.86^{e}±0.39	1.04
Cholesterol (mg)	125.50^{a}±1.07	94.86^{b}±0.67	68.12^{c}±2.54	32.58^{d}±1.46	ND ^{e}	2.59

[a]Data are expressed as the mean±standard deviation. Values followed by the same letter [(a-e)] are not significantly different (P≤0.05).

[b]By difference.

[c]LSD, least significant difference.

[d]ND, refers to not detected.

Generally, control samples contain considerably lower ash content than those samples formulated with frozen cauliflower ($P \leq 0.05$). Ash content of formulated chicken nuggets ranged from 1.68 to 2.17%. The highest ($P \leq 0.05$) ash content (2.17%) was recorded for chicken nuggets incorporated with 20% cauliflower. However the lowest content (1.68%) was recorded for control sample without cauliflower addition. Ash content of chicken nuggets formulated with 20, 15 and 10% of cauliflower as fat replacer were about 1.29, 1.16 and 1.11 times as great as in control samples which containing 20% chicken skin as source of fat, respectively. Fiber content of formulated chicken nuggets ranged from 0.92 to 3.78%. Nuggets formulated with different levels of cauliflower had higher levels of fiber than control samples. Control samples without cauliflower addition had significantly ($P \leq 0.05$) the lowest level (0.92%) of fiber content. The highest ($P \leq 0.05$) content of fiber (3.78%) was recorded for chicken nuggets formulated with 20% cauliflower followed by those samples formulated with 15% cauliflower (3.09%). This finding attributed to the fact

that cauliflower is a good source of dietary fiber. In this regard, Ahmed and Ali, 2013 reported that blanched cauliflower florets contain 11.52 -12.74% crude fiber on a dry weight basis. Carbohydrate content of nugget samples varied from 4.62 to 9.43%. Control sample had lower levels of carbohydrates than those samples formulated with different levels of cauliflower. Carbohydrate content of chicken nuggets incorporated with 20, 15, 10, and 5% of frozen cauliflower as fat replacer were about 2.04, 1.67, 1.29 and 1.12 times as high as in control samples containing 20% chicken skin as source of fat, respectively. Carbohydrate content of blanched cauliflower florets was estimated to be about 50% of the total dry weight (AHMED AND ALI, 2013). Energy content (Kcal) of formulated chicken nuggets ranged from 112.56 to 208.42 per 100g. The highest content of energy (208.42 Kcal) was recorded for control sample formulated with 20% of chicken skin as source of fat. The lower values of calories were recorded for those samples formulated with different levels of cauliflower. Calories (Kcal) of chicken nuggets incorporated with 20, 15, 10, and 5% of cauliflower as fat replacer were about 1.85,1.65, 1.34 and 1.14 times as low as in control samples which containing 20% chicken skin as source of fat, respectively. Foods with a lower level of fat provide fewer calories than foods with a higher level of fat. These findings attributed to the fact that one gram of lipids provides 9 kcal, while one gram of protein or carbohydrates provides 4 kcal. Cholesterol content (mg/100 g) of formulated chicken nuggets ranged from not-detectable level to 113.89. Control samples formulated with chicken skin contain the highest levels (113.89 mg/100 g) of cholesterol. Skin of poultry has the greatest cholesterol level compared with poultry meat or poultry fat. In this regard, Mendez-Lagunas *et al*., 2015 reported that the cholesterol content of chicken skin was 131 mg/100 g of raw wet tissue. BRAGAGNOLO (2009) reported also that raw poultry meat has approximately 27 to 90 mg cholesterol/100 g, while cooked poultry meat has around 59 to 154 mg/100 g. Samples formulated with various levels of cauliflower had significantly ($P \leq 0.05$) lower levels of cholesterol than control samples without cauliflower addition. Cholesterol level of the formulated chicken nuggets can be arranged in the decreasing order as follows: control samples > nuggets with 5% cauliflower > nuggets with 10% cauliflower > nuggets with 15% cauliflower > nuggets with 20% cauliflower. As cauliflower florets are vegetarian diets, they are eaten as cholesterol free diets. Several studies proved that incorporation of vegetarian based foods into diets could promote health and reduce the risk of cholesterol and heart disease (SADLER, 2004). Moisture content of cooked nuggets was considerably lower than that of raw (un-cooked) for all the nugget samples. This decrease in water content may be attributed to the fact that frying process resulted in water expulsion from chicken samples where the frying temperature is higher than the boiling temperature of water. Moisture content of cooked chicken nuggets ranged from 51.74 to 56.24%. The lowest moisture content was observed for cooked control nuggets formulated without cauliflower addition. Cooked nuggets formulated with different levels of cauliflower contain higher level of moisture than control samples. This finding attributed to the capacity of cauliflower fibers to hold water during frying process. Fat content of cooked-fried chicken nuggets ranged from 6.85 to 20.31%. Cooked control samples contain significantly ($P \leq 0.05$) the highest level of fat (20.31%). Fried items uptake fat during frying process, which is a health concern as excessive lipid consumption can contribute to obesity and heart disease (WOLFRAM 2003). Cooked nuggets formulated with different levels of frozen white cauliflower have lower amounts of fat than control samples formulated with 20% chicken skin. Fat content of cooked chicken nuggets formulated with 5, 10, 15 and 20% of frozen white cauliflower were about 1.22, 1.58, 2.03 and 2.96 times as low as in control samples formulated with 20% chicken skin as source of fat, respectively. This finding attributed to the fact that certain cruciferous

vegetables of the genus *Brassica* including cauliflower, contain little fat and energy (MUKHERJEE *et al.*, 2008). A critical element of deep-fat fried food is the high level of fat that is absorbed during the frying process, reaching in some cases up to 40% of the total weight of fried product. Several investigations have reported that excess consumption of fat is a key dietary contributor to coronary heart disease and perhaps cancer of the breast, colon, and prostate (KOENE *et al.*, 2016). No significant differences ($P \geq 0.05$) in protein content were observed among cooked chicken nuggets. Ash content of cooked nuggets varied from 1.79 to 2.51%. Increased ash content was noticed in all the cooked nuggets when compared to raw chicken nuggets. Losses of moisture, occurring during frying process resulted in higher ash content in cooked nuggets as compared to the uncooked nuggets Cooked nuggets formulated with different levels frozen white cauliflower contain greater levels of ash than control samples without cauliflower addition. No significant ($P \geq 0.05$) differences in ash content were observed among cooked samples formulated with various amounts of cauliflower. Fiber content of cooked nuggets ranged from 1.08 to 3.84%. As expected nuggets formulated with frozen white cauliflower had higher levels of fibers than control sample without cauliflower addition. Fiber content of cooked nuggets formulated with 20, 15, 10, and 5% of frozen white cauliflower were about 3.55, 2.84, 2.41 and 1.62 times as high as in control samples containing 20% chicken skin as source of fat, respectively. Incorporation of dietary fiber into foods may be an effective power for enhancing functional and nutritional properties as reported earlier, There is a dramatic rise in the demand of food items with high levels of fiber and low levels of lipids as they are very efficient in lowering of fat absorption by the product, particularly fatty acids and cholesterol (BORDERIAS *et al.*, 2005), that could be useful in reducing obesity. Cooked chicken nuggets formulated with frozen white cauliflower had significantly ($P \leq 0.05$) higher values of carbohydrates than control samples without cauliflower addition. The highest value (10.38%) of carbohydrates was recorded for nugget samples incorporated with 20% of frozen cauliflower. Energy content (Kcal) of cooked chicken nuggets ranged from 182.86to 282.78. As expected, control samples are higher in energy content than those samples formulated with frozen white cauliflower. In the current study, reducing the content of chicken skin from 20% to 0% and replacing it with frozen-blanched cauliflower caused significant ($p \leq 0.05$) decreases in energy content of cooked nuggets. At a 25% chicken skin replacement, the energy content was reduced by 10.94%, while the nuggets sample in which 100% chicken skin was replaced, the energy content was reduced by 35.33%. The inverse relation between fat content and energy content was observed in different types of meat products formulated with dietary fiber (MÉNDEZ-ZAMORA *et al.*, 2015; KEENAN *et al.*, 2014). Cholesterol content (mg/100 g) of cooked nuggets varied from not-detectable concentration to 125.50. Frying process caused significant ($P \leq 0.05$) increases in cholesterol content of formulated chicken nuggets. The cholesterol content in the control (cooked) sample was the highest, amounting 125.50 mg / 100 g. Cholesterol levels were higher in fried chicken nuggets compared to un-fried samples. These increases in cholesterol content may attributed to the loss of moisture content during frying process, which leading to changes in cholesterol levels (PADIANI *et al.*, 2002; TURHAN *et al.*, 2007). In this regards, MEDINA *et al*. (2015) reported that frying process caused significant increases in cholesterol content of the refrigerated nuggets; they added also that such changes in cholesterol as a consequence of frying could also be related to the slight dehydration caused by the heat treatment. The cholesterol content of chicken nuggets decreased with respect to the incremental addition of frozen cauliflower as a chicken skin substitute ($P \leq 0.05$), cholesterol content of cooked chicken nuggets supplemented with 5, 10 and 15% of frozen white cauliflower were about 1.32, 1.84 and 3.85 times as low as in

control samples incorporated with 20% chicken skin as source of fat, respectively. Cholesterol has not been detected in nugget samples formulated with 20% frozen white cauliflower as fat replacer. Incorporation of hydrated potato flakes as a type of carbohydrates-based fat replacers caused significant reduction in cholesterol content of beef patties (ALI *et al.*, 2011).

3.4. Physicochemical characteristics of cooked chicken nuggets formulated with different levels of frozen cauliflower

3.4.1 pH

No significant differences in pH values between control samples and those samples incorporated with different levels of frozen cauliflower. This means that the reduction in chicken skin content and the addition of frozen white cauliflower did not affect ($P \leq 0.05$) the pH of formulated chicken nuggets (Table 5). KIM *et al.*, 2015 showed that the pH has not been significantly altered for chicken nugget formulated with various contents of chicken skin and wheat fiber mixture. The value of pH affects the ability of meat and meat products to retain moisture. Water-holding capacity (WHC) of meat reaches a minimum when the pH value is at the isoelectric point of meat proteins. WHC is an important factor for meat products as it affects both the yield and the quality attributes of the end product (YOGESH *et al.*, 2013).

Table 5. pH, cooking yield and color measurements of cooked chicken nuggets formulated with different levels of frozen cauliflower (n= 5)

Parameter	Control	Formula 1	Formula 2	Formula 3	Formula 4	LSD at 0.05
pH	6.1^{a}±0.01	6.1^{a}±0.01	6.1^{a}±0.03	6.1^{a}±0.02	6.1^{a}±0.04	0.029
Cooking yield	93.44^{b}±1.02	94.28^{ab}±0.90	95.60^{a}±0.51	96.4^{a}±0.38	96.64^{a}±0.28	1.24
L (lightness)	69.55^{a}±1.2	67.10^{ab}±1.8	66.60^{ab}±0.90	64.90^{b}±2.2	64.80^{b}±0.60	2.67
a (redness)	4.50^{b}±0.1	4.61^{b}±0.3	4.61^{b}±0.4	4.73^{b}±0.2	7.74^{a}±0.30	0.50
b (yellowness)	29.50^{a}±0.9	29.50^{a}±1.10	30.00^{a}±0.75	30.05^{a}±1.3	30.10^{a}±0.80	1.80

Data are expressed as the mean±standard deviation. Values followed by the same letter are not significantly different ($P \leq 0.05$).
LSD, least significant difference.

3.4.2 Cooking yield

Table 5 illustrates the impact of substitution of chicken skin with different proportions of frozen white cauliflower on the cooking yield of chicken nuggets. Cooking yield of formulated chicken ranged from 93.44 to 96.64%. Generally, these high values of cooking yield may be attributed to the fact that significant amounts of fat were absorbed from frying media during cooking (frying) process (MELLEMA, 2003). Cooked chicken nuggets formulated with frozen white cauliflower had significantly ($P \leq 0.05$) higher cooking yield values than control samples without cauliflower addition. The lowest value (93.44%) of cooking yield was recorded for control samples. The addition of frozen cauliflower had

positive effects for the cooking yields of the nuggets (Table 5). These increases in cooking yield may be attributed to the ability of cauliflower florets fibers to hold moisture and lipids during frying process. It was proved that the yield percentages of cooking depends on cooking temperature, time of cooking, the amounts and type of additive, as well as the type of fat and dietary fiber in the meat products (CHOI *et al.*, 2014; KEENAN *et al.*, 2014; MÉNDEZ-ZAMORA *et al.*, 2015).

3.4.3 Color measurement of chicken nuggets formulated with different levels of frozen cauliflower

Measurement of texture and colour using instrumental methods can provide quantified indicators for determining processing operations to improve and enhance quality properties of finished products (NGADI *et al.*, 2007). The surface color is one of the major physical attributes that determine consumer's acceptability of finished products (SYUHAIRAH *et al.*, 2016). The L* values of formulated cooked nuggets ranged from 64.80 to 69.55. The L* value of control chicken nuggets was higher ($P \leq 0.05$) than chicken nuggets formulated with different levels of frozen cauliflower (Table 5). The lightness values of nuggets were clearly affected ($P \leq 0.05$) by the percentage of incorporated fats. Colour lightness of nuggets significantly ($P \leq 0.05$) reduced with decreasing the content of chicken skin (Table 5). The results showed also that the increase in level of cauliflower incorporation results in linear decrease of the lightness values of nugget samples. The lower values were recorded for nugget samples incorporated with 15 and 20% of frozen white cauliflower. However, those samples formulated with 5 and 10% of frozen white cauliflower as fat replacer showed slight decreases in lightness values,which might be due to the presence of moderate amount (15 and 10%) of chicken skin. The a* value of formulated cooked nuggets ranged from 4.50 to 7.74. The addition of frozen cauliflower at 5, 10 and 15% as fat replacer had no significant effect on the a* value of the formulated samples. Nuggets samples formulated with 20% cauliflower as fat replacer had significantly the highest value of redness (a* value). Redness may not be a desirable colour in fried food products in general (KROKIDA *et al.*, 2001). However redness in cooked meat products is a desirable factor for consumer preferences (YOGESH *et al.*, 2013). No significant ($P \geq 0.05$) differences in b* value (yellowness) were found among formulated chicken nugget samples (Table 5).

4. CONCLUSION

In conclusion, sensory evaluation results revealed that chicken nuggets supplemented with different levels of cauliflower had overall palatability that was similar to control samples with 20% chicken skin. Ash, fiber and carbohydrates contents in chicken nuggets formulated by incorporating 20% of frozen cauliflower were higher compared to control samples without cauliflower addition. It was observed also that the addition of cauliflower reduced the fat content of reformulated nuggets. The highest cooking yield was found in the sample containing 20% of cauliflower as fat replacer.

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PAPER

MICROENCAPSULATION OF *ELAEAGNUS MOLLIS* OIL TO ENHANCE THE OXIDATION STABILITY OF POLYUNSATURATED FATTY ACIDS: THE INTERACTION BETWEEN FATTY ACIDS AND WALL MATERIALS

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ABSTRACT

Our study deals with the protective effect of gelatin-acacia as a wall material on *Elaeagnus mollis* oil (EMO), the core material with over 45% polyunsaturated fatty acids. We found that 2.0% of the gelatin-acacia complex was an ideal concentration for facilitating the microencapsulation of EMO. The ideal ratio of EMO/wall materials would be 1:1. As a result, the microencapsulated EMO successfully underwent the spray-drying treatment because of close hydrogen bonding between EMO and the gelatin-acacia complex. Moreover, no spoilage was observed for the heated EMO microcapsules during storage.

Keywords: polyunsaturated fatty acids, storage stability, appropriate ratio, hydrogen bonds

1. INTRODUCTION

The seeds of *Elaeagnus mollis*, a tree species naturally distributed in northern China, are annually harvested to produce edible woody oil by local people (WANG *et al*., 2012). *Elaeagnus mollis* oil (EMO) is reported to contain higher levels of polyunsaturated fatty acids (PUFAs), especially linoleic acid, which can be helpful in preventing heart diseases (ZHANG, 2008; LIU, Low & Nickerson, 2015; KAN *et al*., 2017). The local population not only utilizes the EMO but also sells it for monetary benefits. However, one hurdle for the EMO storage and usage is its shorter shelf life owing to the presence of higher PUFAs levels (TEIXEIRA *et al*., 2004).

It is known in general, that essential PUFAs such as omega-3 and omega-6 are more susceptible to oxidation and subsequently produce undesirable flavors (AUGUSTIN *et al*., 2010; JOHNSON and DECKER, 2015). Hydrogenation is the most feasible means to settle the problem of high PUFA-containing oils, but the process of oil hydrogenation yields more trans fatty acids (ZHAO *et al*., 2018).

Microencapsulation is an alternative technology adapted in the food industry because of its safety (AZIZ *et al*., 2014; GOMEZ *et al*., 2016; SHAMAEI *et al*., 2017; ZHOU *et al*., 2017). Thus, they might provide the effective solution of preventing PUFA oil-mediated oxidation spoilage. Gelatin and acacia are usually selected as preparation material for microcapsules due to their excellent emulsifying and film-forming properties (UMESHA *et al*., 2013; LIU *et al*., 2015; ANVARI *et al*., 2015; HCF *et al*., 2013). However, the difference in PUFA content strongly influences the microencapsulation efficiency of the wall materials (SHAMAEI *et al*., 2017; ZHOU *et al*., 2017). So design the appropriate wall material concentration and wall material/core material ratio to improve the microencapsulation efficiency of EMO as a representative of PUFA content over 45% should be significant.

Therefore, the objectives of this study were to (1) examine the effect of gelatin-acacia in EMO protection and deduce the optimal concentrations of gelatin-acacia, as well as the EMO/gelatin-acacia ratio; (2) investigate the interaction between EMO and gelatin-acacia; and (3) check the tolerance of EMO microcapsules towards heat treatment and their storage stability.

2. MATERIALS AND METHODS

2.1. EMO preparation

Supercritical carbon dioxide extraction (SPE-ED, Applied Separations, Allentown, USA) was used to produce EMO. Seeds of *Elaeagnus mollis* were dried in an electro-thermostatic drying oven (GHG-9420A, Yi-heng Science, Shanghai, China). Then, 200 g seed powder *of Elaeagnus mollis* (d = 0.42 mm) was fed to a 300-ml extraction vessel. The extraction of EMO by supercritical carbon dioxide technique was carried out at 38°C for 6 h with a pressure of 30 Mpa and solvent flow rate of 3.0 L/min.

2.2. Concentration design of gelatin-acacia

Gelatin and acacia (Jinchun Biochemical Technology Ltd, Shanghai, China) were dissolved in distilled water and heated to 50°C until complete dissolution. Gelatin and acacia were mixed in a ratio of 1:1 (v/v). Gelatin-acacia mixtures of 1.0%, 1.5%, 2.0%, 2.5%, and 3.0% were blended with EMO. The ratio of each of the gelatin-acacia complex and EMO sample

was 3:2. Each combination of gelatin-acacia and EMO was emulsified using Ultra-Turrax homogenizer (FA 25, Fluko Ltd, Shanghai, China) at 12000 r/min for 4 min. The emulsions, kept at 40°C, were adjusted to pH of 4.0 and then stirred for 20 min until coacervation. Next, the pH of the emulsions was adjusted to 6.0 after their temperatures dropped within the range of 10°C and 20°C. At this moment, 0.25 g transglutaminase (calculated as gelatin per gram) (Leveking Bio-Engineering Ltd, Shenzhen, China) was added to the emulsions. These emulsions were stirred for 3 h to complete the cross-linking and form microcapsules. Those microcapsules were kept for a while to remove some of the supernatant, and then filtered with a vacuum pump, and washed with an appropriate amount of distilled water to obtain the wet capsules, which were then freeze-dried to obtain the microcapsule products. The dry microcapsules obtained were used to determine the optimal concentration of gelatin-acacia.

2.3. Ratio design of EMO/gelatin-acacia

The ratios of EMO to the mixtures of gelatin-acacia (used at a concentration of 1.5%) were designed according to the ratios of 1:2, 2:3, 1:1, 3:2, and 2:1. The combinations of EMO and gelatin-acacia were emulsified using Ultra-Turrax homogenizer (FA 25, Fluko Ltd, Shanghai, China) at 12000 r/min for 4 min. They were then kept at 40°C, were adjusted to a pH of 4.0, and stirred for 20 min until coacervation. Moreover, the emulsions' pH values were adjusted to 6.0 after their temperatures lowered between 10°C and 20°C. Next, 0.25 g transglutaminase (calculated in gelatin per g) was added to the emulsions to complete the cross-linking process. The microcapsules after cross-linking were then freeze-dried to obtain the dry microcapsule products, which were used to determine the optimal ratio of EMO/gelatin-acacia.

2.4. Morphological observation

The shapes of the EMO microcapsules were observed by a polarizing microscope (XSP-1813, Shanghai optical instrument factory, Shanghai, China), and their images were captured using a digital camera (DSC-W800, Sony, Japan).

2.5. Microencapsulation efficiency

One gram of EMO microcapsules were dissolved in 50 ml of petroleum ether for 1 min and repeated the operation for two times then went through filtration. The filtrate was then evaporated and dried at 60°C (KAN *et al*., 2017) until constant weight was achieved to obtain the surface oil. The total oil referred to amount of the oil that was used to prepare encapsulation products. The Microencapsulation efficiency (ME) was calculated as follows (XIAO *et al*., 2014):

$$ME = (1 - (\text{surface oil} / \text{total oil})) \times 100\ \% \qquad (1)$$

2.6. Microencapsulation yield

Microencapsulation yield was calculated according to the formula adapted from previously published literatures follows XIAO *et al*. (2014):

$$MY = (W_1/W_2) \times 100\ \% \quad (2)$$

Where W_1 represents the weight (g) of the freeze-dried microcapsules products, and W_2 stands for the initial weight (g) of core and wall materials used.

2.7. Thermal stability analysis of EMO microcapsules

2.7.1 Spray-drying treatments

Gelatin-acacia encapsulated EMO and non-encapsulated EMO were treated via spray dryer (B290, Buchi Ltd, Switzerland) for the evaluation of heat resistance. Non-encapsulated EMO treated with spray-drying was segregation but not as powder (MAURER *et al*., 2016). Spray-drying parameters were carried out using the following condition settings: feed rate of 7.5 ml/min, inlet temperature of 140°C, and outlet temperature of 80°C.

2.7.2 Polyunsaturated fatty acid analysis of EMO and EMO microcapsules

The PUFA composition and content of EMO and EMO microcapsules after spray-drying were performed by Gas Chromatography and Mass Spectrometry (GC-MS) (GC-MS-2010, Shimadzu corporation, Shimadzu, Japan) with the help of silica capillary column Rtx-5MS (0.25 mm × 30 m × 0.25 μm). The operation conditions were as follows: injection temperature of 280°C, sample quantity of 1 μl, Split ratio of 40:1, He as carrier gas, and speed of 1 ml/min. The PUFAs in EMO were identified by more than 95% matching with the retention time of MS database from a commercial library (NIST ver. 2011 library & NIST11s ver. 2011 library). The concentration of PUFAs was calculated by area ratio method. The test of PUFAs in spray-dried EMO microcapsules referred to the EMO extracted from the spray-dried microcapsules. The EMO was extracted from the spray-dried powder by petroleum ether. The extraction process was repeated until no EMO was leached then the extracted EMO went through filtration. The filtrate was then evaporated to remove the petroleum ether.

2.7.3 Micro-structure observation

Micro-structure of spray-drying EMO microcapsules were visualized and obtained via scanning electron microscopy (SEM). The powder of spray-dried EMO microcapsules was pasted onto a piece of double sided tape, and then a thin gold layer was sprayed onto it. SEM (S-3400N II, Hitachi High technologies, Tokyo, Japan) was operated at 5 kV. The imaging visuals of representative samples were chosen for taking pictures.

2.7.4 Analysis of vitrification temperature of EMO microcapsules

DSC (DSC100, TA instruments, New Castle, USA) was employed to determine the vitrification temperature of spray-dried EMO microcapsules. Samples, placed into DSC100, were heat-treated from 25°C up to 90°C at an increasing rate of temperature i.e. 5°C/min for DSC analysis.

2.7.5 Interaction between gelatin-acacia and EMO

All samples from gelatin, acacia, spray-dried EMO microcapsules, EMO, and the blank were characterized by FTIR spectroscopy (Spectrum100D, PerkinElmer Company, America) in the wavelength range of 0 to 4000 cm^{-1}. The experimental data were explained according to the published references to determine the interaction between gelatin-acacia and EMO (XIAO *et al*., 2014; BUĞRA, 2012; MAJI and HUSSAIN, 2010).

2.7.6 Computational docking

All computational work was performed by Discovery Studio 2017R2 software (Accelrys Software Inc., USA). The simulation model of interaction between gelatin-acacia and EMO was built using the "Receptor-Ligand Interaction" module. The structural details of gelatin were uncertain but it shared similarity to collagen (HONGO *et al*., 2001; JIRAVANICHANUN *et al*., 2006; ZONG *et al*., 2005). Therefore, the structure of collagen adhesin from *S. aureus*was used as an alternative template in this simulation. Linoleic acid, being the most abundant PUFA in EMO, was used during simulation while alpha-L-arabinopyranose was chosen as the representative of acacia. The structure of linoleic acid (Pubchem CID: 5280450), alpha-L-arabinopyranose (Pubchem CID: 439731), and collagen adhesin from *S. aureus* (PDB ID: 2F68) were downloaded from Pubchem database.

2.8. Storage stability of EMO microcapsules

2.8.1 Effect of temperature on the oxidation of EMO microcapsules

Spray-dried EMO microcapsules and EMO (as control) were stored without light at 4°C, 20°C, and 37°C. The peroxide value (PV) of all samples was determined each week for 5weeks. PV was determined according to AOAC (1995) method. First of all, the EMO from the spray-dried EMO microcapsules was extracted by petroleum ether. Next, 3.0g EMO and EMO from the spray-dried EMO microcapsules were placed into Erlenmeyer flasks. Then, 30 ml chloroform-acetic acid mixture (2:3 v/v) and 1.0 ml KI solution, each, were added to the flasks to mix with non-encapsulated EMO or extracted EMO from spray-dried microcapsules. The mixtures, after being left for 5 min in a dark place, were then blended with 100 ml distilled water. The solutions were titrated with sodium thiosulfate and starch indicator. PV was calculated using the following formula:

$$PV = (A \times N \times 1000)/E \quad (3)$$

Where A represents the volume (ml) of sodium thiosulfate solution required for the samples, N represents the normality of thiosulfate, and E means the mass (g) of the EMO or EMO which extracted from the spray-dried EMO microcapsules weighed.

2.8.2 Effect of light on the oxidation of EMO microcapsules

Spray-dried EMO microcapsules and EMO (as control), which were stored at room temperature, were treated with the presence and absence of light (darkness). Each week, the PV of all samples was evaluated until the 5th week. PV was determined as described in the previous section.

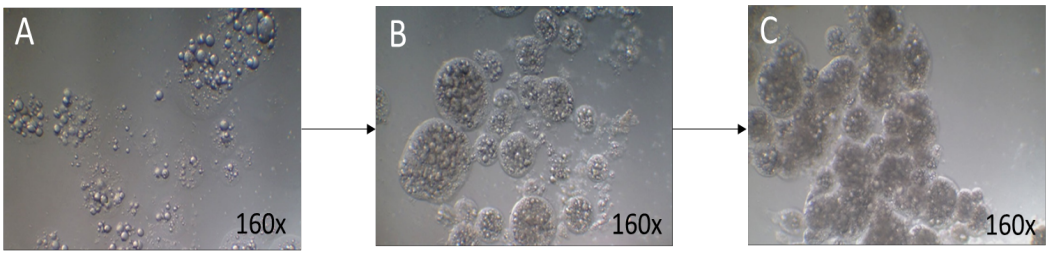
2.9. Statistical analysis

All experiments and analyses were carried out in triplicate, and the values were represented as the mean values. Microsoft Office Excel 2010 and SPSS (17.0) system software were used for data analysis.

3. RESULTS AND DISCUSSION

3.1. The optimal ratio of gelatin-acacia on EMO

Fig. 1 shows the morphological properties of EMO microcapsules in the presence of various gelatin-acacia concentration complexes. It was seen that if the concentration of gelatin-acacia was 1.0% (Fig. 1 A), the EMO microcapsules were irregularly shaped, and the microencapsulation efficiency (ME) and microencapsulation yield (MY) of EMO were 56.43% and 44.94%, respectively (Fig. 1 D). Use of 1.5% gelatin-acacia concentration raised the ME and MY values of EMO (Fig. 1 D). Besides, the EMO microcapsules formed a globular structure. The maximal ME (69.85%) and MY (67.19%) of EMO occurred when the gelatin-acacia concentration was 2% (Fig. 1-D). In this case, EMO microcapsules were homogeneous and multinuclear in shape. Higher amount of EMO was encapsulated and no adhesion took place between EMO microcapsules (Fig. 1 B). However, ME and MY of EMO decreased if gelatin-acacia concentration was further increased to 2.5% (Fig. 1 D). Use of 3.0% gelatin-acacia concentration caused the ME of EMO to quickly fall to 51.39% (Fig. 1 D). The appearance of EMO microcapsules was highly irregular and they adhered strongly to each other (Fig. 1 C).
Fig. 2 deals with the effects of EMO/gelatin-acacia ratio on the morphological properties of EMO microcapsules. It was seen that 1:2 of EMO/gelatin-acacia achieved a good ME and MY score 70.74% and 45.12%, respectively (Fig. 2 D). However, this was accompanied by an irregular shape, and no sufficient EMO was encapsulated (Fig. 2 A). A 2:3 ratio of EMO/gelatin-acacia enabled ME of EMO to reach the maximum (72.92%) (Fig. 2 D). Nevertheless, the EMO microcapsules were unable to achieve the morphological spherical structure. Interestingly, when the ratio was 1:1, we observed a multinuclear outer-shape of EMO microcapsules with homogeneous shell thickness and appropriate oil content (Fig.2-B). The ME and MY of EMO were 72.01% and 49.67% respectively (Fig. 2 D). Upon using 3:2 of EMO/gelatin-acacia, the shape became irregular, and excessive oil was entrapped. When this ratio was 2:1, the highest MY of EMO occurred but the oil secreting from the shell caused the microcapsules to get aggregated (Fig. 2 C-D).



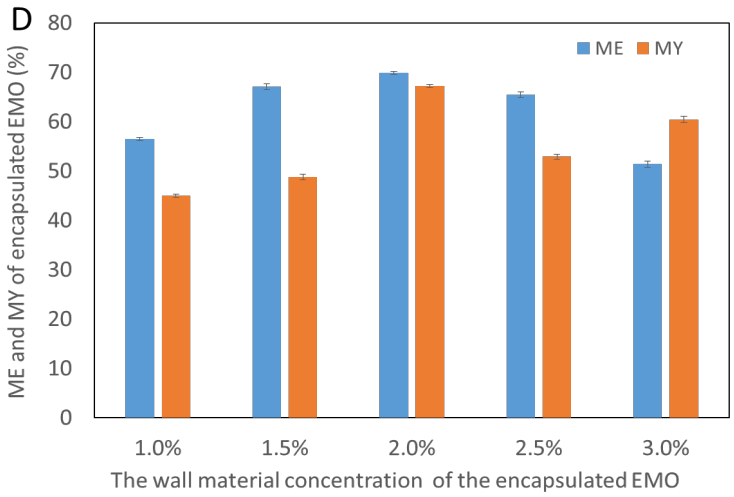


Figure 1. Gelatin-acacia microcapsules observed by polarizing microscope (160×magnification) as a function to determine the optimal concentration: (A) 1.0% of gelatin-acacia, (B) 2.0% of gelatin-acacia, (C) 3.0% of gelatin-acacia, (D) shows the microencapsulation efficiency (ME) and microencapsulation yield (MY) of *Elaeagnus mollis* oil (EMO) microcapsules prepared with different concentration of gelatin-acacia.

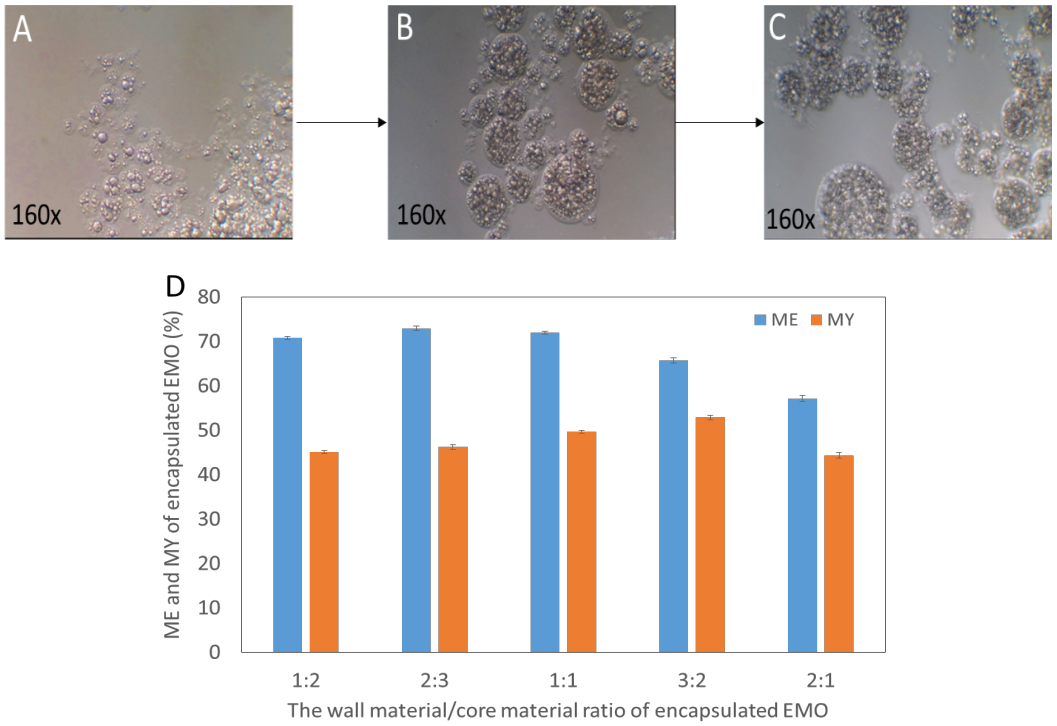


Figure 2. Gelatin-acacia microcapsules observed by polarizing microscope (160×magnification) as a function to determine the optimal ratio of EMO/gelatin-acacia: (A) EMO/gelatin-acacia in 1:2, (B) EMO/gelatin-acacia in 1:1, (C) EMO/gelatin-acacia in 2:1, (D) shows the microencapsulation efficiency (ME) and microencapsulation yield (MY) of *Elaeagnus mollis* oil (EMO) microcapsules prepared with different ratio of EMO/gelatin-acacia.

An appropriate gelatin-acacia combination and proper EMO/gelatin-acacia proportions are clearly required for successful encapsulation. In case of the oil containing more than 45% of PUFAs, the experimental data showed that high ME and MY of EMO could hardly be achieved with lower wall material concentration (Fig. 2 D). The resulting wall would be quite weak, reflecting poor storage ability. If gelatin-acacia concentration was too high, globular shape was hardly formed due to strong adherence between microcapsules (Fig.1-C). Researchers have demonstrated that increase in the wall materials' concentration leads to enhanced MY and ME of oil, though the spherical multinuclear microcapsules would gain more irregularity (XIAO *et al*., 2014; DONG *et al*., 2007). Of course, large amount of oil existing in emulsion also results in aggregation among microcapsules because of high surface oil content. AZIZ *et al*. (2014) confirmed this finding and proposed/speculated that the ratio of oil/wall influences the internal structure of microcapsules. The experiment results demonstrated that increase in EMO/gelatin-acacia ratio for a given wall material concentration caused the EMO microcapsules to enlarge with weaker walls (Fig. 2 C). Therefore, 2.0% gelatin-acacia complex as a carrier plus 1:1 ratio of EMO/gelatin-acacia significantly minimizes the loss of the woody oil.

3.2 Thermal stability analysis of EMO microcapsules

3.2.1 Polyunsaturated fatty acids change before and after heat stress

To evaluate the tolerance of the EMO microcapsules towards elevated temperature, firstly spray-drying was used to process them. The fatty acids of EMO and spray-dried EMO microcapsules are illustrated in Table 1. 11 kinds of fatty acids had been identified in EMO and EMO microcapsules. 4 kinds of those fatty acids were PUFAs, and their relative content was up to 46.10%. Omega-6 was the main PUFA in EMO, and its relative content was 45.09%. The content of Omega-6 of EMO microcapsules was 44.99% upon heating. The PUFA content showed no significant different between EMO and EMO microcapsules (p>0.05). Data from PUFAs composition change proved that 2% of the gelatin-acacia and 1:1 of EMO/gelatin-acacia ratio ensured that the composition and content of PUFAs of EMO were unaffected upon heating.

3.2.2 Micro-structure observation

Micro-structure observation of EMO microcapsules has been shown in Fig. 3. It was seen that the shape of EMO microcapsules was spherical. Moreover, there were visible wrinkles over their surface, indicating that wall materials perfectly protected core materials. The wrinkles appeared due to the high temperature in the drying chamber, wherein the very fine atomized droplets are subjected to a maximal drying rate to withdrawal of water from them (RAJABI *et al*., 2015; QV *et al*., 2011). This keeps a very low water level, protecting the oil from quick oxidation.

3.2.3 Vitrification of EMO microcapsules

The glass transition refers to the phenomena where a supercooled, malleable liquid or rubbery material changes into a disordered solid glass upon cooling, or vice versa (RAJABI *et al*., 2015). DSC analysis revealed that the vitrification temperature of EMO microcapsules was around 62°C (Fig. 4). Previous report by LAINE *et al.* (2010) also proposed that the vitrification temperature of gelatin was 62°C with Aw of 0.24. This

confirmed the protective effect of gelatin. Normally, the storage temperature of PUFA-enriched products ranges between 0°C and 30°C (QV *et al*., 2011). Thus, the EMO encapsulated by gelatin-acacia, which was able to resist harsh heat-treatment such as spray drying, possesses good stability during longer storage periods.

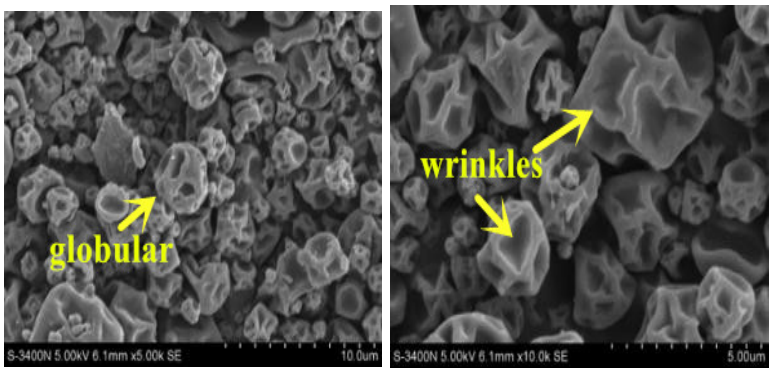


Figure 3. Scanning electron microscopy micrographs of *Elaeagnus mollis* oil microcapsules after heat treatment was observed at 5000 and 10000 magnification.

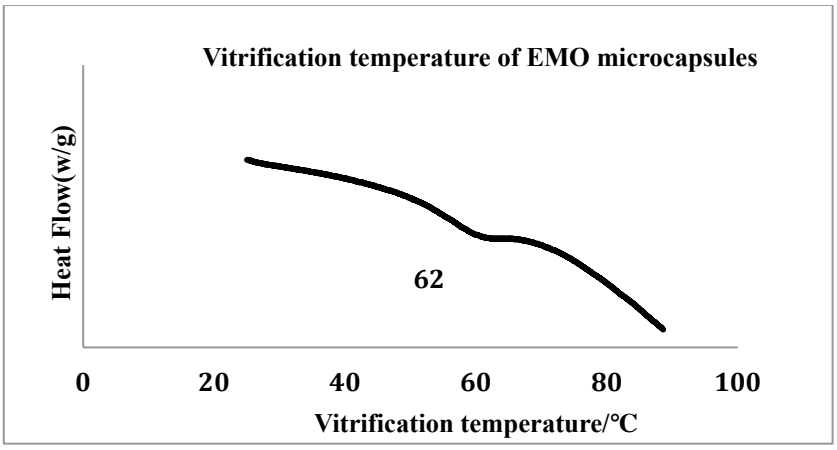
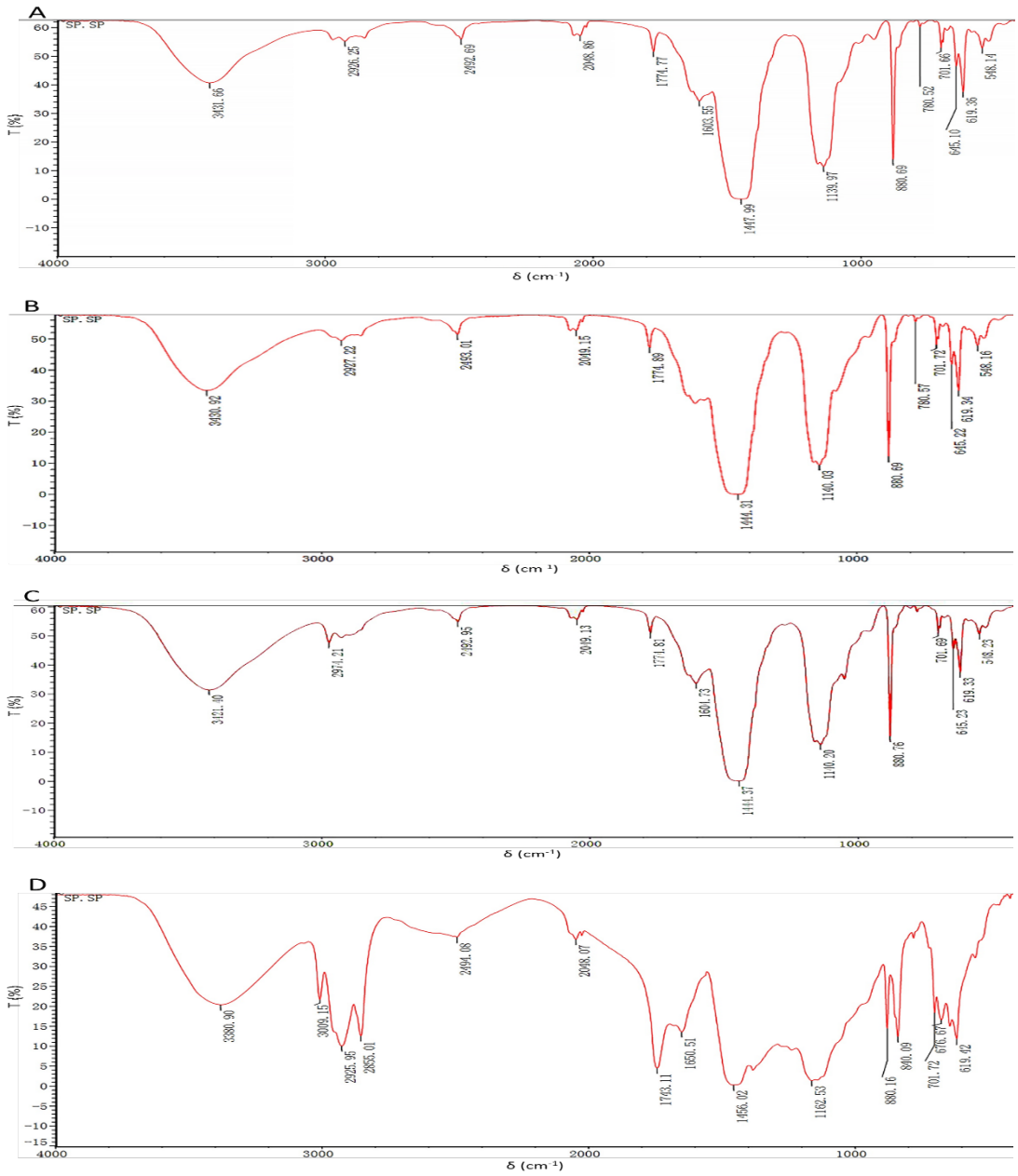


Figure 4. The vitrification temperature of spray-dried *Elaeagnus mollis* oil microcapsules which determined by DSC.

3.2.4 The interaction between gelatin-acacia and EMO

An appropriate ratio of gelatin-acacia complex and EMO/gelatin-acacia enabled EMO with 45% of PUFAs to have a heat-resistant property. The interplay between gelatin-acacia and EMO is noteworthy and requires adequate discussion. Gelatin, acacia, EMO, EMO microcapsules, and the blank microcapsules were detected by FTIR (Fig.5). The strong absorption peak of EMO, found at wavelength of 2925 cm^{-1}, 2855 cm^{-1} was due to CH_2 asymmetric stretching vibration and CH_2 symmetric stretching vibration. In the microcapsules, the carbonyl stretching bands shifted to 2974 cm^{-1}, indicating that no chemical changes occurred between EMO and gelatin-acacia (Fig. 5 A-B-C). The FTIR spectrum at 1740 cm^{-1} to 1755 cm^{-1} comprised the absorption peaks of characteristic molecule

groups such as -COOR and C=O (BUĞRA, 2012; MAJI and HUSSAIN, 2010). The wavelength of 1744 cm^{-1} in the FTIR spectrum of gelatin, acacia, EMO microcapsules, and EMO remained unchanged (Fig. 5 A-B-C-D). Therefore, EMO was encapsulated successfully by gelatin-acacia coupled with physical interaction.



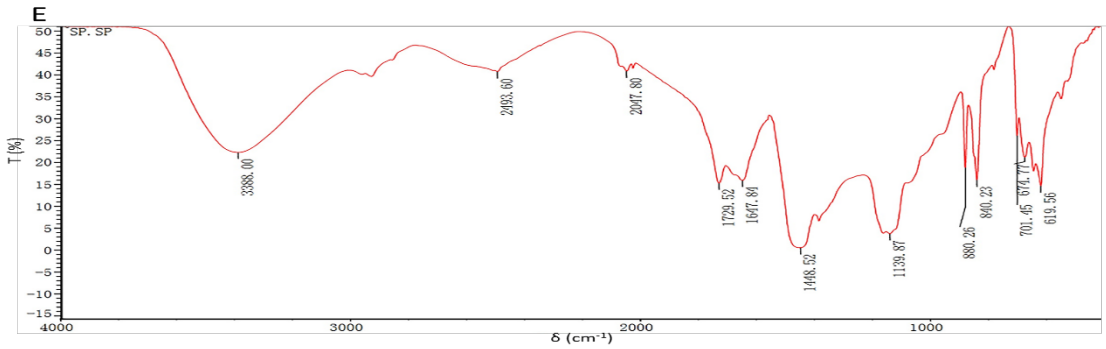


Figure 5. Comparison analysis of infrared spectroscopy of (A) acacia, (B) gelatin, (C) *Elaeagnus mollis* oil microcapsules, (D) *Elaeagnus mollis* oil, and (E) blankmicrocapsules as a function to indicate the interaction between gelatin-acacia and *Elaeagnus mollis* oil.

Moreover, the simulation among linoleic acid, collagen adhesin from *S. aureus*, and alpha-L-arabinopyranose demonstrated a possible interaction among EMO, gelatin and acacia in their original states. The hydrogen and oxygen atoms of C18 side chain of linoleic acid and *S. aureus* collagen adhesin were attracted to each other with hydrogen bonds (Fig. 6 A). The hydrogen bonding was also found between hydrogen side chain of alpha-L-arabinopyranose and *S. aureus* collagen adhesin (Fig. 6 B). Bumps were found at many sites between the same side chains of linoleic acid and alpha-L-arabinopyranose, indicating strong hydrogen bonding (Fig. 6 C). The carbonyl stretching and vibration in the microcapsules may be due to the hydrogen bonding between EMO and acacia rather than chemical changes (Fig. 6 D). The simulation results confirm the interaction between EMO and gelatin-acacia to be physical, and the PUFAs of EMO remained unchanged even after heating.

3.3. Storage stability of EMO microcapsules

3.3.1 Effect of temperature and light on EMO and EMO microcapsules

Temperature and light are selected to study the protective effect of microcapsules on EMO during the storage in the present research, because the temperature and exposure to light are usually considered as main factors affecting the storage stability of PUFAs (ZHOU *et al*., 2017). Data from Fig. 6 demonstrated that the PV of EMO increased significantly at 37°C, indicating that temperature had a significant influence on EMO storage stability. On the contrary, the PV of EMO protected by gelatin-acacia increased gradually. When stored at 4°C and 20°C, both the PVs of the encapsulated EMO relatively held stability for the first 3 weeks (Fig. 7 A-B). The PV only increased to 15 meq/kg oil after the encapsulated EMO was incubated at 37°C for 5 weeks (Fig. 7 C). Clearly, use of gelatin-acacia to entrap EMO retarded the PUFAs from quick oxidation, weakening the influence of storage temperature on the oil quality.
The fast growth in the PV of EMO indicated that light was another factor affecting the stability of the PUFAs (Fig. 7 D-E). The change in the PV of EMO protected by gelatin-acacia was very low, even when it was exposed to light. Therefore, the light-resistant ability was enhanced in EMO microcapsules. Therefore, the EMO microcapsules are able

to maintain a stable status after being exposed to 4°C, 20°C, 37°C, and light during their storage.

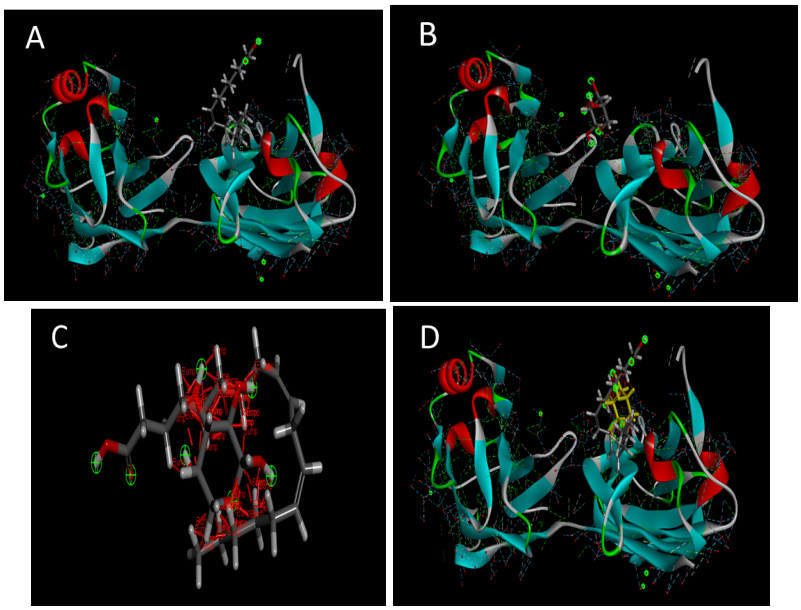
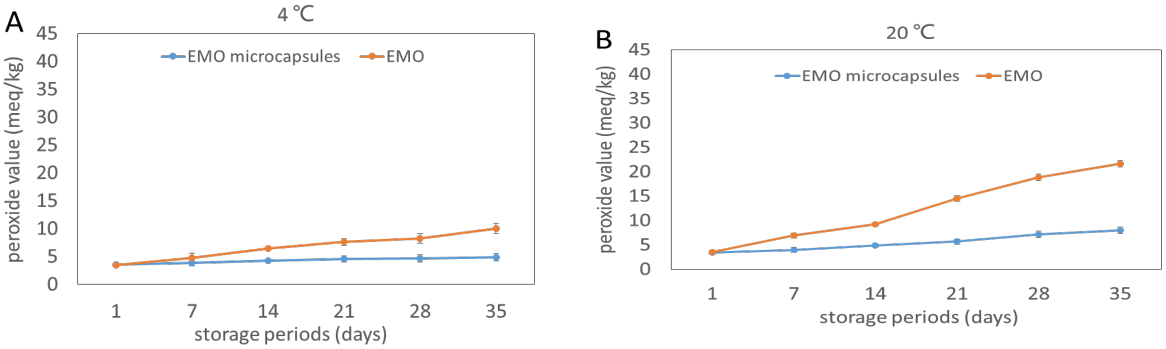


Figure 6. Model interaction among linoleic acid, collagen adhesin from *Staphylococcus aureus*, and alpha-L-arabinopyranose; (A) hydrogen bonds between linoleic acid and collagen adhesin from *S. aureus*; (B) hydrogen bonds between alpha-L-arabinopyranose and collagen adhesin from *S. aureus*; (C) hydrogen bonds and bumps between linoleic acid and alpha-L-arabinopyranose; (D) interaction between linoleic acid, collagen adhesin from *S. aureus* and alpha-L-arabinopyranose. Hydrogen bonds are shown as green colour in side chain of linoleic acid and side chain of alpha-L-arabinopyranose and surface of collagen adhesin from *S. aureus*. Bumps are coloured as red between linoleic acid and alpha-L-arabinopyranose.



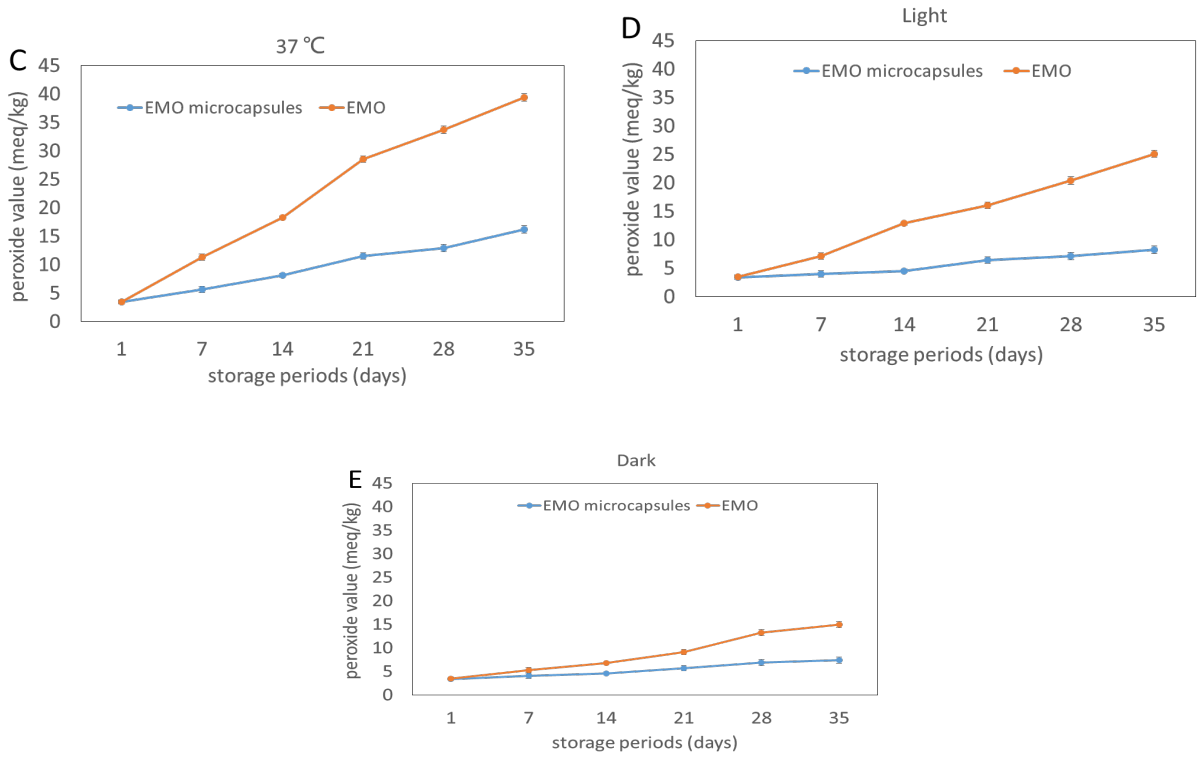


Figure 7. The stability assessment of spray-dried *Elaeagnus mollis* oil microcapsules with different temperatures as influential factors: (A) storage at 4°C, (B) storage at 20°C, and (C) storage at 37°C; The stability assessment of spray-dried *Elaeagnus mollis* oil microcapsules with light as influential factor: (D) storage in light and (E) storage in dark.

4. CONCLUSIONS

In summary, a gelatin-acacia combination could be applied in preventing PUFAs from oxidation in the food industry. The microencapsulated EMO with 45% PUFAs was able to tolerate heat stress and maintain long-term storage. However, either more wall material or higher oil concentrations would cause the aggregation of microcapsules, reducing the microencapsulation yield of EMO. Thus, the optimal concentration of the gelatin-acacia combination was 2.0%, and a 1:1 ratio of EMO to gelatin-acacia would guarantee the maximal EMO to be encapsulated. Moreover, the microencapsulation of EMO with gelatin-acacia combination involves only hydrogen bonding and no chemical bonding. The microencapsulation of EMO with gelatin-acacia combination might provide a safe way for the manufacturing of PUFA-containing products.

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PAPER

EVALUATION OF PHYSICOCHEMICAL, ANTIOXIDANT AND ANTIMICROBIAL PROPERTIES OF CHICKEN SAUSAGE INCORPORATED WITH DIFFERENT VEGETABLES

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ABSTRACT

The physicochemical, antioxidant, antimicrobial and nutritional features of Capsicum, carrot, spinach, purple cabbage and oyster mushroom incorporated in chicken sausages were investigated in this study. The analysis of microstructure showed that sausage with vegetables had a less dense microstructure mainly due to the consequent increase in moisture content, decrease in fat content and decrease in protein density. The highest DPPH value and total phenolic content were demonstrated by the sausages incorporated with purple cabbage while incorporation of spinach gave the highest total flavonoid content. During the end of storage study, the peroxide value in sausages incorporated with purple cabbage significantly ($P<0.05$) delayed lipid oxidation compared to the control from 1.62meq/kg to 0.58meq/kg. The microbial activity was reduced with the incorporation of vegetables, which subsequently would help to extend the shelf-life of the samples. The results suggest that vegetables used are potentially useful in improving the nutritional quality in chicken-based sausage-like product.

Keywords: hysicochemical properties, antioxidant properties, antimicrobial properties, vegetables, chicken sausage

1. INTRODUCTION

Changes in lifestyle and eating habits of human beings, has been shown by researchers and health organizations (World Health Organization (WHO)), Food and Agriculture Organization (FAO) to be the major cause of increases of diseases like, obesity, cancer, cardio vascular failures, (Who and Organization, 2003). Nowadays people are showing greater interest in foods that contain bioactive or functional components, which will give additional benefits to their health status (COFRADES *et al*., 2008b).

Chicken meat and its products have experienced increasing popularity and become widely spread all over the world. Chicken sausage is one of the popular foodstuffs among these products (BARBUT, 2016), while continually eating processed meat products will affect health status and at the same time will increase cancer risk (Chan *et al*., 2008). thus the demands have initiated to an extensive research on the meat industry to incorporate health-enhancing ingredients. In order to achieve this goal, application of suitable agents possessing both antioxidant and antimicrobial activities may be useful for maintaining meat safety status, quality, extending shelf-life and preventing economic loss (YIN and CHENG, 2003).

Many researches have been conducted to explore the feasibility of using non-meat ingredients to promote a healthier meat sausage product and much research has indicated that lipid oxidation and microbial growth in meat products can be controlled or minimized by using either synthetic or natural food additives (COFRADES *et al*., 2008a) Various synthetic antioxidants, are commonly used to delay the development of rancidity in food products (MARTINEZ-TOME *et al*., 2001). However, consumers are concerned about the safety of synthetic food additives. This concern has led to arouse a great interest in natural additives (POKORNÝ, 1991). Natural agents possessing antioxidant and antimicrobial properties have the advantage of being readily accepted by consumers, as they are considered natural.

Capsicum, carrot, spinach, purple cabbage and oyster mushroom can provide several health benefits in daily diet. Capsicum is the second-most consumed vegetable worldwide and is characterized by its high levels of vitamin C (ascorbic acid), pro-vitamin A (carotene) and calcium. In fact, intakes of 50-100 g fresh capsicum could provide 100% and about 60% of the recommended daily amounts of vitamin C and A, respectively. Capsicums are also rich in carotenoids, compounds with antioxidant and anticarcinogenic capacity (Marti *et al*., 2011). The carrots are the unique roots, contain other compounds, such as phenolic compounds and organic acids and have a characteristic flavor due to the presence of terpenoids and polyacetylenes, therefore, it has nutritional property for human health (RAEES *et al*., 2015). Also spinach is an extremely nutritious vegetable, rich both in core nutrients and phytochemicals. The major micronutrients in spinach are vitamins A (from β-carotene), C, K and folate, and the minerals, calcium, iron and potassium. Spinach also provides fibre and is low in calories (HEDGES and LISTER, 2007). Purple cabbage is a rich source of anthocyanins, minerals, vitamins, oligosaccharides and a number of bioactive substances that provides a positive impact on human health (SYUHAIRAH *et al*., 2016). Oyster mushroom has a great nutritional value since they are an excellent source of protein, with an important content of essential amino acids and fiber, and poor in fat and also they provide a nutritionally significant content of vitamins (B_1, B_2, B_{12}, C, D, and E) and often regarded as an ideal and healthy food for people (REIS *et al*., 2012).

To overcome the disadvantages of replacing synthetic antioxidants and colorants with natural ones in meat and meat products, focus of the current study is on antioxidant and

antimicrobial effects and color properties of plant based materials such as Capsicum, carrot, spinach, purple cabbage and oyster mushroom. Therefore, the objective of the present study was to determine the physicochemical, nutritional, antioxidant and antimicrobial properties of chicken sausage added with Capsicum, carrot, spinach, purple cabbage and oyster mushroom.

2. MATERIALS AND METHODS

2.1. Materials

Minced chicken meat (MCM) and five types of vegetables; capsicum (*Solanaceae annuum*), carrot (*Daucus carota*), spinach (*Spinacia oleracea*), purple cabbage (*Brassica olerace*) and oyster mushroom (*Pleurotus sajor-caju*) were purchased from a local market in Penang, Malaysia. The trichloroacetic acid was purchased from R&M Chemicals, UK, malondialdehyde, boron trifluoride-methanol (Merck, Germany), thiobarbituric acid, cathechin (Sigma-Aldrich, USA) and all other chemical reagents used for the experiments were of analytical grade.

2.2. Preparation of chicken sausage

Mechanically Deboned Chicken Meat (MDCM) and five types of vegetables; spinach, purple cabbage, carrot, capsicum and oyster mushroom were purchased from a local market in Penang, Malaysia. In our previous research, we were conducted with various percentages (30%, 40% and 50%) of vegetables in the sausage formulations. Based on last study results, sausages formulated with 40% vegetables were selected as the best formulation (SYUHAIRAH *et al*., 2016). The selection of the best formulation was based on the result of physical properties of the sausages prepared. As shown in Table 1 the sausages with the selected formulation were prepared and all ingredients were mixed for 5 min using a mixer (Robot Coupe®, Blixer 3, France).

Table 1. Formulations of sausage preparation.

Ingredients (g)	Samples/100g	
	Control	V40
Minced chicken meat	75	45
Vegetables	-	30
Tapioca flour	10	10
Spices	4	4
Palm oil	1.8	1.8
Fresh egg white	3	3
Cold water	3	3
Salt	1.7	1.7
Sugar	1.35	1.35
Sodium thiosulphate	0.15	0.15

Ratio of vegetables: chicken meat = 40:60 Vegetables: Capsicum (CP), carrot (C), spinach (S), purple cabbage (PC), oyster mushroom (OM).

The batter was then stuffed manually into 2.5 cm diameter cellulose casing. Sausages were steamed in a steamer (Electric and Steamer, Model RS-6, 0881, China) until their internal temperature reached 72±2EC (measured using a thermocouple probe) and held for approximately 30 min. The steamed sausages were promptly cooled in ice water for 15 min, the casing was peeled and vacuum-packed. The prepared sausage samples were kept in the freezer at -18EC prior to analyzes.

2.3. Scanning Electron Microscope (SEM)

Scanning electron microscope (SEM) Microstructure characteristics of the samples were measured according to the procedure described by Andres *et al*. (2006). Small pieces of sausages of 0.5cm in diameter and 0.2-0.3cm thick were used for analysis. The samples were dehydrated under vacuum (sputtering), allowing surface and cross-section visualization. Micrographs of the samples were obtained by scanning electron microscope (SEM 505, Philips, Netherlands)

2.4. Chemical composition of sausages

2.4.1 Moisture determination

Moisture content in the samples was determined by using AOAC Method number 960.39 (William 2000) – Oven Drying Method. The moisture content was calculated using the following equation:

$$\text{Moisture content } (\%) = \frac{\text{weight of wet sample (g)} - \text{weight of dried sample (g)}}{\text{weight of wet sample (g)}} \times 100$$

2.4.2 Protein determination

The amount of protein content in the percentage of protein content was determined by multiplying the percentage nitrogen of sample with a factor of 6.25. The percentage of protein was calculated as below:

$$\%(\text{w/v}) \text{ Nitrogen} = \frac{(\text{mL HCL of sample} - \text{mL HCL of blank}) \times \text{HCL molarity} \times 14 \times 100}{\text{Weight of sample (g)}} \times 100$$

$$\%\text{Protein content} = \%\ \text{Nitrogen} \times 6.25\ (\text{conversion factor})$$

2.4.3 Fat determination

The amount of fat content in sausage was determined based on AOAC Method number 960.39 (William 2000) – Soxhlet Method. The percentage of crude fat was calculated as below:

$$\%\text{fat (dry basis)} = \frac{Weight\ of\ flask\ with\ fat\ (g) - Weight\ of\ flask\ (g)}{Weight\ of\ sample\ (g)} \times 100$$

2.4.4 Ash determination

The amount of ash in sausages was determined by using AOAC Method number 945.38 (William 2000) – Dry Ashing Method. Percentage of ash content was calculated using the equation as below:

$$\%(\mathrm{w/w})\ \mathrm{Ash} = \frac{Weight\ of\ crucible\ with\ ash\ (g) - Weight\ of\ crucible\ (g)}{Weight\ of\ sample\ (g)}$$

2.4.5 Crude fiber determination

The amount of crude fiber in sausages was determined by using AOAC Method number 962.09 (William 2000)– Acid/alkaline Hydrolysis Method. Percentage of crude fiber content was calculated using the equation as below:
%(w/w) Crude fiber=
Where, S = Weight of sample (g), C = Weight of filter paper (g), A = Weight of crucible (g) + filter paper (g) + dried precipitate B = Weight of crucible (g) + ash (g)

2.5. Antioxidant composition

2.5.1 DPPH radical scavenging activity

DPPH radical scavenging activity was determined using a method described by Tangkanakul *et al*. (2009). Percentage of DPPH scavenging activity (%SA) was calculated from the equation (1-X/C)*100, where X is absorbance of extract and C is absorbance of control.

2.5.2 Total phenolic content

Total phenolics were determined using the method adapted from SINGLETON and ROSSI (1965). The results were expressed as mg gallic acid equivalent (GAE)/100g food.

2.5.3 Total flavonoids content

Total flavonoid content was determined according to the colorimetric method described by ZHISHEN *et al.* (1999), The total flavonoid content was expressed in mg of catechin equivalent per gram of sample (mg CE/g).

2.6. Storage test

The sausages with the selected formulation were used for the storage test. The samples were stored in a refrigerated incubator at 4±1°C and took three samples from each packaging at pre-determined time (0, 7, 14 and 21days of storage), and evaluated properties of the sausages for lipid oxidation and microbial activity.

2.6.1 Microbial analysis

Bacterial counts were carried out using pour plate method as described by Andrés *et al*. (ANDRES *et al*. 2006) for every seven days of refrigerated storage (4°C) for 0, 7, 14, 21

days. The initial dilution was made by aseptically blending 10g of the sample with 90mL peptone solution (1g/L) in Stomacher at 200 rpm for 1 minute. A series of sample dilution was prepared until 10^{-4} and plated duplicate with Plate Count Agar (PCA) for the total mesophilic aerobic count (incubated at 37ºC for two days), Potato Dextrose Agar (PDA) was used for yeast and mould count (incubated at 30ºC for five days). Data was expressed as log colony forming units (CFU)/g sample.

2.6.2 Peroxide value

Measurement of peroxide value (POV) was determined according to the SALLAM *et al*. (Sallam *et al*. 2004). The POV was calculated and expressed as milliequivalent peroxide per kg of sample:

$$\text{POV (meq/kg)} = \frac{S \times N}{W} \times 100$$

Where, S = Volume of titration (mL) N = Normality of sodium thiosulfate solution W = Weight of sample (kg).

2.7. Statistical analysis

The results were analyzed using the two-way statistical analysis of variance (ANOVA), followed by Duncan multiple range test in the experiments of preliminary study. The results of chemical analysis and shelf life determination were compared using one-way ANOVA, followed by Duncan multiple range test. All data were processed using SPSS package (SPSS 21.0 for Windows, SPSS Inc, Chicago, Illinois, U.S.A) and expressed as mean value±standard deviation. Statistical significance was indicated at 95% confidence level.

3. RESULTS AND DISCUSSION

3.1. Scanning Electron Microscope (SEM)

The microstructures of sausages incorporated with 40% of vegetables were observed by using scanning electron microscope (SEM). Overall, the microstructures of samples were not visually different for each type of vegetables added as shown in Fig. 1. In general, all sausage samples had the microstructure consist of course protein gel matrix and fat with numerous holes, which formed a porous network structure. The fat globules observed and integrated in the matrix, bound by strands of 75 protein network. Meanwhile, fine strands and sheets within the protein matrix showed the presence of fiber in the sausage samples (Fig. 1e, f). Control sausage presented a denser network structures of a honeycomb-like appearance (Fig. 1a). Similar finding on the microstructure were also reported by AYO *et al*. (2008). Thus, consistent with the results that shows higher hardness and tougher sausage texture as presented in our previous work (SYUHAIRAH *et al*., 2016). According to CARBALLO *et al*. (1996), a less dense matrix microstructure, softer and chewy texture formed by sausage incorporated with vegetable is mainly due to consequent increase in moisture content, decrease in fat content and protein density. Based on the result, sample OM40 as in Fig. 1b shows a packed protein gel matrix compared to the rest of sausages

with vegetable. This suggests due to the presence of high fiber and fat content as reported in Table 3.

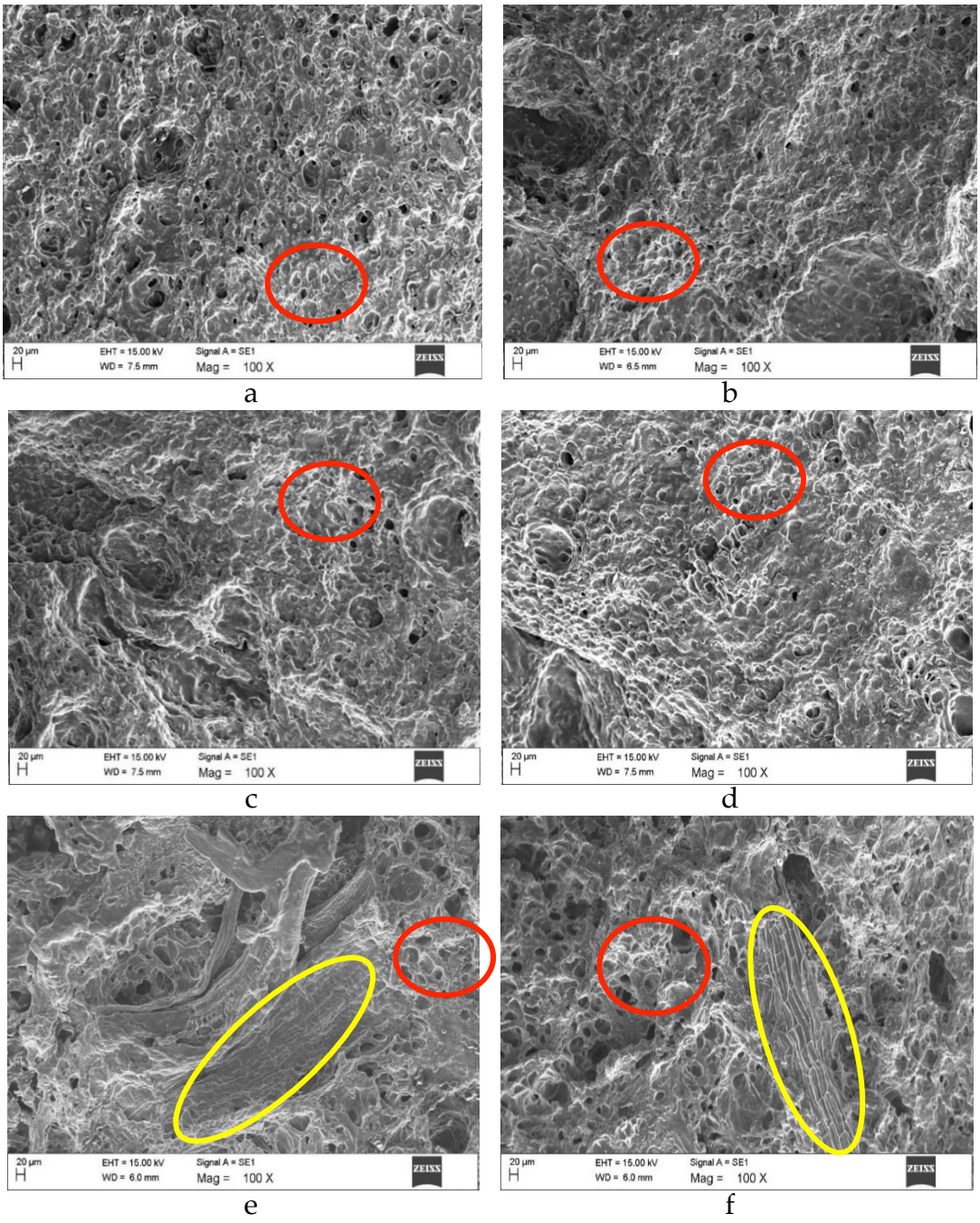

Figure 1. Scanning electron micrograph of sausages with 40% incorporation of vegetables at 100x magnifications: (a) Control (b) Oyster mushroom (c) Capsicum (d) Carrot (e) Spinach (f) Purple cabbage. Fat globules (), fiber strands ().

The sample OM40 however, yielded the lowest hardness value (3.73 kg) (SYUHAIRAH *et al*., 2016). This is probably due to smooth and velvety texture of oyster mushroom, as the alignment of myofibrillar proteins induced by incorporation of vegetables is depending on the original texture of vegetables and fiber content. As a result, a varied gel matrix or network is developed and water retained in the network might be different. Thus, the disposition and aggregation of protein filaments contributed to the difference in water-holding capacity as well as the textural properties.

3.2. Chemical composition

3.2.1 Moisture content and Fiber content

The moisture and fiber content of sausages are as shown in Table 2 Based on the results, the moisture content was significantly higher ($P<0.05$) for all sausages incorporated with vegetables (64.52-66.46%) as compared with the control sample (58.13%). This is most probably due to the incorporation of vegetables in sausage formulation, which provided a significant ($P<0.05$) amount of fiber content in the products. According to CHOI *et al*. (2010), addition of dietary fiber increases the moisture content of meat emulsion systems, providing higher water retention and improves emulsion stability. Additionally, GARCıA *et al*. (2002) indicated that the high moisture content of sausage by adding dietary fiber is due to high water retention of the fiber. These results agree with those reported by CHOI *et al*. (2012), who found a significant increase of moisture content in chicken frankfurter when added with pumpkin fiber. Similarly, LEE *et al*. (2009) showed that the addition of kimchi fiber also increased the moisture content of sausages produced.

3.2.2 Fat analysis

Based on the fat analysis, as shown in Table 2 control sausage (14.15%) yielded the highest fat content followed by sample OM40 (7.11%) > CP40 (6.79) > C40 (6.52) > S40 (6.41) and PC40 (5.84) significantly. The added vegetables may have affected fat content, as moisture content relatively increased (Choi *et al*. 2012). These findings are in tandem with the idea of Yang *et al*. (2007) who reported that replacing pork loin with hydrated oatmeal and tofu at 15% significantly lowers the amount of fat content. Similar 78 findings were reported by ALESON-CARBONELL *et al*. (2005) and CHOI *et al*. (2012) with addition of lemon albedo in dry-cured sausage and pumpkin fiber in chicken sausage. Moreover, the highest fat content in sample OM40 compared to other sausage samples with vegetable added is probably due to high fat content in oyster mushroom. Based on the USDA National Nutrient Database for Standard Reference (GEBHARDT *et al*. 2008), the comparison between different types of vegetables used in this study indicated that oyster mushroom contains the highest fat content (0.41g/100g) while the raw purple cabbage contains the lowest fat content (0.16g/100g).

3.2.3 Protein content

Table 2 shown Protein content was significantly ($P<0.05$) higher in control sausage (14.87%) compared to other sausages incorporated with vegetables. The main reason for that is due to the substitution of 40% chicken meat with vegetable, which lead to significant loss of protein and fat in the samples. (TROUTT *et al*. 1992) reported a similar trend in low fat beef patties containing polydextrose and oat flour as texture modifying

ingredients. The replacement of lean meat by apple pomace in mutton nugget further supported the results (HUDA *et al*. 2014).

3.3.3 Ash content

Ash content was significantly (P<0.05) higher with the incorporation of vegetable which suggested that due to significant (P<0.05) increase in fiber content compared with control (Table 2). Studied by FERNANDEZ-GINES *et al*. (2004) observed that the ash content increases significantly with the addition of dietary fiber such as incorporation of dietary fiber from lemon albedo in low-fat sausage. Similar results were obtained by CHOI *et al*. (2012) who studied the physicochemical properties of reduced fat frankfurters by partially substituting pork back fat with a *makgeolli* lees fiber.

Table 2. Moisture, fat, protein and fiber of sausages containing chicken and 40% vegetables.

Sample	Moisture	Fat	Protein	Fiber	Ash
Control	58.13±0.35^{d}	14.15±0.18^{a}	14.87±0.23^{a}	0.80±0.01^{f}	2.90±0.00^{d}
CP_{40}	64.54±0.35^{c}	6.79±0.06^{bc}	9.80±0.35^{c}	1.21±0.02^{e}	2.93±0.01^{c}
C_{40}	64.52±0.50^{c}	6.52±0.32^{c}	10.13±0.12^{bc}	2.46±0.04^{c}	2.96±0.01^{a}
S_{40}	65.56±0.38^{b}	6.41±0.37^{c}	10.13±0.12^{bc}	2.75±0.08^{b}	2.95±0.00^{b}
PC_{40}	66.46±0.18^{a}	5.84±0.34^{d}	9.73±0.23^{c}	2.30±0.03^{d}	2.95±0.00^{b}
OM_{40}	65.50±0.63^{b}	7.11±0.30^{b}	10.33±0.31^{b}	2.94±0.07^{a}	2.96±0.01^{a}

CP= capsicum, C= carrot, S= spinach, PC= purple cabbage, OM= oyster mushroom Mean±SD lowercase within the column indicate significantly different (P<0.05).

3.3. Antioxidant composition

3.3.1 DPPH radical scavenging activity, total phenolic content and total flavonoid content

The use of antioxidant helps to minimize rancidity, retard the formation of toxic oxidation products, maintain nutritional quality and increase the shelf life of food products (Fukumoto and Mazza 2000). Incorporation of capsicum, carrot, spinach, purple cabbage and oyster mushroom would help to deliver the benefits of high antioxidant properties of these vegetables into sausage samples. Therefore, numerous studies have been widely used to determine the antioxidant contents. In this study, DPPH radical scavenging activity, total phenolic content and total flavonoid content of methanolic sausages extract were analysed and the results are tabulated in Table 3. Based on the result, the highest DPPH value was demonstrated with the sausages incorporated with purple cabbage (87.38%SA) followed by capsicum (86.89%SA) with no significant difference. Meanwhile, sausage with spinach, oyster mushroom, carrot and control recorded (63.82%SA), (48.24%SA), (45.28% SA), and (42.21%SA) respectively, with significant differences. The high DPPH value in PC40 and CP40 samples indicates the higher ability of antioxidant compounds in these samples to lose a hydrogen and possibly acting as a primary antioxidant. Both samples also possibly have better reaction with free radicals, particularly of the peroxy radicals, which are the major propagator of the autoxidation chain of fat, thereby, abort the chain reaction. Osman and Milan (2006) reported that the DPPH value

in raw purple cabbage was the highest (>80%SA) compared with bitter melon, paprika, mulberry leaves, grape peel, onion peel and red beet. The result found in this study is also in line with MATSUFUJI *et al*. (2007) who reported that the red capsicum has higher DPPH value (>80%SA) compared with green, yellow and green capsicum.

Phenolic compounds such as flavonoids, anthocyanins, and carotenoids are the major antioxidant components found in plants, which are free radical scavengers not only because of their ability to donate hydrogen atoms or electrons but also due to their stable radical intermediates (SHAHIDI *et al*. 1992). It was observed that PC40 recorded the highest phenolic content as well as antioxidant capacity. Meanwhile, control sausage had the lowest phenolic content and antioxidant capacity. The results demonstrated that the highest contribution of phenolic compounds provided by the incorporation of purple cabbage to the sausages, in comparison with the possible contribution provided by the chicken as in the control sausage. This showed that the radical scavenging effect of the sausages' extracts positively correlate with the total amount of phenolic compounds. Previous studies by BOO *et al*. (2012) also present positive correlation between the quantity of phenolic compounds and the DPPH free radical scavenging effect in extracted samples. Therefore, this study stipulated that incorporation of vegetables helps to improve the nutritional quality of new formulated sausages.

Flavonoids can be classified as anthocyanidins, flavanols (catechins), flavones, flavanones and flavonols, which are responsible for the orange, red and blue colour in fruits and vegetables (Lin and Tang 2007). However, to screen phenolic-rich, including flavonoid-rich fruits and vegetables, the value is not dependent on their colour. The total flavonoid content in sausage samples are in the order of S40 (6.24 mgCE/g) > OM40 (5.70 mgCE/g) > PC40 (5.11 mgCE/g) > CP40 (4.63mgCE/g) > C40 (4.30 mgCE/g) > control (2.93 mgCE/g). The highest flavonoid content in sample S40 is probably due to the high concentration of flavonoid in spinach. PANDJAITAN *et al*. (2005) reported that the total flavonoid in mature commercial cultivar of spinach was 12.7mgCE/g. Nonetheless, it is worth to note that the antioxidant in vegetables vary considerably due to several factors; genetics, cultivation practices, environmental, growing conditions, maturation, storage, and processing.

Table 3. Total phenolic content, DPPH and total flavonoid of sausages containing chicken and 40% vegetables.

Sample	Parameters		
	DPPH (%SA)	TPC (GAE/100g)	Flavonoid (mg CE/g)
Control	42.21±1.61^{e}	14.37±0.08^{e}	2.93±0.09^{f}
CP_{40}	86.89±1.33^{a}	21.18±0.02^{b}	4.63±0.03^{d}
C_{40}	45.28±0.41^{d}	15.51±0.13^{d}	4.30±0.06^{e}
S_{40}	63.82±0.79^{b}	17.23±0.33^{c}	6.24±0.27^{a}
PC_{40}	87.38±1.14^{a}	23.17±0.34^{a}	5.11±0.12^{c}
OM_{40}	48.24±2.34^{c}	17.24±0.22^{c}	5.70±0.15^{b}

CP = capsicum, C = carrot, S = spinach, PC = purple cabbage, OM = oyster mushroom.
Mean±SD. Lowercase within the column indicate significantly different (P<0.05).

3.4. Storage test

3.4.1 Peroxide value

The peroxide value (PV) is used as an indicator of the primary oxidation in sausage samples. Based on the result in Fig. 2, a gradual increase in PV was observed for all samples throughout the storage ($P<0.05$) from day 0 until day 21. Control sausage had significant ($P<0.05$) highest PV compared to sausages containing 40% vegetables as the storage time increased from 2.89 at day 0 to 6.00 at day 21, indicating that lipid oxidation was most extensive in this sample. This was expected as the control sample had the highest fat content (14.15%). Moreover, sausages incorporated with vegetables had a lower PV comparative to the control sample probably due to free radical scavenging antioxidants interfere with the initiation or propagation steps of lipid oxidation reactions by scavenging lipid radicals and forming low-energy antioxidant radicals that do oxidation of unsaturated fatty acids (MAQSOOD and BENJAKUL, 2010). Between samples incorporated with vegetable, S40 had the lowest initial PV followed by OM40, C40, CP40 and PC40 respectively. However, at the end of storage period, samples PC40 and CP40 showed the lowest PV. This suggested that possibility of hydroperoxide formed as a primary oxidation product in sample PC40 and CP40 might have undergone decomposition into secondary oxidation products. A decrease in the level of primary oxidation products is associated to hydroperoxide degradation, producing secondary lipid peroxidation products (BOSELLI *et al.*, 2005). Overall, the result revealed that incorporation of purple cabbage gave the best antioxidative effect in the sausage and thus reduced the oxidation level. Nonetheless, all samples produced had PV within acceptable limits (3.11-6.00 meq/kg) until day 21 of storage. In all cases, PV was lower than 25 meq of active O_2/kg of fat, which is the limit of acceptability for fatty foods (ABDULHAMEED *et al*. 2014).

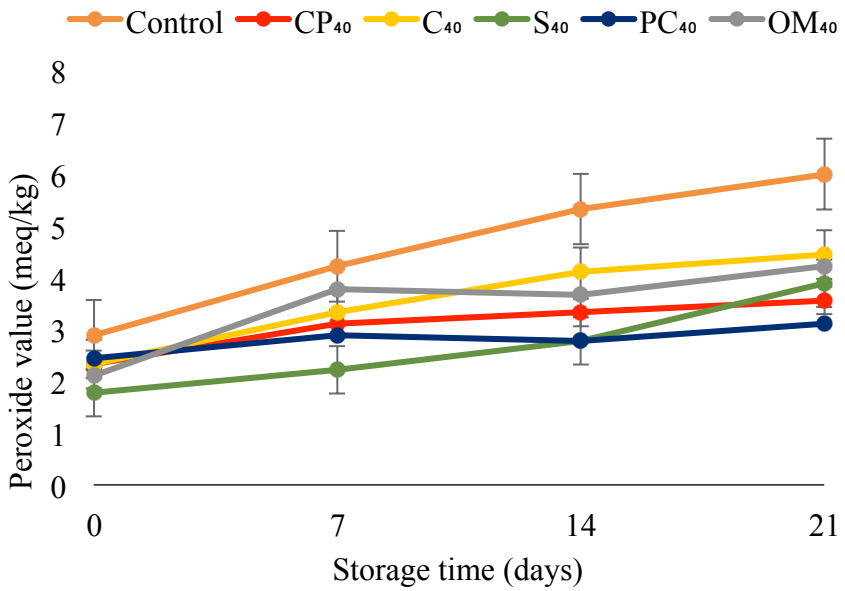

Figure 2. Effects of different vegetable incorporation on peroxide value of sausages stored at 4°C, for 21 days (vertical bars represent standard error).
CP = capsicum, C = carrot, S = spinach, PC = purple cabbage, OM = oyster mushroom.

3.4.2 Microbial analysis

Besides lipid oxidation, the quality attributes of sausages could deteriorate due to microbial growth. This can lead to major public health hazards and economic loss in terms of food poisoning and meat spoilage. Hence, the incorporation of vegetables into sausage formulation anticipated to serve both functions; antioxidant and antimicrobial properties useful for preserving meat quality, extending shelf-life and preventing economic loss (SALLAM *et al*. 2004). Based on the results obtained as in Fig. 3, the initial total plate count (TPC) in sausages was in the range of 1.91-2.36 log10CFU/g and all the samples showed an increased in the count during storage. Samples incorporated with capsicum, carrot, spinach, purple cabbage and oyster mushroom comparatively had lower microbial count. At day 21 of storage, the count for sample CP40, C40, S40, PC40, OM40 were 4.00, 4.22, 4.30, 3.99 and 4.00 log10CFU/g, respectively, which were lower than that of the control (5.05 log10CFU/g). However, the count in all samples was below 6 log10CFU/g, which is the MPL (Maximal Permissible Limit) for APC recommended by Malaysia Food Regulation (Tee 2011).

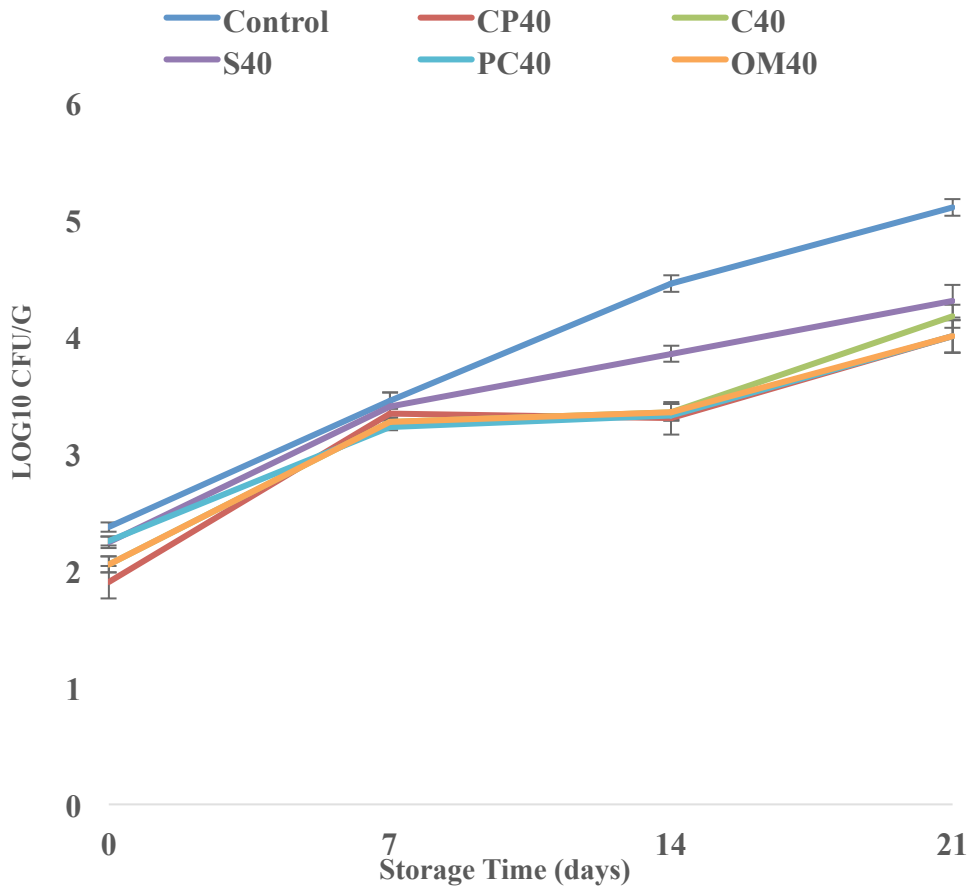

Figure 3. Changes on total plate count in sausage incorporated with different vegetables during storage at 4°C, for 21 days. CP = capsicum, C = carrot, S = spinach, PC = purple cabbage, OM = oyster mushroom.

In this study, PC40, CP40 and OM40 samples had a comparatively lower microbial count than C40 and S40 samples. The difference was probably due the higher phenolic compounds presence in sausage incorporated with purple cabbage, capsicum and oyster mushroom as compared to sausage with carrot and spinach (Table 4). This finding is in agreement with those reported by LIU *et al*. (2009) who found lower TPC in chicken frankfurter added with Chinese mahogany, which has higher phenolic compound than rosemary chicken frankfurter. In addition, the lower TPC in sample PC40, CP40 and OM40 than other sausage samples are probably due to the antimicrobials properties present, which retards bacteria growth. A previous study by VO and Ariyo proved that mushrooms contain some bioactive compounds such as rutin, gallic acid and catechin, which contain a high antimicrobial effect.

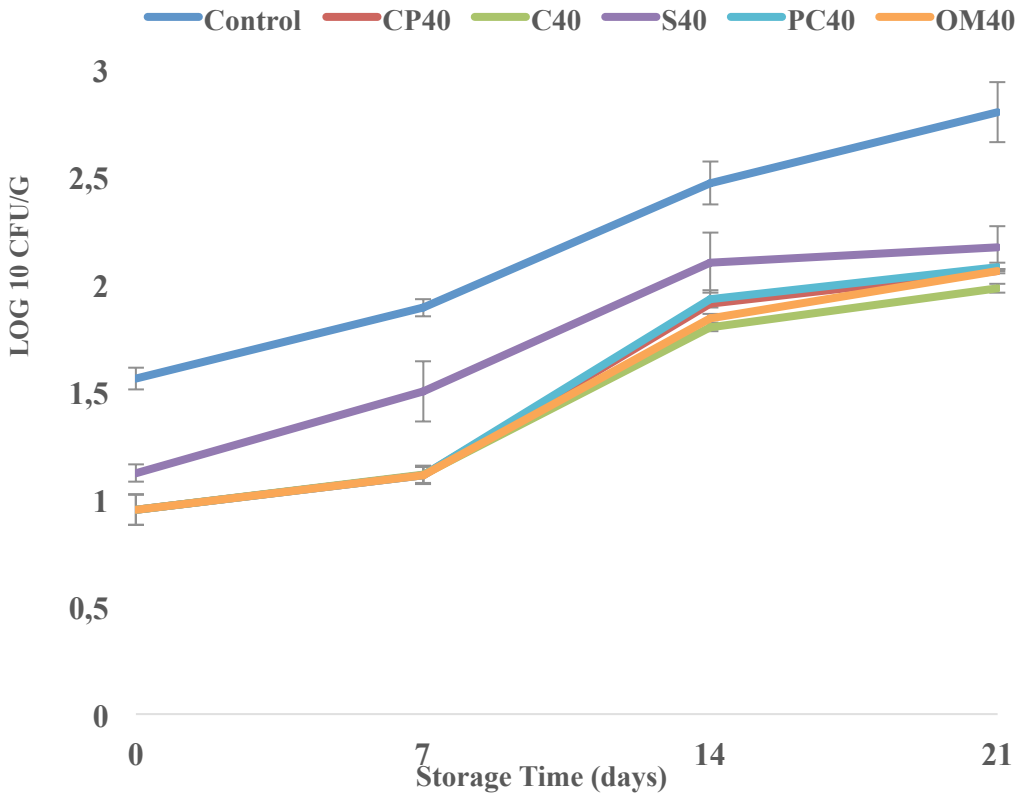

Figure 4. Changes on yeast and mould count in sausage incorporated with different vegetables during storage at 4°C, for 21 days. CP = capsicum, C = carrot, S = spinach, PC = purple cabbage, OM = oyster mushroom.

Yeast and mould results showed an increased in the count during refrigerated storage ranging from 0.95-1.60 log10CFU/g at day 0 to 1.98-2.82 log10CFU/g at day 21 for all sausages formulations (Fig. 4). The trend was similar with the TPC result obtained where incorporation of vegetables in sausage lowered the yeast and mould count. The inhibition of yeast and mould growth was also probably due to the growth of lactic acid-producing bacteria under anaerobic packaging conditions during refrigerated storage (Bradford *et al*. 1993).

4. CONCLUSION

This study unveils that the incorporation of capsicum, carrot, spinach, purple cabbage and oyster mushroom in chicken-based sausage-like product at 30%, 40% and 50% did alter the physicochemical properties of sausages produced. In general, 40% incorporation of vegetables demonstrated more desirable physical properties, which are of particular importance in the case of quality and economic justification of the sausages. The sausages with vegetables had a less dense microstructure with a porous network due to a consequent increase in moisture content, decrease in fat content and protein density. However, the sample with oyster mushroom gave a more closely packed protein gel matrix. The highest DPPH value (87.38%SA) and total phenolic content (23.17GAE/100g) were demonstrated by the sausages incorporated with purple cabbage, which helps to deliver health benefits on human health and retard the formation of oxidation products. Sausage with purple cabbage has the highest stability during 21 days of storage time. The sample had significantly ($P<0.05$) reduced the peroxide value at the end of storage from 1.62meq/kg to 0.58meq/kg compared with the control. Microbial activity results demonstrated that incorporation of vegetables tends to lower the microbial counts, which would help to extend the shelf life of sausages produced. Overall results indicated that the incorporation of vegetables in sausages was potentially helps to deliver both antioxidant and antimicrobial properties, act as a natural preservative and improve quality of the chicken based sausages and reduced production cost.

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PAPER

CO-EXTRUSION OF COLLAGEN CASINGS. EFFECTS OF PREPARATION, BRINING, AND HEATING ON STRENGTH, RHEOLOGY AND MICROSTRUCTURE

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ABSTRACT

Properties of five collagen preparations were investigated to enhance understanding of in-line co-extrusion casing formation. The first study revealed that 30% NaCl and 5 min brining provided maximum strength. The second study showed 100% difference in tensile strength between preparations; when adjusted to % protein, the difference was smaller but still existed. Extrusion force and elastic modulus (G') also varied; appear to be acid dependent. Denaturation temperature of raw dispersions was between 36.7 and 38.9˚C. Upon salt brining, it substantially increased to 63.3 - 65.3˚C. Polarized light microscopy revealed numerus intact segments of connective tissue fibers and some cellulose fibers.

Keywords: casings, collagen, dispersions, films, mechanical properties

1. INTRODUCTION

A sausage casing is an essential component in the transformation of comminuted meat into a finished product. In the past 100 years there have been a number of technologies that helped improve processing, handling and functional properties of casings' uniformity, hygiene, strength, flexibility and stability during storage (OSBURNE, 2000; SAVIC and SAVIC, 2016). Before the early Twentieth Century almost all sausages were produced with natural casings, derived from animal intestines. Although natural casings are still considered the 'gold standard', advances in casing technology have led to different types of engineered casing. Today, casings are produced with various materials, ranging from regenerated biopolymers (e.g., collagen, cellulose) to thermoplastic materials (e.g., polyvinyl alcohol polymers). Manipulation of these materials has made it possible to tailor casings with specific functional attributes (WANG, 1986; BARBUT, 2015).

Until recently, modern casings fabrication required specialized production facilities due to the complexity of the process (SAVIC and SAVIC, 2016; KARMAS, 1974). The fairly recent development of co-extrusion has eliminated the need to prefabricate, and store casings prior to stuffing. Co-extrusion is a continuous operation of sausage production, where a thin layer of casing material is extruded on the comminuted meat" rope" coming out of the stuffer. Immediately after formation, the casing material undergoes stabilization, through dehydration and/or cross-linking to enhance its mechanical strength. A brine application (spray, drip, immersion) dehydrates the casing and allows the material to conform to the shape of the meat. Further stabilization is commonly performed through cross-linking (e.g., employing the aldehyde in liquid smoke) and air-drying as they both help provide the mechanical strength needed during linking, cooking and packaging (MORGAN *et al*., 1988).

Co-extruded casings can be produced with a collagen gel of fibrous and soluble collagenous material or a dispersion of a hydrocolloid gum such as alginate (MORGAN *et al*., 1988; HARPER *et al*., 2013). The collagen is typically derived from cattle hides and is mainly composed of collagen (RATANAVARAPORN *et al., 2008*). Generally speaking, collagen casing production involves corium separation, decalcification and regeneration. During collagen separation, hides are washed and limed (pH 11 to 13) to remove impurities. Calcium is later removed to promote uniform swelling of collagen fibrils and then the material is chopped and ground. Following decalcification, collagen undergoes regeneration where swelling is promoted via the use of acids (SAVIC and SAVIC, 2016). In commercial products, hydrochloric acid (HCl) is the most commonly used swelling agent, but other acids such as acetic are also used (RATANAVARAPORN *et al., 2008*). Overall, this is a lengthy (few weeks) and complicated process involving quite a few steps; some are proprietary. Prior to extrusion (i.e., at the meat processing plant), the material is mixed one last time to reorient the fibers to improve film strength (SAVIC and SAVIC, 2016; BARBUT, 2010).

Although one of the first patents for collagen co-extrusion technology was introduced about 50 years ago, the technology has not been used on a large commercial scale for the following 30-40 years. Only recently large dedicated plants, for in-line co-extrusion, have been constructed. Today there is still very little published information about the topic (e.g., collagen, composition, viscosity, shear thinning) and even basic data about the effect of raw material source (e.g., animal age, breed), processing parameters during extraction (e.g., type of acid used), and conditions during application (e.g., drying, cross-linking). However, there has been some effort made to understand the properties of certain co-extruded collagen films and the effects of different additives (TOMIHATA *et al.,* 1994;

OLDE DAMINK *et al.*, 1995; O'SULLIVAN *et al.*, 2006; WOLF *et al.*, 2006; HARPER *et al.*, 2012; OESCHSLE *et al.*, 2014; OESCHSLE *et al.*, 2016; BARBUT and IOI, 2019). Overall, the properties of prepared collagen dispersions and processing conditions of co-extrusion casing production needs more attention. Today the co-extrusion process is highly automated and as such also helps to improve food safety and reduce waste (OSBURN, 2000; BARBUT, 2015). The main goal of this research was to evaluate different processing conditions that can be used to partially dehydrate collagen films. The study is believed to be the first to look at the differences between commercially prepared collagen dispersions that are used in the production of co-extruded meat products. Differences were evaluated by studying the collagen dispersions and films' mechanical, microstructure and thermo-mechanical properties. The results should help researchers and manufacturers to better understand co-extrusion through material selection and process controls.

2. MATERIALS AND METHODS

2.1. Collagen dispersions

Collagen dispersions were evaluated and identified as Collagen 1 through 5 (C1, C2, C3, C4, C5), as they are proprietary blends (see explanation in the Introduction). Information that was provided to the researchers, by the two major manufacturers in the world, and compositional analysis can be found in Table 1 (note: manufacturers did not want to provide any specific information on product number/identity as some of the dispersions are still being modified). Protein content was determined in triplicate by the Dumas method (FP528, Leco, St Joseph, MI) using a nitrogen factor 6.25. Moisture content is reported as the moisture lost when drying (Oven 650G, Fisher Scientific, Toronto, ON) from 5 g samples heated at 105˚C for 24 h.

Table 1. Commercial collagen dispersion specifications and the work needed for extrusion (i.e. measurement of resistance to flow) through a 7mm die: C1 (Collagen 1), C2 (Collagen 2), C3 (Collagen 3), C4 (Collagen 4) and C5 (Collagen 5).

Collagen	pH	Moisture Content		Protein (%)	Swelling Agent[a]	Work of Extrusion[b] (Nm)	Work of Extrusion[b] (Nm/%protein)
		Dispersion (%)	Film (%)				
C1	2.06	93.6	74.4	5.14	-	5.16±0.19	1.00±0.04
C2	2.21	95.7	77.8	3.57	-	3.19±0.21	0.89±0.06
C3	2.01	93.6	74.5	3.68	HCl	4.04±0.05	1.10±0.01
C4	2.67	94.1	74.7	4.37	HCl/Acetic Acid	3.63±0.01	0.83±0.01
C5	2.04	93.7	76.9	4.82	-	4.04±0.13	0.84±0.03

[a]Swelling agent as reported by the manufacturer (see text). [b]n = 9.

2.2. Partially dehydrated film formation

The method of film formation was adapted from HARPER *et al.* (2013) who worked with alginate solutions. Briefly, collagen dispersions were first cooled to 4˚C to reduce the

adhesiveness during film formation. The collagen dispersions were also degassed using a vacuum packaging machine (Multivac Canada Inc., Woodbridge, ON) at 7.3 kPa for 25 s, 50 s and 75 s, consecutively (settings 4, 6 and 8, respectively). This was performed to remove gas bubbles that were incorporated during processing, as they can create weak spots in the films. Following the degassing stage, dispersions were mixed to assure homogeneity of the samples (dispersions were mixed by rolling the material, placed in plastic bags, 10 times in adjacent directions).
Later, approximately 3 g portions of the collagen were rolled on a stainless steel board between two layers of plastic sheets with a stainless steel roller. The roller had a recess of 0.50 mm in order to achieve uniform film thickness. The top plastic sheet was removed and the film was then placed in a salt bath on the remaining plastic sheet. The first study was performed with one collagen sample (C2), to evaluate the effects of brine concentration and contact time on the textural properties of the films (note: C2 was selected as it is one of the most popular dispersions used by the industry). Brine solutions consisted of 15, 20, 25 and 30% NaCl in deionized water. Films were immersed in the brine for 1.0, 2.5, 5.0 and 10.0 min intervals. After formation, films were covered again with a plastic sheet to prevent further dehydration before testing.
After establishing the salt concentration and time (see data and conclusions in Discussion), the second study evaluated all five dispersions and their films, which were dehydrated in 30% NaCl brine for 5 min. These conditions ensured that the films were strong enough to be removed from the plastic sheet and tested.

2.3. Extrusion force of collagen dispersions

The collagen dispersions were evaluated by using a texture analyzer (TA-XT2i, Texture Technologies Corporation, Scarsdale, NY) with a forward extrusion fixture (TA 93, Texture Technologies). Approximately 50 g samples were loaded into the cell (100 mL capacity) fitted with a 7 mm opening die, and brought to 4˚C. The plunger compressed the dispersion at a rate of 1 mm/s. From the generated force-distance curve (n = 9 per dispersion) the work of extrusion was calculated once the readings had stabilized (i.e., after pushing down about 10-35 mm).

2.4. Mechanical properties and film thickness

A standard method for testing film's tensile properties (ASTM, 2010) was performed on the partially dehydrated films. Films were evaluated by using the texture analyzer (TA-XT2i, Texture Technologies) equipped with grippers that were set at a distance of 50 mm, trigger force of 5 g, test speed of 2 mm/s, and an overall test distance of 25 mm. The film's thickness was determined by measuring each film at the top, center and bottom using a digital micrometer (Testing Machines Inc., Islandia, NY). The films were cut into 75 mm × 25 mm strips (JDC Precision Sample Cutter, Thwang-Albert Instrument Comp., Philadelphia, PA). The average thickness and width of the films were used for the tensile stress calculations. From the generated stress–strain curve, the tensile strength (maximum stress the film endured prior to breaking) and the percent elongation (maximum elongation the film reached prior to breaking) were determined. A total of eighteen films were tested for each of the treatments (six films per each of the three trials).
A puncture test was also performed with the texture analyzer. In this test, a 5 mm diameter ball probe was used to puncture films mounted in a film extensibility fixture, which has circular opening of 10 mm diameter (TA-108S5, Texture Technologies). The test

speed was 1 mm/s and the trigger force was set to 5 g. The distance to puncture and work of puncture were determined from the generated force - distance curve. A total of eighteen films were tested for each of the treatments (six films per trial).

2.5. Light microscopy

Collagen dispersions were prefixed in 10% neutral buffered formalin for 10 h at room temperature and then dehydrated in 70% isopropanol for 2 h, 95% for 1 h, and 100% for 4 h. The dehydrated samples were dipped in xylene, prior to embedding in paraffin. Samples were cut into 4-6 μm cross sections. Masson stain was used to differentiate collagen from other meat proteins. In another set of sections, Periodic-Acid Schiff (PAS) stain was used to differentiate carbohydrates, specifically cellulose fibers.
A light microscope (Olympus BX 60, Olympus Corporation, Centre Valley, PA) was used to examine the samples. Representative images (a total of six images per treatment) were taken using Image Pro Plus (Version 6.0, Media Cybernetics Inc., Bethesda, MD) software.

2.6. Rheology of film forming dispersion

Rheological analysis was performed on the collagen dispersions using a Bohlin CS50 (Malvern Instruments Ltd, Worcestershire, UK) with a 25 mm DIN coaxial cylinder bob and cup fixture. The bob was lowered into 13 g of collagen that was preloaded into the bottom of the cup. Excess collagen was removed and mineral oil was then applied on the top to keep the exposed surface from drying. The temperature of the collagen was increased from 20° to 55°C at 1.25°C/min, held for 2 min and returned to 20°C, at the same rate. Continuous oscillating shear (1 Hz and 0.0012 strain) was applied during testing. Test parameters were determined in a pre-trial to evaluate the linear range. The elastic modulus (G') was recorded (Bohlin Zetasizer Series software, version 6.32, Malvern Instruments Ltd.) and used to determine the changes in stiffness of the dispersions (HELARY *et al.*, 2009).

2.7. Differential Scanning Calorimetry (DSC)

The melting profiles of the collagen dispersions and partially dehydrated films were evaluated using a differential scanning calorimeter (DSC Q2000, TA Instruments, New Castle, DE). Samples (~10 mg) were placed in anodized-aluminum hermetically sealed pans. Temperature was ramped from 20° to 80°C at a rate of 5°C/min. Samples were then held at 80°C for 2 mins and then cooled back to 20° at 5°C/min. The same thermal profile was used to rescan samples for identifying reversible peaks. The melting behavior was studied between 30° and 80°C by integrating the endothermic peak (TA Universal Analysis 2000 Software, TA Instruments) to determine the onset temperature, melting temperature and enthalpy. A total of three dispersions or films were tested for each of the treatments.

2.8. Amino acid analysis

Collagen dispersion's amino acid profile was analyzed (Advanced Protein Technology Center, Toronto, ON). Approximately 0.01 g of each collagen dispersion was weighed. HCl (6N) and norleucine (internal standard) were added to each sample and then

hydrolyzed for 24 h at 110˚C. After hydrolysis, an aliquot was transferred to another tube for derivatization.

2.9. Experimental design and statistical analysis

The experiment used to compare the different dispersions was designed as a complete randomized block with three independent trials. Each trial consisted of six measurements, per dispersion, for the mechanical properties of the films (tensile and puncture tests). The statistical analysis was performed using SAS Version 9.2 (SAS Inst., Cary, NC). A General Linear Model was used for the analysis of variance (ANOVA). The film type means and interactions were compared by using Tukey's multiple comparison analysis ($P \leq 0.05$). For setting up dehydration conditions (salt concentration and time) the results from dispersion C2 are reported as averages and standard deviations.

3. RESULTS AND DISCUSSION

3.1. Mechanical properties

During formation and stabilization of co-extruded casings, the newly produced sausage is exposed to a variety of different stresses (e.g., moving on a conveyer belt, vibration). It is therefore crucial to impart sufficient strength shortly after extrusion so that the sausages can also undergo subsequent treatment, such as smoking, drying and cooking (KOBUSSEN *et al.,* 2012). In an industrial setting, the newly formed casings are stabilized by first removing some of the water through brining. Dehydration is driven by osmosis, which helps to increase the density of the collagen fibers and thus shortening the distance among collagen molecules to help improve the mechanical stability of the casing (KOBUSSEN *et al.*, 2000; VISSER, 2012). In the first study we established the best conditions for the brining procedure by evaluating the effects of brine concentration (15 to 30% NaCl) and brining time (1 to 10 min) using a dispersion that is widely used by the industry (Collagen 2). The overall settings used were based on guidelines described in several industry protocols (KOBUSSEN *et al.,* 2000). In order to evaluate the mechanical properties of the wet films, (i.e., as applied to the sausage), tensile and puncture tests were performed (Fig. 1).

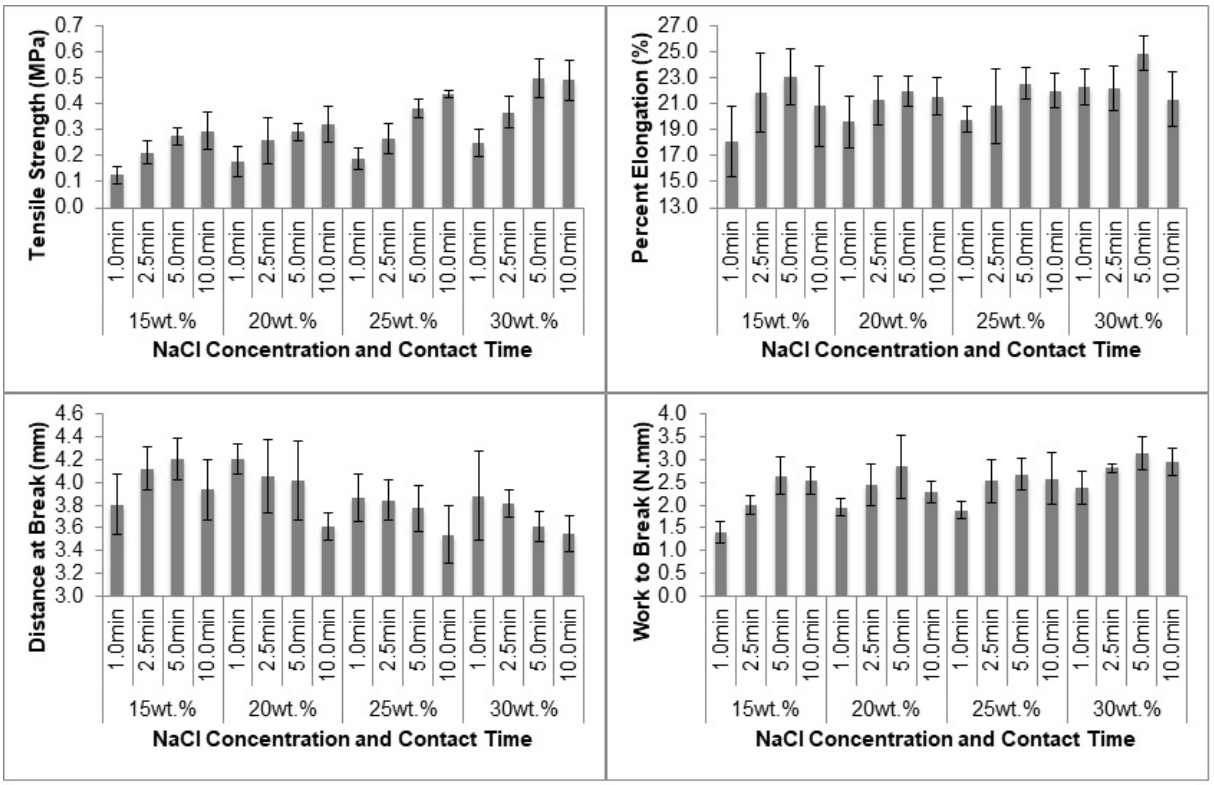

Figure 1. Mechanical properties of collagen films (Collagen 2) produced with increasing concentration of NaCl and contact time (n=18).

Mechanical testing demonstrated that there were no significant interactions (P>0.05) between the brine concentration and contact time. Overall, there were some significant differences (P<0.05) in the film's mechanical properties when modifying the salt concentration or contact time (Tables 2 and 3). Tensile strength, percent elongation and work to break increased with raising salt concentration and contact time. This indicates that there is a need for a certain level of dehydration to stabilize the collagen network in the films. These results also demonstrate that processors would be able to modify the mechanical properties of their casings by altering the concentration or exposure time.

Table 2. Mechanical properties of collagen films (Collagen 2) produced with increasing concentration of NaCl. Means were averaged across contact times; 1.0, 2.5, 5.0, 10.0 min.

Concentration (%)	Tensile Strength[1] (MPa)	Percent Elongation[1] (%)	Distance to Break[2] (mm)	Work of Break[2] (Nmm)
15	0.23^{c}	21.0^{a}	4.0^{a}	2.2^{b}
20	0.26^{bc}	21.1^{a}	4.0^{ab}	2.4^{b}
25	0.32^{b}	21.3^{a}	3.8^{b}	2.4^{b}
30	0.40^{a}	22.7^{a}	3.7^{b}	2.8^{a}

[1]Tensile test.

[2]Puncture test.

Means, within a column, followed by a similar letter are not significantly different (P>0.05).

Table 3. Mechanical properties of collagen films (Collagen 2) produced with increasing contact times to NaCl. Means were averaged across concentrations; 15, 20, 25, 30% NaCl.

Time (min)	Tensile Strength[1] (Mpa)	Percent Elongation[1] (%)	Distance to Break[2] (mm)	Work of Break[2] (Nmm)
1.0	0.19^{c}	20.0^{b}	3.9^{a}	1.9^{b}
2.5	0.28^{b}	21.5^{ab}	4.0^{a}	2.4^{a}
5.0	0.36^{a}	23.1^{a}	3.9^{ab}	2.8^{a}
10.0	0.39^{a}	21.4^{ab}	3.7^{b}	2.6^{a}

[1]Tensile test.

[2]Puncture test.

Means, within a column, followed by a similar letter are not significantly different (P>0.05).

Studying the effects of dehydration conditions helped us to select the best conditions for processing the different commercially prepared dispersions. The higher salt concentration was selected for the evaluations because it provides the most strength and thus helps avoid damaging the casings during further processing. Overall, the film produced with 30% salt had the highest tensile strength and work of puncture (P<0.05), across all contact times (Table 2). This is also the salt concentration currently used in a number of the industrial settings. Although this salt concentration is prepared at the beginning of the day, processors must ensure that it is maintained, because over time it will be diluted (i.e., moisture coming out from the casings; (KOBUSSEN *et al.*, 2012)). The dehydration effect can also be seen in the data concerning moisture content in the films after exposure to the brine solution (Table 1). Table 3 shows that there was no significant difference in mechanical properties between 5 and 10 min. Therefore, a 5 min exposure time was selected for use in the follow up evaluation (second study). It should be mentioned that under commercial conditions, exposure times have been reported to be even shorter (BONTJER *et al.*, 2011). This is usually due to production pressures. In any case, the products are not rinsed after the industrial brining process so high salt concentration remains on the surface.

In the second study, the properties of the actual films produced from the other commercial collagen dispersions were investigated. Overall, there were some significant differences in the tensile strength and percent elongation among the five preparations (Table 4). Films produced with C4 had the lowest tensile strength and percent elongation of the protein films (0.15 MPa and 16.33%, respectively). As will be discussed below, these observations may be correlated to the sample's protein content, collagen structure, acid used for swelling, and the overall dispersions' composition.

The mechanical properties of the partially dehydrated films were also compared after adjusting for protein concentration (Table 5), to examine the effect of protein content (i.e., important for processors dealing with cost per unit). These data reveal differences in the tensile strength, distance at break and work to break; therefore, it would appear that differences among the dispersions are influenced by more than just protein content (assuming protein content affects mechanical properties linearly). It should be noted that meat processors use the dispersions as they arrive to the plant (i.e., no dilution or ingredients added) and therefore cost per unit is critical for them.

Table 4. Mechanical properties of partially dehydrated films: C1 (Collagen 1), C2 (Collagen 2), C3 (Collagen 3), C4 (Collagen 4) and C5 (Collagen 5).

Collagen	Tensile Strength[1] (MPa)	Percent Elongation[1] (%)	Distance at Break[2] (mm)	Work to Break[2] (Nm)	Thickness (mm)
C1	0.33^{a}±0.03	24.38^{a}±3.82	6.12^{a}±0.51	3.72^{a}±0.88	0.43^{a}±0.01
C2	0.29^{ab}±0.05	18.57^{ab}±2.42	5.57^{a}±0.41	2.75^{ab}±0.48	0.37^{b}±0.00
C3	0.19^{cd}±0.02	19.22^{ab}±2.35	5.26^{a}±0.47	1.99^{b}±0.41	0.41^{a}±0.01
C4	0.15^{d}±0.03	16.33^{b}±1.37	5.33^{a}±0.42	1.89^{b}±0.34	0.41^{a}±0.02
C5	0.24^{bc}±0.02	23.42^{a}±1.81	5.91^{a}±0.24	1.81^{b}±0.26	0.42^{a}±0.01

[1]Tensile test.

[2]Puncture test.

Means, within a column, followed by a similar letter are not significantly different (P>0.05).

Table 5. Comparing mechanical properties of partially dehydrated films: C1 (Collagen 1), C2 (Collagen 2), C3 (Collagen 3), C4 (Collagen 4) and C5 (Collagen 5) based on adjusting percent protein.

Collagen	Tensile Strength[1] (MPa/%protein)	Percent Elongation[1] (%/%protein)	Distance at Break[2] (mm/%protein)	Work to Break[2] (Nm/%protein)
C1	0.046^{b}±0.00	3.30^{a}±0.52	0.83^{c}±0.07	0.50^{ab}±0.12
C2	0.061^{a}±0.01	3.94^{a}±0.51	1.18^{a}±0.09	0.58^{a}±0.10
C3	0.035^{bc}±0.00	3.51^{a}±0.43	0.96^{bc}±0.09	0.36^{bc}±0.07
C4	0.029^{c}±0.01	3.12^{a}±0.26	1.02^{ab}±0.08	0.36^{bc}±0.07
C5	0.037^{bc}±0.00	3.57^{a}±0.28	0.90^{bc}±0.04	0.28^{c}±0.04

[1]Tensile test.

[2]Puncture test.

Means, within a column, followed by a similar letter are not significantly different (P>0.05).

Meat processors must also consider the extrusion properties of the dispersion so that they can adjust the co-extrusion equipment. It is interesting to note that today most collagen suppliers do not provide that information. In the current study, the forward extrusion test was performed to provide insight into whether the five dispersions show differences in flow behaviour. Overall, the values varied by as much as 60% (Table 1). It appeared that samples with lower pH (C1, C3 and C5) required a higher work of extrusion. When the values were adjusted to %protein, C1 and C3 were still the highest. It may suggest that a greater degree of conformational changes increases the stiffness of the dispersion. The conformational changes discussed are a result of lowering the pH from 5 to 2 (away from isoelectric point of 8.26 and 4.56 for collagen and collagen hydrolysate, respectively), which has been reported to increase fiber hydration and swelling (WOLF *et al.*, 2006; OESCHSLE *et al.*, 2014). OESCHSLE *et al.* (2014) demonstrated that collagen entanglement depends strongly on the pH as well as acid used, and indicated that entanglement increases with lowering pH values below the isoelectric point.

Lower tensile strength and elasticity could also be the result of excessive alkaline modification during corium separation. If alkaline modification, or liming, is not controlled then the extracted collagen may be of low molecular weight, which will not

contribute as much to the regeneration of collagen structures (SAVIC and SAVIC, 2016). These authors also indicated that intact fibrillar structures produce higher strength and elasticity in collagen casings.
Commercially prepared collagen dispersions are sometimes modified by the addition of functional ingredients, such as fillers (e.g., cellulose), plasticizers (glycerol), cross-linking agents (smoke condensate), as well as colourants (BARBUT and IOI, 2019). The combination of collagen (commonly used at 3 to 8%) and other modifiers (ranges from 0 to 10%) typically results in dispersions with 3 to 25% dry matter (KOBUSSEN *et al.*, 2000). With such a wide range, one can expect quite a lot of variation among dispersions. In the dispersions evaluated here (representing some of the most commonly used by the industry) dry matter ranged from 4.3 to 6.4% (Table 1).

3.2. Light microscope imaging

Light microscopy was used to identify and characterize the homogeneity and condition of the fibers within the collagen dispersion. As previously discussed, collagen dispersions are mixtures of soluble and insoluble collagen. The proportion is affected by the origin of the material, method of extraction (e.g., extent of liming) and processing (e.g., degree of chopping). Masson trichrome stain was used to differentiate collagen from other proteins, and also help study the condition of the collagen fibers in relation to their mechanical and thermo-mechanical properties. Since cellulose was added to some of the samples, PAS stain was also used to identify and characterize carbohydrate fillers (CARSON, 1997) found in the dispersions. Overall, cellulose fibers are one of the most commonly used additives, as the fibers can contribute to the film's strength and elasticity. MATHEW *et al.* (2012) observed that physical entanglements of cellulose nanofibers could increase the tensile strength of dried collagen films. It appears that some of the dispersions studied here contained a certain amount of cellulose (see micrographs below).
The micrographs (Fig. 2) show some differences in the proportions of long and short fibers, as well as the homogeneity of the collagen network (i.e., shown as areas of varying stain intensity throughout the network) among the dispersions. Collagen 3 (C3) appeared to have the greatest homogeneity and as previously mentioned required the highest work of extrusion (1.10 J/% protein) (Table 1). Therefore, the work of extrusion may be attributed to the homogeneity. HELARY *et al.* (2009) also reported that areas of non-homogeneity affect the mechanical properties of collagen hydrogels. As can be seen, collagens C2 and C5 appeared to have more small circular pockets, which may be small air bubbles that stayed within the dispersions.
All of the dispersions show insoluble fibers (some show typical birefringence, as can be seen on the right side of Fig. 2), of pretty much similar size range and morphology. The fibers are suspended in the soluble collagen network (i.e., background stained blue by the Masson stain). Some of the insoluble fibers were identified as cellulose because they had a ribbon-like morphology with twists down their length (REDDY and YANG, 2004; CRANSTON and GRAY, 2008). The ribbon-like fibers also picked up the PAS carbohydrate stain, providing further evidence that they are cellulose (Fig. 3). It is interesting to note that the stained material on the interface between the collagen matrix and cellulose appeared to be darker (Fig. 3). This may indicate that collagen might have developed interfacial interactions with some of the cellulose fibers. Overall, commercial collagen dispersions often have 0.5% cellulose fiber. Since there does not appear to be major differences in the cellulose concentration, the differences in mechanical properties may not be attributed to the addition of cellulose.

3.3. Thermo-mechanical Properties

Understanding collagen melting temperatures is very important to the meat processors because they often cook the sausages in the co-extruded casings. The rheological tests demonstrated that between 30˚C and 40˚C all of the dispersions start to display a rapid decrease in elasticity (Fig. 4). It was observed that the collagen samples with higher pH (Table 1; C4 and C2) began to lose their elasticity at a higher temperature. This may be attributed to the fact that collagen undergoes significant conformational change as one lowers the pH from 5 to 2. As previously mentioned, conformational changes result in increased hydration and swelling (WOLF *et al.*, 2006), therefore this may have reduced the thermal stability of these dispersions.

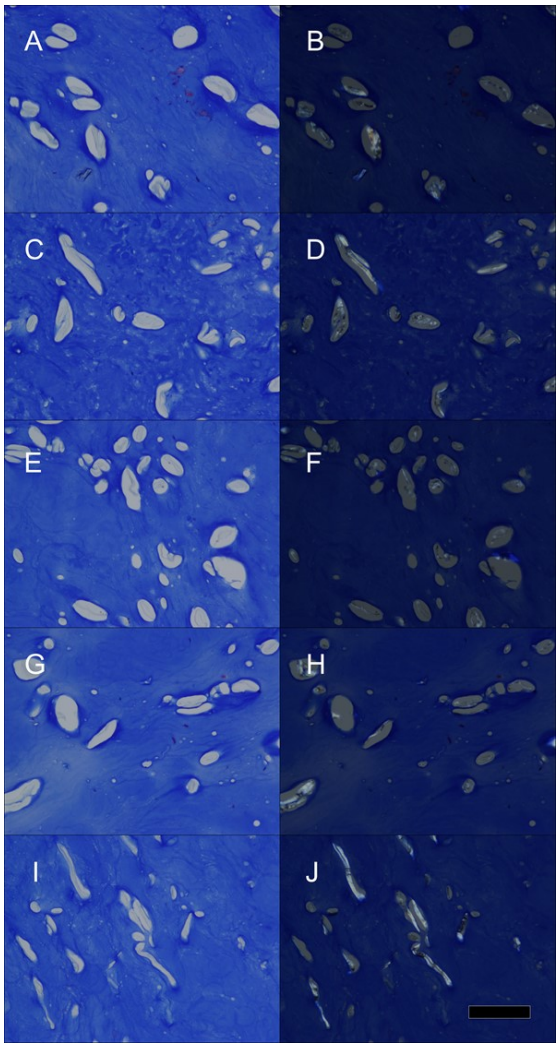

Figure 2. Light micrograph images of commercial collagen dispersions: Collagen 1 under regular illumination (A); Collagen 1 under polarized light (B); Collagen 2 under regular illumination (C); Collagen 2 under polarization (D); Collagen 3 under regular illumination (E); Collagen 3 under polarized light (F); Collagen 4 under regular illumination (G); Collagen 4 under polarized light (H); Collagen 5 under regular illumination (I); Collagen 5 under polarized light (J). Black bar represents 100 μm.

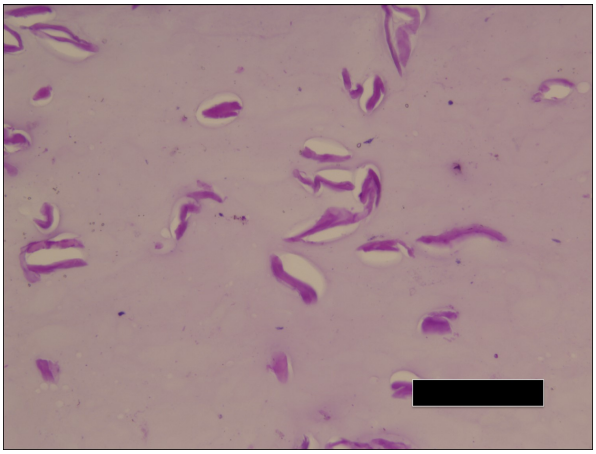

Figure 3. Light microscope image of a commercial collagen dispersion stained with periodic acid schiff. See text for explanation. Black bar represents 100 μm.

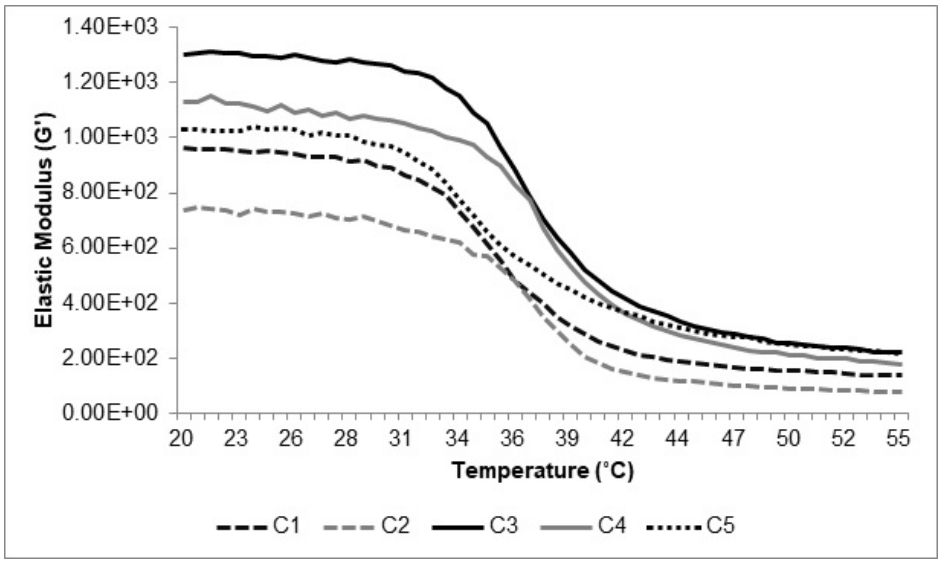

Figure 4. Rheological thermographs (20 to 55˚c at 1.25˚c/min) of five commercial collagen dispersions: C1 (Collagen 1), C2 (Collagen 2), C3 (Collagen 3), C4 (Collagen 4) and C5 (Collagen 5).

DSC scans were performed on the collagen dispersions and partially dehydrated films. The collagen dispersions exhibited an endothermic peak that started between 33.5 to 35.4˚C, with a maximum at 36.7 to 38.9˚C, and had a denaturation enthalpy of approximately 3.1 to 5.3 J/g (Table 6). Once the initial DSC run was completed the samples were cooled down to 4˚C, before a secondary run was performed to look for irreversible changes. The second run of all dispersions resulted in no endothermic peaks, indicating that irreversible denaturation occurred. This is similar to FRIESS and LEE's (1996) observation of insoluble collagen fibers.

The DSC denaturation temperatures are in the same range as the rheological measurements discussed above. Similar to the rheological observations, the collagen with

lower pH displayed lower thermal stability in the DSC thermograms. The dispersions with a pH closer to 2 (Collagens C1, C3 and C5) appeared to have slightly lower denaturation temperatures. As mentioned before, conformational changes at a lower pH may result in a greater hydration (WOLF *et al.,* 2006). GIOFFRE *et al.* (2011) observed similar thermal denaturation behavior when the pH of wet gelatin films was decreased.
The range of onset temperature values corresponds to the rheological transitions observed (Fig. 4) and therefore confirms that the collagen in these dispersions has been significantly modified during the preparation procedure (extraction of collagen from hides, the liming process, and the follow up chopping and acidification procedures). It should be mentioned that in native collagen samples denaturation transitions are seen in the 60˚C range (BERNAL and STANLEY, 1986).
DSC was also performed on partially dehydrated films (brined with 30% salt) to see if there was any effect on the thermal stability of the brined collagen. Overall, dehydrating the films greatly increased the stability. The thermograms of the films showed an endothermic peak that started between 58.2 to 60.3˚C, with a maximum at 63.9 to 65.3˚C and a denaturation enthalpy of approximately 1.7 and 4.1 J/g (Table 6). These denaturation temperatures are fairly similar to those reported by BERNAL and STANLEY (1986) who examined native bovine tendons, and reported denaturation at 61.55˚C. GIOFFRE *et al.* (2011) also observed an increase in the denaturation temperature when wet gelatin films were dried. Thus the difference between the denaturation temperature of the raw collagen dispersions and the partially dehydrated films is the result of some protein denaturation, higher salt concentration and a decrease in moisture content (i.e. 95 to 75%; Table 1). Fiber assembly may also help explain the increased thermal stability of the partially dehydrated and salted films. MCPHERSON *et al.* (1986) suggested that stronger association of collagen fiber structure is correlated with increased denaturation temperature. It has also been demonstrated that high ionic strength conditions result in a greater degree of packed collagen fibers and assembly (WILLIAMS *et al.*, 1978). Overall, since the ionic strength is raised during film dehydration (salt migration into the film), there may be collagen fiber assembly, resulting in a higher thermal stability of the films.

Table 6. Analysis of endothermic peaks from differential scanning calorimetry (DSC) thermograms. Five commercial collagen samples were tested as collagen dispersions and partially dehydrated/brined films: C1 (Collagen 1), C2 (Collagen 2), C3 (Collagen 3), C4 (Collagen 4) and C5 (Collagen 5).

Collagen	Treatment	Onset Temperature (˚C)	Temperature of Denaturation (˚C)	Enthalpy ΔH (J/g)
C1	Dispersion	33.54±0.21	36.71±0.51	5.33±0.61
C2	Dispersion	34.59±0.15	38.44±0.06	3.05±0.31
C3	Dispersion	34.26±0.01	38.09±0.08	4.12±0.10
C4	Dispersion	35.41±0.11	38.94±0.02	3.93±0.26
C5	Dispersion	33.45±0.10	37.30±0.21	4.45±0.03
C1	Film	59.90±0.23	64.87±0.12	3.07±0.55
C2	Film	58.40±0.21	63.88±0.57	1.76±0.38
C3	Film	60.32±1.61	65.00±0.68	3.05±0.21
C4	Film	58.22±0.24	63.94±0.61	3.06±0.82
C5	Film	58.30±0.40	65.34±0.37	4.19±0.37

3.4. Amino acid analysis

In addition to the protein content, the protein quality may also influence the mechanical and thermal behaviour of the collagen fibers. Analyzing the amino acid profile (Table 7) was done to examine potential correlations between the protein quality and performance of the collagen dispersions. Collagen fiber's properties vary as a result of the formation of cross-links between overlapped collagen molecules. Cross-links between molecules and fibers can be formed via a number of different mechanisms: Schiff base cross-links from enzymatic oxidation (lysyl oxidase), or through non-enzymatic processes like glycation (glucose, lysine and arginine). In general, lysine, glutamic acid and hydroxyl groups have been classified as reactive groups because they project radially from collagen molecules, thus providing sites for intermolecular and interfibrillar cross-links to occur (AVERY and BAILEY, 2008). Overall, there appeared to be some differences in the amino acid makeup of the dispersions (e.g., Lys, Glu, OH-Pro, Arg), but there were no observable trends.

Table 7. Amino acid profile (relative molecular mass) of commercial collagen dispersions.

Amino Acid	Relative Molecular Mass (%)				
	Collagen 1	Collagen 2	Collagen 3	Collagen 4	Collagen 5
Asx (Asp+Asn)	4.6	5.0	5.1	5.3	4.7
Glx (Glu+Gln)	7.5	6.9	7.8	8.2	6.9
OH-Pro	14.1	13.1	14.3	14.1	12.8
Ser	3.9	3.9	3.9	3.9	3.9
Gly	23.1	22.9	22.4	22.2	23.4
His	1.1	0.9	1.1	1.1	0.9
Arg	10.6	9.5	10.5	10.6	9.4
Thr	1.7	1.8	1.8	1.8	1.8
Ala	7.0	8.0	6.9	6.9	7.8
Pro	15.8	15.1	15.3	15.1	16.1
Tyr	0.6	0.6	0.7	0.7	0.5
Val	1.7	2.1	1.7	1.8	2.1
Met	2.1	1.7	2.1	1.9	1.8
Ile	1.0	1.3	1.1	1.1	1.1
Leu	1.5	2.0	1.4	1.4	1.9
Phe	1.5	2.2	1.9	1.9	2.1
Lys	2.2	2.8	2.0	2.0	2.9

Note: values based on a single determination.

4. CONCLUSION

Manipulating the dehydration conditions (brine concentration; 15 to 30% NaCl, and contact time; 1 to 10 min) resulted in differences in mechanical properties when the brine concentrations were averaged across contact times and contact times were averaged across salt concentrations. This indicates that meat processors can adjust the performance of their casings through the modification of brine concentration and contact time.

The results also show differences among commercial collagen dispersions and provide actual values (currently not available in the literature), as well as some potential explanations for the differences. The mechanical evaluation of dispersions and films demonstrated that there are significant differences in flow behavior of the raw dispersions as well as tensile strength and percent elongation of the partially dehydrated films (i.e., after the common brining process). It was suggested that a higher degree of intact fibers can result in a collagen film with higher tensile strength and elasticity. Collagen dispersions with pH values closer to 2 seem to exhibit lower thermal stability, as conformational changes in the fiber structure occur at lower pH. Furthermore, the process of partially dehydrating the collagen fibers greatly increases the temperature of denaturation.
Overall, this paper provides research and industry personnel with a better understanding of the parameters important for co-extrusion of collagen dispersions into casings, through material selection and manipulation of brining operations.

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PAPER

TEXTURAL, PHYSICAL AND RETROGRADATION PROPERTIES OF MUFFIN PREPARED WITH KAMUT (*TRITICUM TURANICUM* JAKUBZ)

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ABSTRACT

The effects of kamut flour substitution levels (0%, 25%, 50%, 75%, 100%) on muffin properties were investigated. As kamut flour level increased muffins showed lower height and volume and the L value decreased. The crumb hardness increased with increasing kamut level, and the control showed the lowest value in elasticity, chewiness, and brittleness. The increment of kamut flour level resulted the total flavonoid and polyphenol contents, reducing power, and ABTS and DPPH radical scavenging activities. During storage, the avrami exponent decreased between the control to the sample added with 75% kamut. The crumb air cell number decreased, but the area increased with kamut flour level increment. In sensory evaluation, the samples with kamut level 25% and 50% were acceptable. Therefore, muffins with an appropriate level of kamut flour improve the nutritional profile, and quality of baking products.

Keywords: antioxidant, kamut (Khorasan wheat), muffin, physical properties

1. INTRODUCTION

In human nutrition, wheat is an important component of cereal-based foods, and it is one of the most consumed food sources on a global scale (SOFI *et al*., 2013). As the awareness of healthy life increases, healthy food products are more in demand. Following this trend, various wheat varieties such as whole wheat, organic wheat, and ancient wheat, have emerged in the market (ANGIOLONI and COLLAR, 2011; FATMA *et al*., 2017; DINU *et al*., 2018). Among the wheat varieties, kamut (Khorasan wheat, *Triticum turanicum* Jakubz) is one of the noted ancient grains due to the higher content of selenium content and the protein content. Kamut contains 400-1000 ppb of selenium depending on harvesting condition and contains relatively large amounts of 12-18/100 g of protein (WIJNGAARD and ARENDT, 2006; DI LORETO *et al*., 2017). In addition, consuming whole grain and the products from its derivative could be providing antioxidant substances and various cofactors such as Copper, Iron, Zinc and Selenium (BENEDETTI *et al*., 2012).
In recent studies (BORDONI *et al*., 2017; CARNEVALI *et al*., 2014; DI LORETO *et al*., 2017), the whole grain kamut was found to contain higher phytochemical contents than common wheat, and it has been attracting attention due to its nutraceutical properties such as high antioxidant, prebiotic activities, and reduction of irritable bowel syndrome symptoms. In addition, in human intervention studies, a volunteer group that consumed kamut products for 8 weeks demonstrated a significant decrease in total and LDL cholesterol and glucose levels while the control group showed no significant changes.
The popularity of bakery products, especially with health functionalities, is increasing and as the consumption of cereal-based products increase, these products are important for taking essential nutrients in daily life (ALPASLAN and HAYTA, 2006). Among the bakery products, muffins are easy to make into various products depending on the ingredients to be added, so the studies on functional muffins such as legume blended muffin, coffee ground residue water extracts muffin, flaxseed muffin and buckwheat muffin (KIM *et al*., 2016; BAE and JUNG, 2013; KAUR and KAUR, 2018; QIAN *et al*., 2017) are briskly. Also, muffins have high acceptance for the consumer due to sweet taste and soft texture and are characterized by typical pore formation.
Previous studies on kamut have been on antioxidant effects of kamut in the rat liver, sourdough bread, flake and muesli, tortillas and cookies (BENEDETTI *et al*., 2012; CARINI *et al*., 2010; CHANDI *et al*., 2015; CHOI *et al*., 2016; SUMCZYNSKI *et al*., 2015). The present study, therefore, focused on antioxidant, baking, rheological, microstructural, storage and quality characteristics of muffins with whole grain kamut and with the aim to find the optimal addition level of kamut for increasing utilization of kamut and development of functional bakery products.

2. MATERIALS AND METHODS

2.1. Muffin materials

Kamut (KAMUT® International, Ltd., Missoula, USA) cultivated in Canada in 2016 was purchased. Kamut (Khorasan wheat) grains were washed three times and freeze-dried (FD8508, Ilshinbiobase Co., Dongducheon, Korea) at -80℃ for 5 days. Dried kamut grains were ground (RT-04, Wongangbio Co., Taiwan) for 2min, passed through a 40 mesh sieve twice to obtain kamut flour (KF), and stored at -20℃ until use. A soft wheat flour (WF) (Q1, Samyang Co., Asan, Korea), salt (Chungjungwaon, Shinan, Korea), sugar (Beak-seol,

Incheon, Korea), egg (Nature egg, Yeo-ju, Korea), butter (Unsalted Pure New Zealand Butter, Fonterra, New Zealand), milk (Seoul milk, Chung-ju, Korea), and baking powder (Baking soda, Gimpo, Korea) were acquired at a local market.

2.2 Preparation of muffin samples

The muffin formulations are presented in Table 1. Muffins were prepared using a modified method as described by QIAN *et al*. (2017). Five different muffin samples with various ratio of KF and WF [100 WF (CON), 75 WF:25 KF (K25), 50 WF:50 KF(K50), 25 WF:75 KF(K75), 100 KF(K100)] were prepared along with a control sample (100 WF). Butter, salt and sugar were whipped with a blender (KMM020, Kenwood, Havant, England) for 3 min at 40 rpm. The egg was added in two portions and mixed for 5 min at 54 rpm. Flour mix and baking powder were added and mixed at 40 rpm for 2 min. Finally, milk was blended at 40 rpm for 2 min. The muffins were cooled for 1 h at 25℃ after baking and were used for analyses.

Table 1. Formula for a muffin with different levels of KF.

Samples	CON	K25	K50	K75	K100
Wheat flour (g)	200	50	100	150	0
Kamut flour (g)	0	150	100	50	200
Salt (g)	2	2	2	2	2
Sugar (g)	120	120	120	120	120
Egg (g)	70	70	70	70	70
Butter (g)	80	80	80	80	80
Milk (g)	100	100	100	100	100
Baking powder (g)	6	6	6	6	6

CON: Control. Without added KF. K25: 75% WF, 25% KF. K50: 50% WF, 50%KF. K75: 25% WF, 75% KF. K100: 100% KF.

2.3. Physicochemical measurement

2.3.1 Moisture and Brix degree

The moisture content was measured at 105℃ in 5.0 g of crumb parts of muffins with a moisture analyzer (MB, OHAUS, Zurich, Switzerland). The sugar content was measured with a digital refractometer (PR-201 α, Tokyo, Japan) having a range of 0-60% by stirring 5.0 g of sample and 50 g of distilled water for 5 min (SONG *et al*., 2017).

2.3.2 Batter measurement

The batter specific gravity was measured at 25℃ by standard methods of analysis (AACC, 2000). The baking loss and baking yield were calculated according to the following formulas using the batter weigh.

$$\text{Baking loss (\%)} = [(\text{batter weight} - \text{muffin weight})/\text{batter weight}] \times 100$$
$$\text{Baking yield (\%)} = (\text{muffin weight}/\text{batter weight}) \times 100$$

2.3.3 Physical properties

Muffin weight was measured with a digital scale (EB-2200HU, Dong-il Shimadzu Corp., Seoul, Korea). Muffin volume was measured by the method of seed displacement (Pyler, 1979). Specific volume (mL/g) was determined by dividing the muffin volume (mL) by muffin weight (g). Muffin height was measured as the vertical distance from the bottom to the top of the muffin center using vernier calipers.

2.3.4 Appearance

The color values of both crumb and crust were measured by a spectrophotometer (CR-400, Konica Minolta Co., Ltd, Tokyo, Japan). The color values were shown as lightness (L), redness (a), yellowness (b) and total color difference (‧E). The ‧E was calculated by the following equation. The appearance and cross section of muffins were captured by a digital camera (X-T20, Fujifilm, Tokyo, Japan).

$$\Delta E = \sqrt{(L_{\text{sample}} - L_{\text{standard}})^2 + (a_{\text{sample}} - a_{\text{standard}})^2 + (b_{\text{sample}} - b_{\text{standard}})^2}$$

2.4. Textural analysis of muffin crumb

Texture Profile Analysis (TPA) was performed on muffins at a 25℃. The samples (20 mm × 20 mm × 20 mm) were measured by a two-bite compression test using rheometer (Compac-100Ⅱ, Sun Scientific, Tokyo, Japan). In this measurement, the cylindrical probe (20 mm diameter) was mounted and operated at 1.0 mm/s. Hardness (N), springiness (%), cohesiveness (%), chewiness (g) and brittleness (g) were determined. Hardness refers to the maximum force with the maximum peak of the first compression. Springiness is the deformation rate between the first compression and the second compression, defined as the ratio of distances (d1: the maximum distance of the first bite; d2: the distance to the deformed sample surface in the second bite). Cohesiveness is the strength of internal bonds and defined as the ratio of area. Chewiness is calculated by multiplying the hardness value by the cohesiveness value. Brittleness, also called fracturability, is a measure of force at the first peak.

$$\text{Springiness (\%)} = d_2/d_1 \times 100$$
$$\text{Cohesiveness (\%)} = A_2/A_1 \times 100$$

2.5. Antioxidant activity properties

2.5.1 Antioxidant compound extraction

The muffins were freeze-dried at -80℃ for 48 h and ground for 1 min (40 mesh). The muffins were defatted with hexane at a ratio of 1:5 w/v (3 min, 3 times), dried at 45℃ for 5 h, and extracted in a water bath (BS-20, Jeio Tech, Seoul, Korea) at 185 rpm for 1 h at 40℃

with 70% methanol at a ratio of 1:10 w/v. The sample extracts were filtered using Whatman filter (No. 4) and kept at 4℃ for subsequent experiments.

2.5.2 Total polyphenols content

Total polyphenols content was determined by the Folin-Ciocalteu method. Briefly, 50 μL of 0.9 N Folin-ciocalteu's reagent (Merk KGaA, Darmstadt, Germany) and 150 μL of 20% sodium carbonate solution (Merck KGaA, Darmstadt, Germany) were added sequentially to the extracted samples (20 μL) mixed with distilled water (790 μL). After incubating for 2 h incubation at 25℃, the absorbance of the mixed sample was read at 700 nm using an ELISA microplate reader (Apollo11LB913, Berthhold Thechnolosies Co., Ltd., Bad Wildbad, Germany). The total polyphenol content (μg GAE/g) was converted to gallic acid equivalents.

2.5.3 Total flavonoids content

Total flavonoids content was examined by the method of ZHANG *et al*. (2017). Briefly, 150 μL of 5% sodium nitrie (Junsei Chemistry) was added to 1 mL of samples and incubated in the darkroom at 25℃ for 6 min. Subsequently, 0of 10% $AlCl_3$ was added to the mixed samples and incubated in the darkroom at 25℃ for 5 min. Finally, 1 mL of 1 N NaOH was mixed, and the absorbance of the sample mixture was read at 520 nm using an ELISA microplate reader (Apollo11LB913, Berthhold Thechnolosies Co., Ltd., Bad Wildbad, Germany). The total flavonoids content (μg QE/mg) was converted to quercetin equivalents.

2.5.4 Reducing power

Reducing power of the extracted samples was determined by a modified OYAIZU (1986) method. Briefly, 250 μL of 0.2 M phosphate buffer, the mixture of sodium phosphate monobasic solution and sodium phosphate dibasic solution (1:2), and 250 μL of 1% potassium ferricyanide solution were added to 250 μL of samples and incubated for 30 min at 50℃. Subsequently, 250 μL of 10% trichloroacetic acid solution was mixed. Finally, 500 μL of distilled water and 100 μL of 0.1% $FeCl_3$ were added to the sample supernatant (500 μL) and the absorbance of the sample mixtures was read at 700 nm using an ELISA microplate reader (Apollo11LB913, Berthhold Thechnolosies Co., Ltd., Bad Wildbad, Germany).

2.5.5 DPPH radical scavenging assay

DPPH assay refers to the method of JOUNG *et al*. (2017). The 200 μmol DPPH reagent was added to the diluted samples extracts (10, 12.5, 20, 25, 50, 100 mg/mL) and reacted in the darkroom at room temperature for 30 min. The absorbance of the sample mixtures was read at 520 nm using an ELISA microplate reader (Apollo11LB913, Berthhold Thechnolosies Co., Ltd., Bad Wildbad, Germany).

2.5.6 ABTS radical scavenging assay

The ABTS assay was measured with reference to the method of ZHANG *et al*. (2017). The ABTS reagent with the absorbance of 1.5 at 405 nm was added to the diluted samples (10, 12.5, 20, 25, 50, 100 mg/mL) and reacted in the darkroom at room temperature for 60 min. The absorbance of the sample mixtures was read at 405 nm using an ELISA microplate reader (Apollo11LB913, Berthhold Thechnolosies Co., Ltd., Bad Wildbad, Germany).

2.6. Air cells determination

Muffins were cut at the height of muffin mold (2 cm), and images of the bottom half were obtained using a digital camera (X-T20, Fujifilm, Tokyo, Japan). By pore size, air cell number and area were calculated using the ImageJ software (MARCET *et al*., 2015).

2.7. Microstructure of batter and muffins

2.7.1 Batter microstructure

A drop of batter was placed on a microscope glass slide and covered with a cover glass. The cover glass was used to apply constant force (1 kg) to equalize and thinly spread the batter layer. The batter samples were observed using a microscope (TS100, Nikon, Tokyo, Japan). The Infinity Capture V6.5.6 for Windows software was utilized with the Infinity lite camera (Lumenera, Ottawa, Canada).

2.7.2 Crumb microstructure

Scanning electron microscopic (SEM) studies were examined using the JSM-6701F (JEOL Ltd., Tokyo, Japan). Sample preparation for SEM was according to the modified method of SHIN *et al*. (2018). The crumb samples were freeze-dried (FD8508, Ilshinbiobase Co.), and pieces of samples (size 2 × 4 mm) were placed separately on aluminum specimen mount using Nem tape and conductive graphite (Ted Pella Inc., California, USA). Mounted samples were coated with Au using the JSM 670-1F (JEOL Ltd., Tokyo, Japan) at 10 mA for 2 min. Each sample was observed at 10 kV and 1.16×10^{-4} torr vacuum.

2.8. Retrogradation kinetics of Avrami model

Retrogradation of the muffin was analyzed with reference to BERSKI *et al*. (2018). Muffin samples were stored at 25℃ for 35 days, and hardness was measured on a rheometer (Compac-100Ⅱ, Sun Scientific, Tokyo, Japan) at the date of production and every week after preparation. According to the Avrami equation, the retrogradation status of muffins was measured by analyzing the alteration of hardness with the storage period. Avrami equation was as follows:

$$\theta = e^{-kt^n} \quad (1)$$

Where θ is the amorphous part remaining after a certain time (t), k is the rate constant, n is the Avrami exponent, and t is the storage period.

The operating conditions for the rheometer were a TPA test using the cylinder probe No.1 (Φ 20 mm), 2 × 2 × 2 cm sample size, 120 mm/min table speed, 66.67% distance, and 10 kg max weight.

$$(E_L\text{-}E_t) / (E_L\text{-}E_0) = e^{-kt^n} \quad (2)$$

Where E_0 is the hardness of the initial period ($t = 0$), E_t is the hardness after a certain time (t) and E_L is the greatest hardness value to be reached theoretically (the hardness value of a muffin stored at 25℃ for 35 days).
The following equation was obtained by taking a common logarithm of equation (2) as:

$$\log \{- \ln (E_L\text{-}E_t) / (E_L\text{-}E_0)\} = \log k + \mathrm{n} \log t \quad (3)$$

Where n is the Avrami exponent (1 ~ 4 values, depending on the crystallization status), and k is rate constant.

2.9. Sensorial evaluation

The muffin samples were baked 3 h before sensory evaluation. The samples were divided into four parts and supplied with water to each panelist at once in a white plastic plate. The sensory evaluation was completed by 51 panelists from Korea University (age range 20-60). The panels evaluated the muffins based on their appearance, flavor, texture, taste and overall acceptability using a hedonic scale of 9 points (9 score is "I like very much" and 1 score is "I dislike very much").

2.10. Statistical analysis

The statistical analysis of results was completed using the SPSS (IBM SPSS Statistics 23, International Business Machines Corporation, New York, USA) program. Significant differences were assessed by one-way ANOVA (analysis of variance) followed by Duncan's multiple range test at a 95% significance level (P<0.05).

3. RESULTS AND DISCUSSIONS

3.1. Muffin physicochemical characteristics

The physicochemical features of muffins are shown in Table 2 (Fig. 1.) The results suggest that the addition of KF significantly affected the moisture content and brix degree (P<0.05). Muffin crumb moisture content was the highest in K100 (28.18%) and the lowest in CON (24.78%). Similar to the description by GURPREE *et al*. (2015), this is due to the water absorption of KF. With increasing KF replacement level, muffin moisture content tended to increase. As the result of comparing the water binding capacity of KF and WF, the water binding capacity of KF (225.09%) showed a higher value than that of WF (198.13%) (data not shown). As the water binding capacity results showed, the moisture content of muffins was significantly increased with the addition of KF. The sugar content of muffins was expressed in brix degree, which is the basic criterion to evaluate sugar contents. Muffin sugar contents diminished significantly (P<0.05) as the KF substitution

increased. Due to the Maillard reaction, which is the reaction between the reducing sugar and the amino acids. The Brix degree represents the content of soluble solids, and the reducing sugar involved in the Maillard reaction is also included in the soluble solids. As the KF replacement level increased, the muffin lightness value decreased and became significantly darker, suggesting that the number of soluble solids was reduced by increasing the nonenzymatic browning reaction. According to a study by ZHANG *et al*. (2016), the brownish or yellowish color of bakery products with high sugar content were caused by the Maillard reaction, which is mainly found in the baking process. The baking loss significantly decreased with increasing KF substitutions (P<0.05). The lowest baking loss was found in K100 (10.67%). The baking yield was the lowest in CON (83.43%) and the highest in K100 (89.33%), and it significantly increased as the KF substitution ratio increased. The results are similar to the study of KOTOKI and DAKA (2010), suggesting that the water binding capacity affects the water content of muffin crumb, which may affect baking yield and baking loss. The specific gravity of the batter was the lowest in CON (0.94), and it significantly increases with the increased in KF substitution levels. The muffin height significantly decreased as the KF substitution rate rose in the batter (P<0.05). The CON muffin had the highest volume (121.83 mL) and specific volume (2.89 mL/g), and these were significantly decreased as the KF substitution ratio decreased. While the CON muffin showed the lowest weight (42.11 g), it significantly increased with the increasing KF substitution percentage.

The lightness (L), yellowness (b), redness (a), and color difference (‣E) values of both crust and crumb are shown in Table 3. Crust L, a and b were the lowest in K100, and the L and b values of crust were the highest in CON. The crust color was affected by the Maillard or caramelization reaction between proteins and sugar. Crumb L and b values decreased significantly, while the a value increased significantly as the KF substitution increased (P<0.05). The crumb color value was affected by the color of the raw material, which was not heated sufficiently to drive the Maillard or caramelization reaction (MARCHETTI *et al*., 2018). The crust ‣E values of CON and K25 were lower than other samples. As the KF proportion increased, the crumb ‣E values increased significantly (P<0.05).

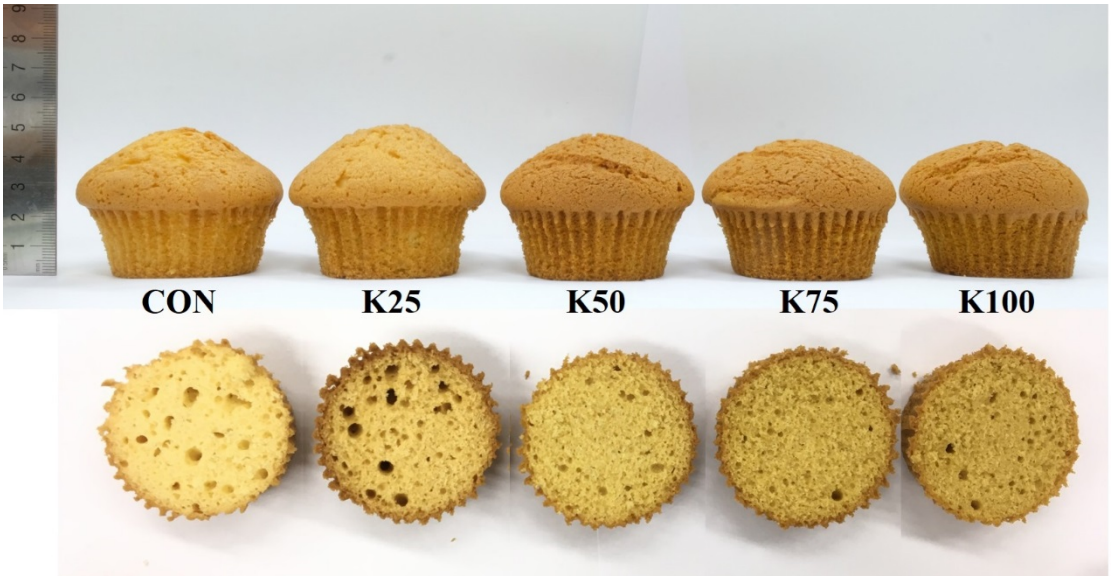

Figure 1. Photograph of muffins and cross section of muffin crumbs with different levels of KF.

Table 2. Some physicochemical properties of muffin with different levels of KF.

Samples	Moisture (%)	Brix degree	Baking loss (%)	Batter yield (%)	Specific gravity	Height (mm)	Volume (mL)	Specific volume (mL/g)	Weight (g)
CON	24.78±0.29 [1)d]	3.03±0.20[a]	16.57±0.20[a]	83.43±0.20[d]	0.94±0.00[e]	48.56±0.43[a]	121.83±2.02[a]	2.89±0.05[a]	42.11±0.07[d]
K25	25.95±0.17 [c]	2.63±0.06[b]	14.22±0.28[b]	85.78±0.28[c]	0.95±0.01[d]	47.24±0.42[b]	117.5±1.50[b]	2.72±0.03[b]	43.16±0.09[c]
K50	26.92±0.43 [b]	2.50±0.00[bc]	11.85±0.03[c]	88.15±0.03[b]	0.96±0.00[c]	47.26±0.47[b]	114.23±0.76[c]	2.58±0.02[c]	44.45±0.02[b]
K75	27.30±0.45 [b]	2.40±0.00[c]	11.66±0.13[c]	88.34±0.13[b]	1.04±0.00[b]	46.64±0.21[b]	107.83±1.26[d]	2.43±0.03[d]	44.33±0.10[b]
K100	28.18±0.22 [a]	2.33±0.06[c]	10.67±0.09[d]	89.33±0.09[a]	1.04±0.00[a]	45.3±0.33[c]	90.33±1.26[e]	2.01±0.03[e]	45.04±0.05[a]
F-value	46.567[***]	23.100[***]	591.725[***]	591.725[***]	616.542[***]	28.328[***]	226.868[***]	317.512[***]	782.063[***]

[1)]The data are mean±SD in triplicates.
[a-e]Different superscripts indicate there are significant differences between values in the same row according to Duncan's multiple range test at $p<0.05$.
[*]$p<0.05$, [***]$p<0.001$.

Table 3. Color measurement of muffin crust and crumb with different levels of KF.

Samples	Crust				Crumb			
	L[2)]	a	b	ΔE	L	a	b	ΔE
CON	48.51±0.44[1)a]	8.99±0.05[b]	15.21±0.06[a]	50.90±0.54[b]	78.51±0.29[a]	-3.74±0.32[d]	24.26±0.42[a]	28.68±0.35[e]
K25	47.78±0.10[b]	8.75±0.16[bc]	15.57±0.54[a]	51.74±0.28[b]	67.68±0.34[b]	-1.02±0.12[c]	23.05±0.27[b]	36.42±0.50[d]
K50	42.87±0.59[d]	9.64±0.07[a]	14.17±0.57[b]	56.11±0.56[a]	61.45±0.40[c]	-0.79±0.09[c]	22.02±0.82[c]	41.63±0.31[c]
K75	44.02±0.30[c]	7.80±0.18[d]	13.13±0.32[c]	55.08±0.99[a]	55.77±0.16[d]	0.97±0.11[b]	20.98±0.12[d]	45.68±0.20[b]
K100	43.67±0.40[c]	8.53±0.21[c]	12.85±0.75[c]	56.03±0.23[a]	52.04±0.32[e]	1.57±0.07[a]	19.89±0.22[e]	49.45±0.36[a]
F-value	125.283[***]	60.174[***]	17.172[***]	53.359[***]	3370.949[***]	455.629[***]	44.380[***]	1557.270[***]

[1)]The data are mean±SD in triplicates.
[2)]L: lightness, a: redness, b: yellowness, ΔE: total color difference.
[a-e] Different superscripts indicate there are significant differences between values in the same row according to Duncan's multiple range test at $p<0.05$.
[*]$p<0.05$, [***]$p<0.001$.

3.2. Textural properties of muffin crumb

The texture of food products is a close factor to the body, such as the feeling of the mouth or fingers. The results of the muffin textural analysis prepared from the various ratio of WF and KF are presented in Table 4. The results showed that KF significantly affected the muffin crumb texture (P<0.05). Hardness was 1.15 N in CON and as the KF ratio increased, the hardness increased significantly (P<0.05), reaching K100 at 3.09 N. The increment in hardness is related to fewer and smaller air cells inside the muffin crumb, and it corresponds to the muffin volume, height, and weight. TESS *et al*. (2015) also mentioned that volume and hardness have an inverse relationship. Springiness is related to aeration and freshness of products, and especially in bakery products, high springiness has close relevance to high quality (MATOS *et al*., 2014). The K100 muffin showed the highest value in springiness, and an appropriate addition of Kamut flour seemed to have a good effect on the muffin textural quality. Cohesiveness is the parameter that signifies the perception linked to the energy required to bite the piece of food and sensory brittleness (SANZ *et al*., 2009). In comparison to CON, the other samples were significantly (P<0.05) decreased in cohesiveness, which suggests that energy was required for the second compression. The chewiness reflects the parameter associated with the energy required for biting activity from a solid form to a swallowable state (TESS *et al*., 2015). CON chewiness showed the lowest value at 0.54 N and it increased significantly (P<0.05) with the increasing KF ratio. K100 chewiness was approximately twice as high as that of CON. Brittleness is related to the muscle motion of biting food, and it has a correlation with hardness (PENG *et al*., 2002). Brittleness in CON was 56.57 g, and it significantly (P<0.05) increased, reaching 214.27 g in K100 and following a similar trend as hardness and chewiness. The previous study carried out by PASQUALONE *et al*. (2011) demonstrated that the crumb firmness of durum wheat bread was slightly lower than Kamut bread, but there was no significant difference.

Table 4. Textural profile analysis of muffin crumbs with different levels of KF.

Samples	Hardness (N)	Springiness (%)	Cohesiveness (%)	Chewiness (N)	Brittleness (g)
CON	1.15±0.12[1)c]	69.65±3.27[c]	67.03±2.63[a]	0.54±0.06[b]	56.57±9.74[e]
K25	1.40±0.15[c]	70.25±1.75[c]	61.56±0.24[b]	0.61±0.05[b]	84.04±3.08[d]
K50	1.73±0.25[c]	75.29±2.11[b]	59.92±0.92[b]	0.78±0.10[b]	110.62±2.52[c]
K75	2.39±0.30[b]	79.86±1.37[a]	54.46±1.48[c]	1.04±0.16[a]	150.87±3.54[b]
K100	3.09±0.60[a]	82.32±0.96[a]	49.17±0.55[d]	1.25±0.24[a]	214.27±3.24[a]
F-value	16.882[***]	21.252[***]	68.269[***]	13.309[**]	426.197[***]

[1)]The data are mean±SD in triplicates.

[a-e]Different superscripts indicate there are significant differences between values in the same row according to Duncan's multiple range test at p<0.05.

[**]P<0.01, [***]p<0.001.

3.3. Antioxidant properties of muffin crumb

The Kamut flour showed significantly higher antioxidant activities than wheat flour. The total polyphenol content was 23.44 μgGAE/mg in kamut flour and 17.74 μgGAE/mg in wheat flour. The total flavonoids content was 27.75 μgQE/mg in kamut flour and 8.44 μg QE/mg in wheat flour. In the results of reducing power, kamut flour (0.84) was about four times higher than wheat flour (0.21). DPPH and ABTS results also showed that kamut flour (129.91 μg/mL and 130.91 μg/mL respectively) had lower IC_{50} values than wheat flour (162.21 μg/mL and 325.79 μg/mL respectively) so that kamut flour had higher radical scavenging activities. SOFI *et al*. (2013) reported a comparison of antioxidant activities between Kamut flour and wheat flour, and Kamut was determined as superior in DPPH and Fe^{2+} chelation. In addition, polyphenols and flavonoids were higher in Kamut. The antioxidant properties of muffins are presented in Table 5. The total polyphenol content and total flavonoid content were determined in terms of gallic acid equivalent and quercetin, respectively. K100 (17.33 μgGAE/mg) was the richest in polyphenols, which was 2.62 times higher than CON. The total flavonoids content was also the highest in K100 (18.54 μgQE/mg), and it increased significantly as KF level increased (P<0.05). The replacement of WF with KF showed an increment in reducing power. The reducing power of extracts is regarded as an indicator of antioxidant activities (KAUR and KAUR, 2018). A significant increment in ABTS and DPPH radical scavenging activities of muffin crumbs was observed as the KF level increased (P<0.05). K100 showed a relatively lower IC_{50} in comparison to CON.

Table 5. Antioxidant activities of muffin crumbs with different levels of KF.

	CON	K25	K50	K75	K100	F-value
Polyphenols (μgGAE/mg)	6.61±0.06[1)e]	8.07±0.12[d]	10.45±0.13[c]	15.30±0.20[b]	17.33±0.08[a]	3953.926***
Flavonoids (μgQE/mg)	9.61±0.25[e]	11.77±0.00[d]	12.80±0.11[c]	16.75±0.33[b]	18.54±0.14[a]	987.048***
Reducing Power	0.23±0.00[d]	0.22±0.00[c]	0.32±0.00[b]	0.36±0.00[a]	0.36±0.00[a]	9770.3***
DPPH (IC_{50}, μg/mL)	243.09±3.79[d]	234.95±1.55[c]	223.95±.6.67[c]	224.90±0.87[b]	213.47±3.48[a]	25.945***
ABTS (IC_{50}, μg/mL)	263.08±2.90[d]	237.89±3.58[c]	223.68±1.91[c]	219.92±6.32[b]	207.34±0.22[a]	104.716***

[1)]The data are mean±SD in triplicates.

[a-e] Different superscripts indicate there are significant differences between values in the same row according to Duncan's multiple range test at p<0.05.

*p<0.05, ***p<0.001.

3.4. Crumb air cells

Table 6 presents the number and area of air cells categorized by a particular size range. Cross section images of muffin crumbs are shown in Fig. 2. During the mixing process, pores are generated in the batter and they grow while baking as CO_2 production results.

Table 6. Air cell number and area in muffin crumbs with different levels of KF.

Samples	Air cells number						Air cells area				
	<1 pixel^2	1-10 pixel^2	10-100 pixel^2	100-1000 pixel^2	1000 pixel^2 <	Total	1-10 pixel^2	10-100 pixel^2	100-1000 pixel^2	1000 pixel^2 <	Total
CON	61	90	87	81	13	332	4.80±2.71	40.80±27.79	331.22±210.10	2336.69±1194.21	2713.51
K25	52	88	123	66	12	341	4.53±2.55	39.36±23.97	265.05±158.10	2230.58±1445.59	2539.52
K50	98	177	187	59	5	526	4.37±2.28	38.63±24.47	222.32±152.39	1723.6±907.82	1988.92
K75	132	214	257	55	2	660	4.29±2.26	33.77±22.74	224.58±113.84	1412.50±433.46	1675.14
K100	129	218	251	53	3	654	4.28±2.32	34.53±22.09	211.47±110.25	1171.33±152.32	1421.61

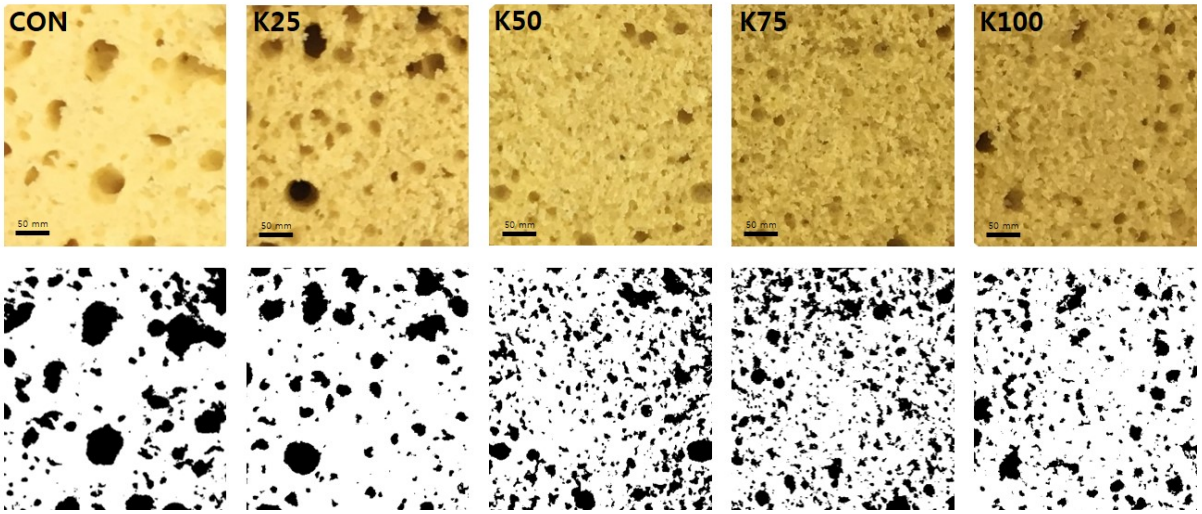

Figure 2. Cellular structure of Muffin crumb with different levels of KF. Top line: scanned images of the cross section of muffin crumb, bottom line: modified images using ImageJ.

As the substitution ratio of WF to KF increases, the number of pores with the size of 100 pixel^2 or less increased, whereas the number of pores decreased over a size of 100 pixel^2. The air cells area result showed the opposite tendency to the air cells number. As the KF substitution rate increased, average air cell size increased in all pore size classes. Therefore, substitution of WF by KF resulted in a tiny air cell and densely structured crumb. The size of air cells is an important factor affecting the texture of final bakery products (GIACOMOZZI *et al*., 2018). The air cells growing during baking affects the tender quality, which is related to the crumb hardness. CON showed a greater number of large air cells than other samples, which had weak crumb hardness. As mentioned earlier, the hardness increased as the pore size decreased and became dense.

3.5. Microstructure of batter and muffins

Batter microstructure is shown in Fig. 3. The micrograph of CON batter shows a uniformly spread air bubble and the air bubble size is relatively large and not concentrated in a particular area. In the former study by RAJIV *et al*. (2011), the control muffin batter showed an even and constant size of air bubble distribution. Micrograph of K25 batter shows relatively small-sized air bubbles appeared. The air bubbles of K25 batter showed closer formation and density compared to those of the CON batter. Micrograph of K50 batter exhibits both large-sized and small-sized air bubbles, and it does not form the uniform distribution. Compare to CON and K25 batter, the smaller air bubbles can be observed to stick together slightly in the K50 batter. The K75 batter micrograph shows unevenly distributed air bubbles, and the small-sized air bubbles are gathered around medium-sized air bubbles. Micrograph of the K100 batter shows an air bubble distribution similar to that of the K75 batter is observed, and the air bubbles are denser.
Fig. 4 presents the scanning electron micrographs. In Figs. 4A-1 (CON), 4B-1 (K25), 4C-1 (K50), 4D-1 (K75) and 4E-1(K100), the muffin crumbs are magnified 100 times, and the change of the pore size, distribution, and matrix surface can be observed. As the KF level increased, the small-sized pores gradually formed and the granular structure on the matrix surface became larger, such that the matrix appeared to be disconnected. Fig. 4A-2 shows the micrograph of the CON muffin crumb prepared entirely with WF, which shows gelatinized starch granules buried under the denatured protein matrix. The microstructure of starch granules embedded in a protein matrix is also described by GAO *et al*. (2018). LEE *et al*. (2001) reported that starch granules can be divided into two types, spherical and lenticular-shaped, which were also observed in the blended wheat flour dough. Fig. 4B-2 displays the micrograph of the K25 muffin crumb, and it shows fewer starch granules and smoother continuous matrix than that of CON. In Fig. 4C-2, which is the micrograph of the K50 muffin crumb, a rather rough and ruptured protein matrix is observed. RAJIV *et al*. (2011) reported that disruption of the protein matrix became greater when wheat was replaced by 60% of finger millet. In Fig. 4D-2, which exhibits the micrograph of the K75 muffin crumb, the gelatinized starch granules are large, and it appears to be coated. Fig. 4E-2 is the micrograph of the K100 muffin crumb, which shows greater and thin starch granules that seem to be entangled in a discontinuous protein matrix. Therefore, when the KF level is increased, the starch granules become greater, and continuity of the protein matrix is lost.

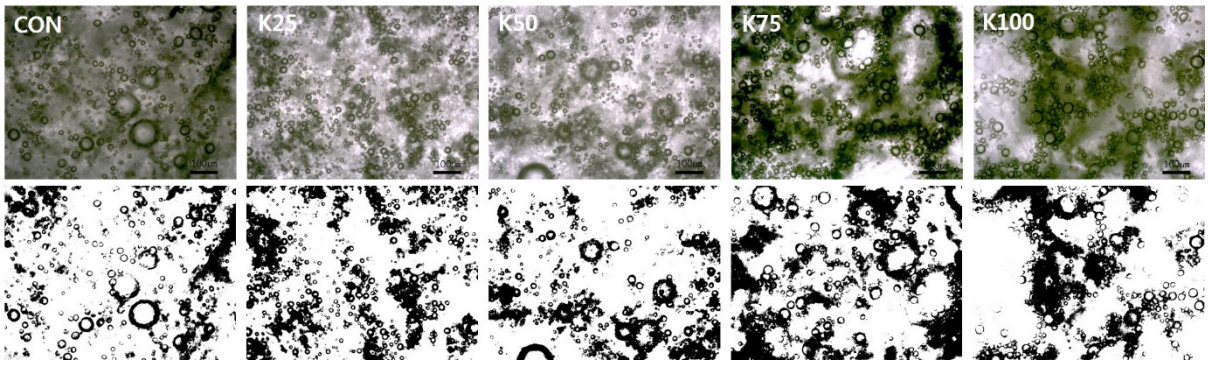

Figure 3. Batter microstructure (×100). Top line: micrograph of muffin batters CON, K25, K50, K75, K100, bottom line: modified images of batter CON, K25, K50, K75, K100 using ImageJ.

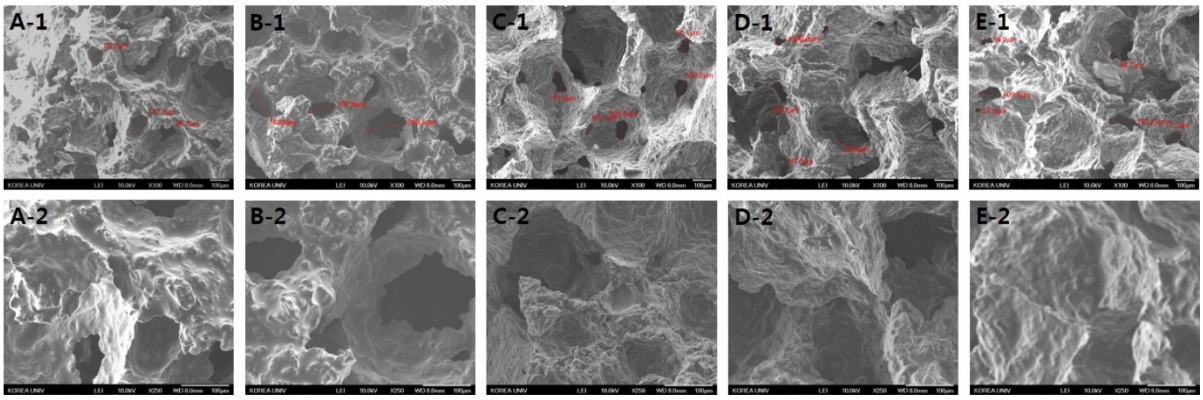

Figure 4. Scanning electron micrograph of muffin crumbs. Top line: SEM micrograph of magnification 100×. Bottom line: SEM micrograph of magnification 250×.

3.6. Retrogradation kinetics

The Avrami equation describes the retrogradation process kinetics and the result is shown in Table 4. Starch retrogradation is an important quality determinant in the staling of bakery products. Avrami exponent (n) indicates the value of nucleation in crystallization, and it depends on the growth rate of crystallites in short storage periods (COLLAR *et al*., 1999). The lower the Avrami exponent, the slower the crystallization rate, which is more effective for delaying retrogradation (KIM *et al*., 2006; S.-S. KIM and CHUNG, 2010; ZHANG *et al*., 2017). Crystallization is an important factor as it is related to the texture and shelf-life of a bakery product. The Avrami exponent showed the highest value in K100, but the value tended to decrease from CON (1.5540) to K75 (0.7669). The rate constant (k) refers to the retrogradation time. The reduction in the rate constant describes delays in retrogradation in the presence of carbohydrates. The rate constant tended to decrease with Kamut replacement from CON (1.8095) to K75 (1.2904), but the K100 (2.1541) showed a sharp increase. According to Avrami kinetic results, muffins prepared only with Kamut had similar values to the CON. CARINI *et al*. (2010) reported that whole Kamut tortillas were very similar to the control group in textural changes during the storage period.

Table 7. Avrami parameters for muffin crumbs with different levels of KF.

AVRAMI	Avrami exponent (n)	Rate constant (k)	R^2
CON	1.554	1.8095	0.9988
K25	1.3208	1.4036	0.9998
K50	1.1402	1.3113	0.9996
K75	0.7669	1.2904	0.9995
K100	1.5774	2.1541	1.0000

3.7. Sensorial evaluation

The sensory evaluation scores for appearance, flavor, texture, sweetness, and overall acceptability of muffins are presented in Table 8. The data showed that the sensory score for appearance, flavor, texture, sweetness, and overall acceptability decreased as the Kamut flour replacement level was over 50%. In the case of appearance and sweetness, except for CON, K25 and K50 scored higher than other samples and became not preferred at levels above 75% KF addition. The lowest appearance score of K100 could be explained due to the dark crumb color by the Maillard reaction between sugar and amino acids, increased brittleness, and small muffin volume. The decreased score in sweetness could be related to a decrement in brix degree. Regarding flavor and overall acceptability, both K25 and K50 were as high as CON. According to statistically analyze result, the CON and both K25 and K50 showed no significant difference in flavor and overall acceptability (P>0.05). It showed that the Kamut replacement had no significant negative effect on product preference at levels below 50%. The texture is one of the important parameters of sensory evaluation of processed foods (ALPASLAN and HAYTA, 2006). The texture was not significantly affected by Kamut flour substitution (P>0.05), unlike the mechanical texture results. In previous studies, the partial or complete replacement of wheat flour with KF provided equal or better sensory characteristics (BORDONI *et al*., 2017), and cooked Kamut grain ranked highly for sweetness among the various wheat varieties (STARR *et al*., 2015). Based on the sensory evaluation results, 25-50% of the Kamut added to muffins is considered as a desirable substitute addition level.

Table 8. Sensory evaluation of muffins with different levels of KF.

	CON	K25	K50	K75	K100	F-value
Appearance	7.18±1.80^{a}	6.76±1.45^{ab}	6.27±1.54^{bc}	5.72±1.39^{cd}	5.49±1.55^{d}	10.435***
Flavor	6.84±1.49^{a}	6.54±1.43^{a}	6.27±1.61^{ab}	5.84±1.54^{bc}	5.53±1.65^{c}	5.948***
Texture	6.10±1.71^{NS}	6.25±1.56	6.11±2.00	5.75±1.62	5.37±1.77	2.161
Sweetness	6.94±1.22^{a}	6.25±1.44^{bc}	6.55±1.63^{ab}	5.86±1.46^{c}	6.00±1.93^{bc}	3.985**
Overall acceptability	6.69±1.69^{a}	6.37±1.57^{a}	6.45±1.80^{a}	5.73±1.42^{b}	5.47±1.74^{b}	5.009**

[1)]The data are mean±SD in triplicates.

[a-e] Different superscripts indicate there are significant differences between values in the same row according to Duncan's multiple range test at p<0.05.

**p<0.01, ***p<0.001.

4. CONCLUSIONS

Replacement of wheat flour by Kamut flour in muffins affected the air bubble distribution in batter and this affected on the muffin volume, weight and height. The air cell distribution became denser as KF level increased so that caused increased harness. In addition, with increasing KF level, the greater starch granules and the less protein matrix continuity could be descripted through the microscopic observation. Textural properties of crumb changed significantly. Addition of KF improved antioxidant capacity. Study of muffin firming kinetics revealed Avrami exponent value of K100 significantly high, but K75 showed the lowest value. All muffins were considered acceptable and the muffin containing less than 50% of Kamut flour level showed a preference score in the sensory evaluation

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EXPERIMENTAL CONTAMINATION OF *CHAMELEA GALLINA* WITH MURINE NOROVIRUS AND EFFECTIVENESS OF DEPURATION

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ABSTRACT

Human Norovirus has been reported as the major non-bacterial cause of human gastroenteritis due to the consumption of contaminated bivalve mollusks. The European legislation established microbiological criteria only for bacteria (*Salmonella* spp. and *Escherichia coli*), while no viruses have still been considered. In this study, samples of *Chamelea gallina* were harvested along the Central Adriatic coasts (Italy) and artificially contaminated with Murine norovirus-1 (MNV-1) up to a final concentration of 10^3 $TCID_{50}$/ml in water. They were subject to a depuration process in a closed-circuit system using both ozone and ultraviolet light. Four experimental trials (100 specimens/trial) were performed and, at the end of depuration, the digestive glands of mollusks were examined by means of two methods – namely, RT-PCR and tissue culture. The results of RT-PCR ranged from $10^{3.17}$ to $10^{4.60}$ $TCID_{50}$/ml, and the constant presence of MNV-1 was confirmed by the tissue culture as well. In conclusion, no significant viral reduction was obtained, but the contaminated bivalve mollusks remained infectious until the end of the depuration treatment. The proper cooking of live bivalve mollusks could be considered the most important preventive measure against this sanitary risk.

Keywords: Norovirus, clams, depuration, tissue culture, RT-PCR

1. INTRODUCTION

Norovirus is a non-enveloped, single-stranded positive RNA virus, a member of the family *Caliciviridae*, and divided into six genogroups (GI-GVI). However, only the genogroups GI, GII and GIV were identified in humans (ILIC *et al*., 2017). Moreover, over 40 genotypes based on the capsid were identified (LEROUX-ROELS *et al*., 2017). A novel GII.17 variant emerged in Asia (China, Japan, Korea and Taiwan) in 2014 (CHENG *et al*., 2017; SUFFREDINI *et al*., 2017) and was also reported in other countries such as Canada, the United States (U.S.), New Zealand as well as some European States – i.e. Germany, Italy, Hungary and Slovenia (CHAN *et al*., 2017). According to the European Union Rapid Alert System for Food and Feed (EU RASFF), the majority of alert notifications involving Norovirus in food were reported by Denmark, France, Italy, the Netherlands and Norway as notifying countries, and France and Serbia as countries of origin, respectively. With regards to border rejection notifications, the main countries of origin were France and Serbia, whereas Italy and Spain submitted to the EU RASFF the majority of them (PAPAPANAGIOTOU, 2017).

Human Norovirus (HuNoV) is reported as the main non-bacterial cause of foodborne outbreaks due to the consumption of live bivalve mollusks. The main clinical symptoms of such an illness, with an incubation period of 10-51 hours, are nausea, sudden onset of vomiting and/or watery non-bloody diarrhea, abdominal or general muscle pain, headache and mild fever (HASSARD *et al*., 2017; JEON *et al*., 2017). In addition, it can lead to more severe conditions, such as dehydratation, hospitalization and potentially death in vulnerable individuals including children and elderly population (TRIVEDI *et al*., 2013; FUSCO *et al*., 2017).

Bivalve mollusks are filter-feeding organisms that can retain and concentrate in their own body not only nutrients but also suspended viruses or bacteria. However, the European Union (EU) Legislation (EC, 2004a) established that sanitary controls of live bivalve mollusks must be based only on the detection of *Escherichia coli* used as an indicator of faecal contamination for the classification of production areas, from which they can be collected. Moreover, Regulation EC No 2073/2005 (EC, 2005) and its amendments reported the absence of *Salmonella* spp. in 25 g of live bivalve mollusks and a range of 230 to 700 MPN/100 g of flesh and intravalvular liquid for *E. coli*. In the U.S. as well, the standards used for shellfish hygiene controls in both growing areas during primary production and for end-products are represented by faecal or total coliforms (CAMPOS *et al*., 2017). On the contrary, viruses are not investigated as vehicles for foodborne disease transmission according to the above mentioned legislations.

Generally, viruses show a higher environmental resistance than bacteria, and depuration is poorly effective on decontamination of live bivalve mollusks (VARELA *et al*., 2016). The most common depuration systems are based on the use of chlorine, ultraviolet light (UV) and ozone. While chlorine can have organoleptic effects in mollusks and cause the formation of chlorinated by-products, UV and ozone have gained popularity in recent years but both of them can be limited because the first is effective in high flow rates (POLO *et al*., 2014a) and ozone is influenced by some parameters such as temperature, salinity, pH and dissolved oxygen (ILIC *et al*., 2017).

The aim of this study is the evaluation of a depuration process in a closed-circuit system using both ozone and UV in clams (*Chamelea gallina*), experimentally contaminated with Murine Norovirus-1 (MNV-1), because it has genetic and pathological features similar to HuNoV and therefore it can be used as surrogate (PREDMORE *et al*., 2015; KIM *et al*., 2017).

2. MATERIALS AND METHODS

2.1. Samples' collection and depuration process

Samples of *C. gallina* (25-32 mm) were harvested along the Central Adriatic coast of Molise region, Italy, in 4 different periods (named A to D) of the year 2015 (from January to October) and put into aquariums containing seawater for acclimation and evaluation of their viability. Then, they were transferred into tanks filled with artificial marine water (Ocean Fish, Prodac International, Padova, Italy) and artificially contaminated for 72 hours with the MNV-1 provided by the Istituto Zooprofilattico Sperimentale delle Venezie (Italy), up to a final concentration of 10^3 $TCID_{50}$/mL in water.
The depuration process was carried out for 72 hours in a closed-circuit system (Tecno Impianti International s.r.l., Riccione, Italy) equipped with UV and ozone. It consisted of 197x72x45 cm tanks with a perlon wool prefilter and hyperactive carbon filter, an active biological filter using *Lithothamnium calcareum* algae and an UV sterilization plant. With regards to the sterilization unit and ozonator, power was 230V and power consumption was 16W and 0.5 A, respectively.
Aliquots of 100 specimens were analyzed at time 0 as negative control, before being placed in the depuration tanks, and further 3 aliquots were examined at intervals of 24, 48 and 72 hours. The clams were washed, opened and their digestive glands were pooled together and homogenized. Then they were analyzed by both tissue culture and RT-PCR according to BAERT *et al*. (2008).

2.2. Preparation of viral stocks

MNV-1 was propagated in monolayers of RAW 264.7 (Mouse Macrophage) purchased from American Type Culture Collection (ATCC-LGC Standards, Milano, Italy) and cultured in 75 cm^2 tissue culture flasks. The cells were maintaned in Dulbecco's Modified Eagle Medium, DMEM (Gibco, New York, USA) supplemented with sterile phosphate buffered saline (PBS, pH 7.4), 1% antibiotics (penicillin, nystatin, gentamicin, streptomycin) and 10% fetal bovine serum (Merck, Darmstadt, Germany). They were incubated at 37°C in a humidified atmosphere of 5% CO_2. For the preparation of viral stocks, the growth medium was removed, the cells were infected with MNV-1 with a virus titer of 10^3 $TCID_{50}$/mL and incubated for 48 hours. When significant cytopathic effects were observed, the supernatant was centrifuged at 500 *g* at 4°C for 30 min. MNV-1 titer was assessed by both RT-PCR assay and traditional virus end point titration according to REED and MÜENCH (1938).

2.3. Tissue culture assay

An aliquot of pooled hepatopancreas (1±0.2 g) was homogenized with sterile quartz sand and diluted 1:10 (w/v) in Antibiotics Antimycotic Solution (100X). The sample was stored at 4°C for 1 hour and centrifuged at 5000 *g* for 10 min. Twenty-four well microplate monolayers of RAW 264.7 were infected with 200 µL of the diluted sample (1:10 and 1:100) in DMEM and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 4-6 days. The presence of MNV-1 was evaluated by means of RT-PCR.

2.4. RT-PCR assay

The glands were pooled and 2±0.2 g were homogenized with 2±0.2 mL of 0.1 mg/mL proteinase K solution (Qiagen, Hilden, Germany), incubated at 37°C with shaking (320 rpm/1 hour) and centrifuged at 3000 *g* for 5 min. Nucleic acids (100 µL of supernatant) were extracted using the Biosprint 96 authomatic system (Qiagen) with the Biosprint 96 One for all vet Kit (Qiagen) according to the manufacturers' instructions. Ten µL of Armored RNA West Nile Virus (HNY1999) (Asuragen, Santa Clara, CA, USA) diluted 1:100 were added to each sample as an internal control to check for any RT-PCR inhibition phenomena. Monolayers of RAW 264.7 infected with10-fold serial dilutions of MNV-1 were used for the devolopment of the RT-PCR assay and the C_t value was < 40. The master mix was prepared by using RNA Ultrasense One-step qRT-PCR system (Invitrogen, Carlsbad, CA, USA) as reported in Table 1.

Table 1. Composition of master mix.

Reagent	C1 [a]	C2 [b]	Vol (µL)
H_2O	5x		1.100
5x Ultrasense reaction mix	50x	1x	4.000
ROX Reference dye (50x)	4.0 µM	1x	0.500
MNV-F	4.0 µM	200 nM	1.000
MNV-R	4.0 µM	200 nM	1.000
MNV-P	20 µM	200 nM	1.000
NS5-2	50 µM	80 nM	0.188
NS5-2F	50 µM	150 nM	0.100
NS5-2R		150 nM	0.100
RNA Ultrasense enzyme mix			1.000
Total			10.0

[a] C1: initial concentration
[b] C2: final concentration

A primer and probe set was selected according to BAERT *et al*. (2008). The sequence of primer pairs and probes was as follows: for MNV-1, the probe was 5'FAM-CGC TTT GGA ACA ATG-3'MGB (MNV-P), the primer Fw was 5'-CAC GCC ACC GAT CTG TTC TG-3' (MNV-F) and the primer Rev was 5'-GCG CTG CGC CAT CAC TC-3' (MNV-R); for HNY1999, the probe was 5'VIC-CCA ACG CCA TTT GCT CCG CTG-3'TAM (NS5-2), the primer Fw was 5'-GAA GAG ACC TGC GGC TCA TG-3' (NS5-2F) and the primer Rev was 5'-CGG TAG GGA CCC AAT TCA CA-3' (NS5-2R). All the primers and probes were purchased from Eurofins MWG Operon (Louisville, USA).
Ten µL of master mix and 10 µL of viral RNA were used for RT-PCR. The assay was performed on a 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) at the following thermal conditions: 50°C for 15 min – 95°C for 2 min – 40 cycles (95°C for 15 sec – 60°C for 1 min).
The analytical sensitivity of RT-PCR was tested analyzing the serial $\log_{10}$ dilutions of the MNV-1 tissue culture $10^{5.9}$ $TCID_{50}$/mL.

2.5. Statistical analysis

The Pearson correlation coefficient with confidence intervals of 95% was used to measure the association between time and viral titer.

3. RESULTS AND DISCUSSION

The presence of MNV-1 in the artificially contaminated clams was observed by tissue culture assay just after 24 hours of exposure, even if the RT-PCR results showed that an interval of 72 hours was the optimum for the viral contamination because the values increased from $10^{3.60}$ (24 hours) to $10^{6.60}$ $TCID_{50}$/mL (72 hours). The analytical sensitivity of RT-PCR resulted $10^{0.9}$ $TCID_{50}$/mL corresponding to 38 C_t value (data not shown).
The results of the different experiments of the depuration process carried out by the tissue culture assay showed values of $3.98x10^4$ $TCID_{50}$/mL for trial A and $1.48x10^4$ $TCID_{50}$/mL for the remaining trials. The viral titer did not vary among the 4 trials, because MNV-1 resulted always vital.
The results of RT-PCR ranged from $10^{3.17}$ to $10^{4.60}$ TCID50/mL (data not shown).
The Pearson correlation coefficient was -0.15 (lower limit = -0.55 and upper limit = 0.29) and therefore not significant.
A similar study was carried out by POLO *et al*. (2014b) in samples of clams (*Venerupis philippinarum*) and mussels (*Mytilus galloprovincialis*) contaminated with MNV-1 and then depurated for 7 days by means of ozone and UV-C radiation for water sterilization. The average reductions compared with the initial levels of MNV-1 were 60.5% for clams and 91.6% for mussels, but they remained still infectious at the end of the process. POLO *et al*. (2014a) as well found the presence of Norovirus in clams and mussels after a depuration process based on water treatment by chlorination. The efficacy of depuration using traditional or closed-circuit system with disinfection by UV was evaluated by SAVINI *et al*. (2009), which reported no statistically significant differences between depurated and non-depurated samples (i.e. *M. galloprovincialis*, *Tapes decussatus* and *Crassostrea gigas*) and indicated that the process was not able to remove Norovirus.
Other studies (LEAL DIEGO *et al*., 2013; IMAMURA *et al*., 2016) showed the failure of depuration process applied on oyster samples using a system based on UV. These results demonstrated that the water exchange could be low and the initial contamination was too high (LE MENNEC *et al*., 2017). Therefore, some measures such as the increasing of depuration time and water circulation as well as the continuous exposure to UV treatment could be able to improve the effectiveness of the process. SOUZA *et al*. (2013) detected MNV-1 in oysters until 96 hours of depuration in a closed system using different UV doses.
The presence of MNV-1 after the depuration process described in the present study demonstrated that these viruses can survive and accumulate in live bivalve mollusks, and therefore they represent a source of foodborne disease for consumers. Recent studies showed that Norovirus strains can selectively accumulate in mollusks due to viral carbohydrate ligands of histo-blood group such as antigens in various tissues of clams, mussels and oysters (POLO *et al*., 2014a; MCLEOD *et al*., 2017). Therefore, the elimination of Norovirus can be difficult using traditional decontamination treatments, because the virus is internalized within the cells of the digestive organs and other tissues of mollusks (KIM *et al*., 2017).

According to the report of EFSA and ECDC (2017), during 2010-2016 the number of reported foodborne outbreaks linked to Calicivirus (including Norovirus) was quite stable, with some differences among the EU member states. In particular, a statistically significant increasing trend was described in Belgium, France, Portugal and the Netherlands, while Austria, Denmark, Estonia and Hungary reported a decrease. A national information system, called SINZoo was developed in Italy aiming at the collection of data regarding food contamination and related zoonoses occurrence (COLANGELI *et al*., 2013). Such a system highlighted 12 positive out of 176 mollusk samples, even if no outbreak linked to Norovirus was described in the year 2017.
The monitoring of viral contamination of mollusks represents an important tool for public health, especially because no legislative standards have been established for viruses. However, according to Regulation EC No 853/2004 (EC, 2004b), each EU member state may adopt national measures in order to amend non essential elements such as additional health standards for live bivalve mollusks in cooperation with the relevant Community Reference Laboratory, including virus testing procedures and virological standards. The multi-annual regional control plan for both Abruzzo and Molise regions (PPRIC 2015-2018) established sampling of mollusks every 2 months for *E. coli* and *Salmonella* spp., and every 6 months for viruses.

4. CONCLUSIONS

The consumption of live bivalve mollusks collected from seawater contaminated with sewage pollution, due to malfunctioning of sewerage system, represents an important risk for human health. In this study, no significant viral reduction was observed, the closed-circuit depuration system was not able to reduce the level of MNV-1 and clams remained still infectious until the end of the experimental design. Therefore, the ingestion of raw or undercooked mollusks should be avoided expecially by some population categories, such as immunocompromised individuals.

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PAPER

PROTEOLYSIS AS A FUNCTION OF DISTANCE FROM SURFACE TO CENTRE IN A SMEAR-RIPENED IRISH FARMHOUSE CHEESE

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ABSTRACT

This study focused on proteolysis in an Irish farmhouse smear-ripened cheese by serial slicing (0.41 mm/slice) the first 2 cm from surface towards the centre of the cheese. Urea-polyacrylamide gel electrophoretograms confirmed higher proteolysis in the outer layers than at the centre. Free amino acid (FAA) analysis confirmed decrease in proteolytic activity from surface to centre. Peptides produced at depths 0.41 mm and 20.5 mm were 720 and 427 from α_{s1}-casein; 691 and 337 from α_{s2}-casein; 807 and 453 from β-casein; 180 and 109 from κ-casein. The study confirms higher proteolytic activity at surface due to action of enzymes of the smear microbiota, than at the centre of cheese and identified the agents responsible for production of many peptides.

Keywords: Gubbeen, smear-ripened cheese, proteolysis, surface, mass spectrometry

1. INTRODUCTION

Smear-ripened, or bacterial surface ripened, cheeses are semi-soft varieties with an orange-red rind. As the name suggests, smear ripened cheeses (e.g., Gubbeen, Reblochon, Tilsit, brick and Taleggio) develop on their surfaces during ripening a viscous, microbial red-orange smear composed of bacteria and yeast (MOUNIER *et al*., 2005; GOBETTI *et al*., 1997). Towards the end of the ripening of the mature red-smear cheese surface, Gram-positive bacteria, yeasts (*Debaryomyces hansenii*) are predominant microorganisms present (BOCKELMANN *et al*., 2002; BRENNAN *et al*., 2004; RITSCHARD *et al*., 2018). This microbial complexity is mainly responsible for the development of the characteristic flavours in the cheeses (CORSETTI, *et al*., 2001; VALDÉS-STAUBER and SCHERER, 1997).
During the first days of ripening of smear-ripened cheeses acid-tolerant yeasts grow and metabolize lactic acid produced by the lactic starter cultures to CO_2 and H_2O, thereby increasing the pH and producing growth factors, such as pantothenic acid, that encourages the growth of the smear bacteria (ELISKASES-LECHNER and GINZINGER 1995; PRILLINGER *et al*., 1999; VALDÈS-STAUBER *et al*., 1997; WYDER and PUHAN, 1999). *Debaryomyces hansenii* is the dominant yeast found in Tilsit cheese (CHURCHILL *et al*., 2003; COGAN *et al*., 2014). The yeasts isolated from the surface of Limburger cheese grew between pH 3.3 and 8.5 (KELLY and MARQUARDT, 1939; REPS, 1993) and *Mycoderma* yeasts isolated from the surface of Brick cheeses grow from pH 3 to 8 (IYA and FRAZIER, 1949; REPS, 1993). Presence of smear organisms on the surface of smear-ripened cheeses is associated with development of its colour, flavour and characteristic aroma (ADES and CONE 1969; RATTRAY and FOX, 1999).
Although *Brevibacterium linens* was considered in the past as the dominant bacterium on red-smear cheese, recent studies have shown a number of other species, particularly of genera *Arthrobacter*, *Brevibacterium*, *Corynebacterium*, *Microbacterium* and *Rhodococcus* (REPS, 1993; ELISKASES-LECHNER and GINZINGER 1995; SEILER, 1986; VALDES-STAUBER *et al*., 1997) are more important at the surface. Several bacterial species more recently isolated during the ripening of the smear cheeses include *Argococcus casei, Arthobacter arilaitensis, Arthobacter bergerei, Brachybacterium alimentarium, Brachybacterium tyroformentans, Corynebacterium casei, Microbacterium gubbeenese, Mycetocola reblochoni, Staphylococcus succinus* subsp. *casei* and *Staphylococcus equorum* subsp. *linens* (BORA *et al*., 2007; BORA *et al*., 2008; BRENNAN *et al*., 2001; IRLINGER *et al*., 2005; PLACE *et al*., 2003; SCHUBERT *et al*., 1996).
Species of bacteria occasionally isolated from smear cheese include *Staphylococcus* spp., *Brevibacterium aurantiacum* in very high numbers, and relatively high numbers of *Psychrobacter* spp. *C. casei*, *C. variabile*, and *M. gubbeennse*. The presence of *S. saprophyticus* in certain varieties (e.g., Gubbeen) distinguishes them from other varieties (Livarot, Reblochon, and Limburger) (COGAN *et al*., 2014; REA *et al*., 2007).
Proteolysis is the key biochemical process in cheese during ripening (FOX, 1993). The complex proteolytic breakdown from surface towards the centre of smear-ripened cheese is a result of the combined action of bacterial and yeast proteolytic enzymes. Proteolysis in this type of cheese depends directly on the extent of penetration of the smear microbiota in the cheese curd and their proteolytic activity and decreases inwards from the surface towards the centre (CHURCHILL *et al*., 2003). Considerable differences in degree of proteolysis are observed, depending on depth from surface to centre of the cheese (CHURCHILL *et al*., 2003). Smear cheese microbiota (bacteria, moulds and yeasts) and their enzyme systems plays a key role in proteolytic, lipolytic and free amino acid catabolic activities in the cheese during ripening (FOX, 1993). Yeasts present in the surface

smear, *D. hansenii and G. candidum,* exhibit a strong extracellular proteolytic and or peptidolytic activity (BAUR *et al*., 2015). An *in situ* study by BOUTROU *et al*. (2006) found *G. candidum* from the surface of the cheese participates in primary proteolysis along with chymosin at pH 5.5 to 6 (CHEN and LEDFORD, 1972; GUEUEN and LENOIR, 1976) during the proteolysis. Rapid hydrolysis of α_{s1}-caseins at the surface, in presence of *G. candidum* and surface microbiota of the cheese was observed also by BOUTROU *et al*. (2006).

Proteolytic enzymes of one of these organisms, *B. linens*, were studied by RATTRAY and FOX (1998). The purified proteinase enzyme from *B. linens* was most active at pH 8.5 and at 50°C with a molecular mass 120 kDa and peptides produced by these proteinases from α_{s1}- and β- caseins were identified (RATTRAY and FOX, 1996, 1997, 1998).

However, there is relatively little literature describing in detail proteolysis and breakdown of caseins in smear-ripened cheese. The current study attempts to characterise proteolysis in a smear ripened variety of Irish farm-house cheese Gubbeen from the surface towards the centre. The study gives an insight to the detail of the proteolysis in smear cheese providing a deeper and more comprehensive insight into the metabolic activity of microbial ecosystems of these varieties.

2. MATERIALS AND METHODS

Commercially ripe samples (300 g) of Gubbeen, an Irish farmhouse smear cheese, product of Gubbeen Farmhouse Products Ltd., Gubbeen House, Schull, Co. Cork. Ireland), were obtained in triplicate from the local market (G1, G2 and G3), made generally as outlined by MOUNIER *et al*. (2005). These were cut in half along the diameter of the cheese wheel. One portion was crumbled and mixed thoroughly (with rind) for compositional analysis and remaining part was used for serial slicing and proteolytic analysis (Fig. 1 A, B, C and D).

2.1. Serial slicing of cheese and peptide extraction

The cheese. Four or five blocks from each cheese were sliced separately and slices taken at the same depth were later pooled. A tissue chopper (McIlwain tissue chopper; Model TC752, Campden Instruments Ltd., Loughborough, Leics, England) with a double end razor blade (Wilkinsons Sword, Edgewell Personal Care, Shelton, CT, USA) was set to give a thickness of 410 mm (0.410 mm) per slice. The blade was cleaned with 70% ethanol before and after each sectioning. Cheese blocks were sliced frozen, at thickness of 0.41 mm/slice, to a depth of 2 cm (Fig 1 A, B, C and D). These slices were collected on aluminium foil sheet with a sterile scalpel and an artist's brush. Slices from multiple blocks from a single cheese were pooled to get a sufficient quantity (10 mg) for analysis and stored frozen in a micro-centrifuge tube. The pooled slices were weighed out (10 mg), then dispersed in 10 mM citrate buffer (pH 5.5), teased using a needle and vortexed (RotaMixer, model # 8768; Hook and Tucker Instruments Ltd., Croydon, England) for 1 min (repeated if required) for uniform mixing. The micro-centrifuge tubes were held in a pre-heated water bath at 40°C for 15 min and centrifuged (Micro-Centaur Centrifuge, MB010.CX1.5, Sanyo, Loughborough, Leics, UK) at 9,600 g for 10 min, cooled for 30 min at 4°C to remove any fat from the supernatant. The supernatant was separated and sufficient 10 % trichloroacetic acid (TCA) solution was added to the supernatant to give final concentration of 2 % TCA in the extracts, which were centrifuged again at 6,700 g for 10

min and cooled at 4°C for 15 min. The clear supernatant was separated and stored frozen in aliquots at -20°C until use. The aliquots were thawed and filtered through 0.22 μm cellulose acetate filters (Sartorious GmbH, Gottingen, Germany) for peptide analysis by mass spectrometry (Q-ToF LCMS) as described below.

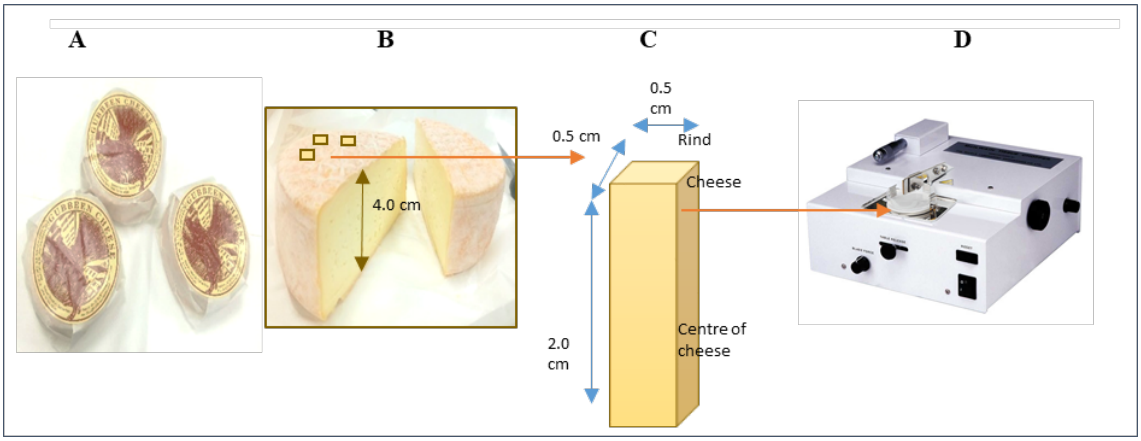


Figure 1. Sampling of smear ripened cheese to depth of 2 cm.
A: Sampling procedure for smear-ripened cheese; B: Cheese cut in halves; C: Blocks cut for slicing; D: McIlwain tissue chopper and slicing mantle.

2.2. Physico-chemical analysis

Compositional analysis of the cheeses comprised of determination of moisture content using an oven-drying method (IDF, 1982). Protein and nitrogen content of the cheeses and pH 4.6-soluble extracts were determined by the macro-Kjeldahl method (N x 6.38; IDF, 1986). Percentage of fat was determined by the Gerber method (IIRS, 1955) and percentage of NaCl was measured by a titrimetric method using potentiometric end-point determination as described by FOX (1963). Each experimental analyses were run in triplicate for each cheese sample. Statistical analyses were performed using R® 16 (R version 3.4.0; the R Foundation for Statistical Computing, University of Auckland, Auckland, New Zealand). Differences in means in between the batches and/or depth were tested by analysis of variance (one way-ANOVA) at significance level, α, of 0.05 (P value $\leq$ 0.05), throughout the study.

2.3. Proteolytic analysis

The pH of a cheese slurry (made from 25 g cheese and 50 g of deionised water) was measured using a calibrated pH meter. The same slurry was used to prepare pH 4.6-soluble and -insoluble fractions of cheeses at all time points as described by KUCHROO and FOX (1982).
Proteolysis in the slices taken from surface to centre of smear-ripened cheeses was studied by urea-polyacrylamide electrophoresis (urea-PAGE) of freeze-dried samples (10 mg/mL) of cheese slices from surface to centre (ANDREWS, 1983; SHALABI and FOX 1987; O'MAHONY *et al.*, 2005). The gels were stained with Coomassie Brilliant Blue G250 (BLAKESLEY and BOEZI, 1977) and de-stained by several distilled water washes.

Individual free amino acids (FAAs) content was determined according to FENELON *et al*. (2000). Frozen extracts of cheese slices taken at depths 0.41 mm, 1.64 mm and 20.5 mm were dispersed in 10 mM citrate buffer, pH 5.5, in 2% TCA, and were used for the analysis of all three batches.
Peptide analysis from cheese extracts (Section 2.1) were conducted at Conway Institute of Biomedical and Biomolecular Sciences, Univesity College Dublin, Ireland, on a quadrupole Orbitrap (Q-Exactive, Thermo Scientific, Waltham, MA, USA) mass spectrometer equipped with a reversed-phase Nano LC UltiMate 3000 HPLC system (Dionex LC Packings, now Thermo Scientific Waltham, MA, USA). Peptide samples were loaded onto C18 reversed phase columns (10 cm length, 75 μm inner diameter) and eluted with a linear gradient from 2 to 97% acetonitrile containing 0.5% acetic acid in 60 min at a flow rate of 250 nL/min. The injection volume was 5 µl. The mass spectrometer was operated in data dependent mode, automatically switching between MS and MS2 acquisition. Survey full scan MS spectra (m/z 300-2000) were acquired in the Orbitrap. The twelve most intense ions were sequentially isolated and fragmented by higher-energy C-trap dissociation.

2.4. Peptide identification

Raw data from the mass spectroscopy (Orbitrap Q-Exactive) were processed using MaxQuant version 1.5.5.1 (COX and MANN, 2008; TYANOVA *et al*., 2016a), incorporating the Andromeda search engine (COX *et al*., 2011). MS/MS spectra were matched to a bovine proteins custom database of previously identified entries (1,059) in the milk proteome in order to identify peptides and proteins. All searches were performed with unspecific digest. The database searches were performed with no fixed modification but with acetylation (protein N terminus) and oxidation (M) as variable modifications. Mass spectra were searched using the default setting of MaxQuant, namely a false discovery rate of 1% at the peptide level. For the generation of ion intensities for peptide profiles, signals of corresponding peptides in different nano-HPLC MS/MS runs were matched by MaxQuant applying a mass accuracy of at least 20 ppm and a maximum time window of 1 min (COX *et al*., 2014). The Perseus statistical software (version 1.5.5.3) (TYANOVA *et al*., 2016) was used to analysis the peptide ion current intensities.

3. RESULTS

3.1. Physico-chemical composition

The physico-chemical composition of three batches (G1, G2 and G3) of commercial smear-ripened cheese was determined (Table 1). pH decreased from 6.3 to 5.7 from surface to centre of the cheese, respectively, in all the batches, as was also reported by MOUNIER *et al*. (2005). Levels of moisture, fat, salt (NaCl), fat in dry matter (FDM), moisture in non-fat solids (MNFS), protein and nitrogen content in cheese and pH 4.6-soluble nitrogen (SN) as a percentage of total nitrogen (TN), are reported in Table 1. Moisture levels were 44 to 46% in agreement with the literature (MOUNIER *et al*., 2005; REA *et al*., 2007; COGAN *et al*., 2014). Salt (% NaCl) was ~2.1 %, with no significant variation between batches. The % fat values were similar in all the batches (47 %) as were the values of % FDM (84% to 86%), %MNFS (83% to 87%). The gross composition was observed to be typical of smear ripened

cheese (COGAN *et al*., 2014). Crude protein (N x 6.38) content of cheese showed a slight variation between the batches G1, G2 and G3 (Table 1).

Table 1. Physico-chemical compositional analysis of mature smear-ripened cheese after ripening.

	% Moisture	%Fat	%Salt	%N	%(Prt)	%pH4.6SN/TN	%MNFS	%FDM
G1	44.11±0.5^{a}	47±0.01^{a}	2.13±0.05^{b}	2.93±0.05^{a}	19.67±0.34^{a}	9.73±0.11^{b}	83.21±0.95^{a}	84.09±0.76^{a}
G2	44.31±1.4^{a}	47±0.02^{a}	2.03±0.05^{a}	3.36±0.15^{b}	21.41±1.00^{b}	6.56±0.66^{a}	83.61±2.81^{a}	84.43±2.22^{a}
G3	46.27±0.7^{a}	47±0.01^{a}	2.07±0.05^{a}	3.59±0.07^{b}	22.07±0.48^{b}	6.41±0.17^{a}	87.33±1.32^{a}	86.85±1.15^{a}

Samples G1, G2 and G3 are separate batches of smear-ripened cheese used for compositional analysis, where %N= Nitrogen%, %(Prt) = crude protein% in smear-ripened cheese, total SN= pH 4.6-soluble nitrogen (SN) as a percentage of total nitrogen, %FDM= fat in dry matter and % MNFS=moisture in non-fat solids, represented by mean ± standard deviation and different superscript letters represent significant difference ($P<0.05$).

3.2. Proteolysis

The ratio between nitrogen soluble at pH 4.60 and total nitrogen (pH 4.6 SN/TN) (%) is an index of proteolysis and, in smear-ripened cheese, is mainly produced by the action of chymosin, plasmin, lactocepins (LacCeps) and enzymes from the smear microbiota on the caseins (ARDO *et al*., 2017). Levels of pH 4.6 SN/TN were higher in batch G1 as compared to batches G2 and G3 (Table 1). Overall % pH 4.6 SN/TN indicated a normal proteolysis at the end of the ripening in the cheese and values from the current study were comparable to those reported by COGAN *et al*. (2014) a range of smear-ripened cheeses.
Proteolysis from surface to the centre of the cheese was also studied by urea-PAGE (Fig. 2). Samples for the gel were prepared from freeze dried slices (0.41 mm/ slice) of smear-ripened cheese from surface to centre (2 cm). Extensive breakdown of α_{s1}- and β-casein was observed in topmost layers up to 1.23 mm, followed by normal patterns of proteolysis typical of internal bacterially ripened varieties, closer to the centre. Action of plasmin was prominent for the break-down of β-caseins into γ-caseins at depth less than 1 cm from the outer surface, as was also observed in other smear ripened cheese varieties like Tilsit at early stages (day 6) of ripening (BOUTROU *et al*., 1999, 2005, 2006; GUIZANI *et al*., 2002; CHURCHILL *et al*., 2003). Extensive degradation of α_{s1}-casein at the surface (Fig. 2) was likely caused by the action of chymosin together with the enzymes from the smear microbiota. Levels of degradation decreased from surface to center of the cheese, due to reduced penetration of smear enzymes. The high extent of degradation of α_{s1}-casein at the surface despite the higher pH (which would reduce chymosin action) suggests a major role of enzymes from smear organisms. The results are similar to those observed by CHURCHILL *et al*. (2003) in Tilsit cheese.

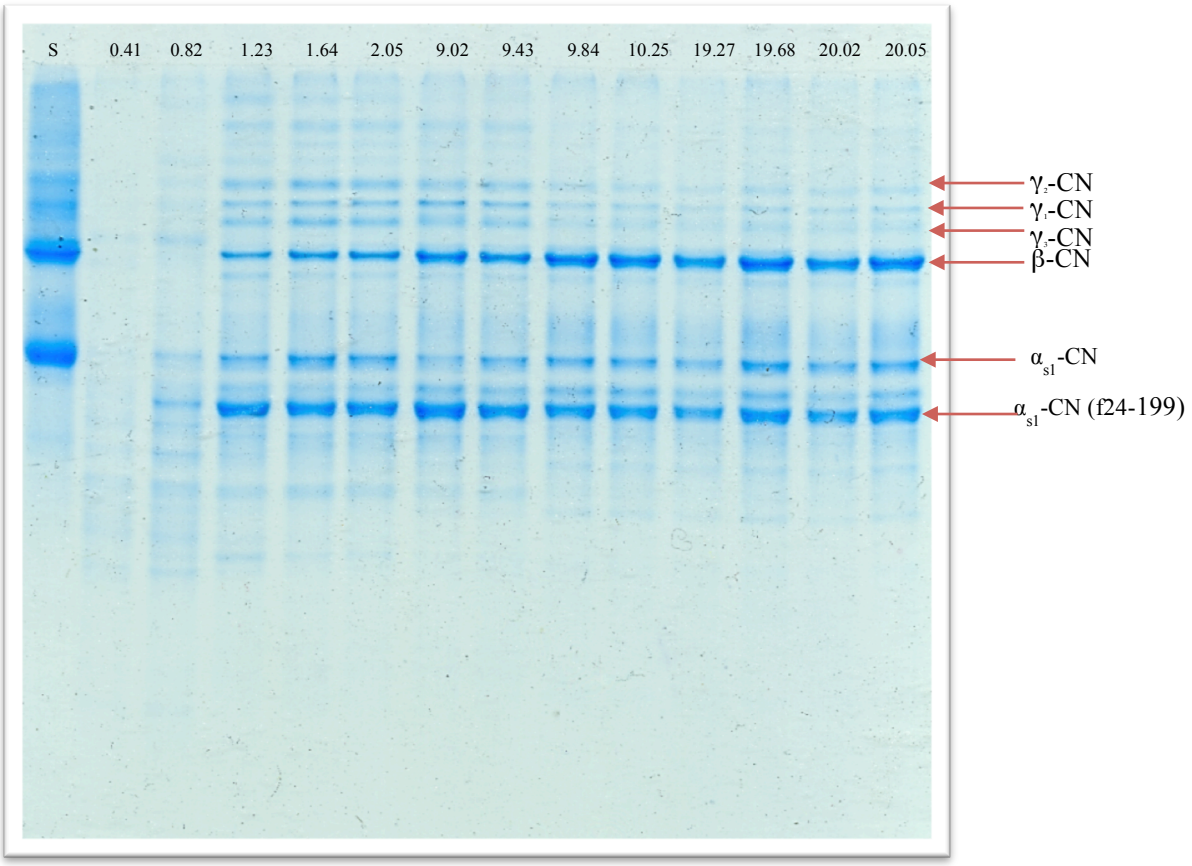


Figure 2. Urea-polyacrylamide gel electrophoretograms of freeze dried samples of sliced smear-ripened cheese from surface to centre (2 cm), standard protein marker, sodium caseinate: (S), numbers above lanes 2-14 indicates depth of smear-ripened cheese in mm at which sample was taken.

Profiles of free amino acids analysed from extracts from cheeses dispersed in 10 mM citrate buffer at pH 5.5 are as shown in Fig. 3. Total amino acid contents observed were 107.19 μg/mg at 0.41 mm, 33.59 μg/mg at 1.64 mm and 33.78 μg/mg at 20.5 mm depth of cheese. Levels of individual amino acids were also found to be higher at the surface than towards centre (Fig. 3). Values at 0.41 mm for glutamic acid were 21.60 μg/mg, glycine were 16.5 μg/mg, proline were 16.56 μg/mg and of alanine were 10.03 μg/mg (Fig. 2). The outer layers of the cheese showed high levels of glutamic acid, glycine, alanine, tyrosine, and proline only particular to the surface of the smear cheese. Higher concentrations of threonine, lysine, phenylalanine and valine were found towards the inner layers of smear-ripened cheese than at the surface. Values were similar also to amino acid profiles of mould ripened cheeses (ZARMPOUTIS *et al*., 1997).

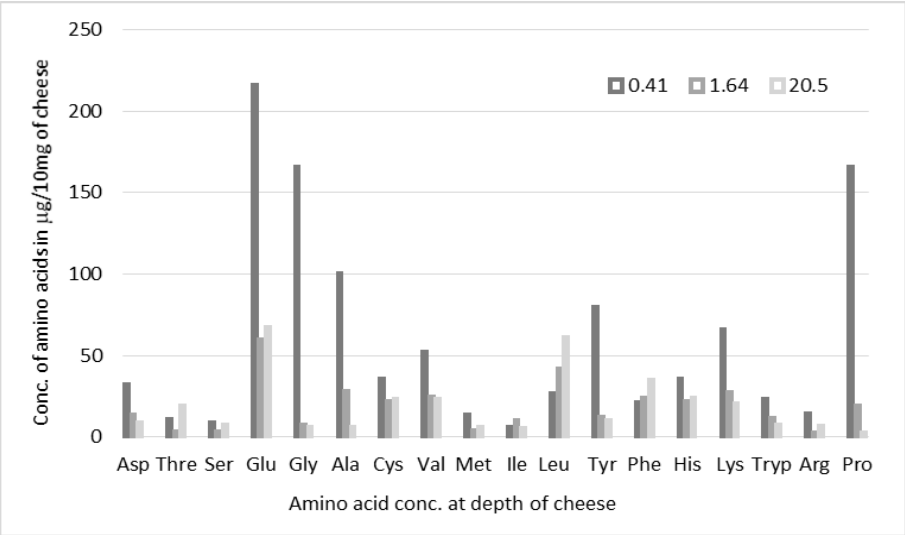


Figure 3. Free amino acid analysis.
Concentration (µg /mg) of free amino acids in 10 mM citrate buffer pH 5.5 dispersed extracts from 0.41 mm, 1.64 mm and 20.5 mm slices of smear-ripened cheese.

3.3. Peptide profiles at different depths of the cheese

Qualitative differences were found in peptides produced at all depths. Mass spectroscopic analysis of peptides in 10 mM citrate extracts at pH 5.5 showed a large difference between the numbers of peptides produced at the outer surface as compared to the centre (depth of ~2 cm) (Fig. 4). In case of the individual caseins, the number of β-casein-derived peptides was highest at all the depths, followed by peptides derived from α_{s1}-casein, α_{s2}- and κ-casein.

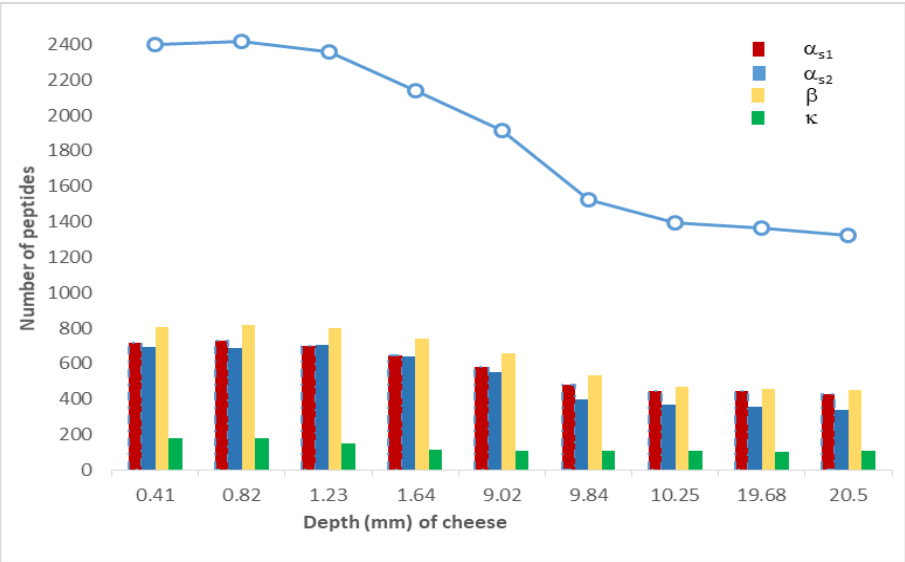


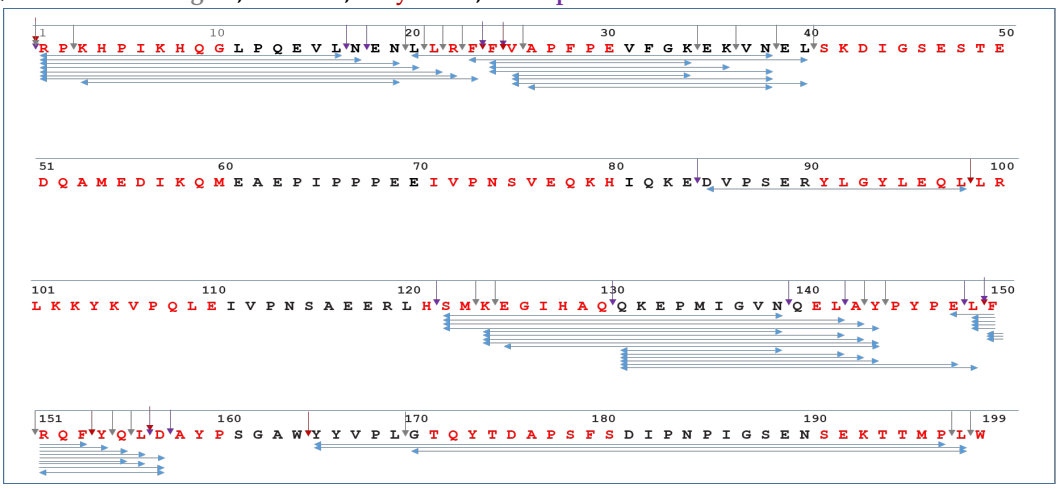
Figure 4. Total caseins at depths in smear ripened cheese.
Graph representing the number of casein derived-peptides as a function of distance from surface of cheese (-O-). Bars indicate number of peptides derived from α_{s1}-, α_{s2}-, β- and κ-casein.

Identification of peptides during ripening was a major objective of this study. So far, very little has been reported in the literature about identification of peptides from smear cheese. The first 150 peptides with highest values of relative intensity at each depth, obtained from LCMS data were considered and sorted into peptides from α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein at depths 0.41 mm, 9.02 mm and 20.05 mm and were plotted on the primary structure of the proteins (Fig. 5 A, B, C and D). Identified peptides were compared with known cleavage specificities of chymosin, plasmin and lactocepins (SINGH *et al*., 1994, 1995, 1997; BREEN *et al*., 1995; FERNANDEZ *et al*., 2005; UPADHYAY *et al*., 2006; ARDO *et al*., 2017). The peptides were also compared with the cleavage specificities of proteinases of *B. linens* on α_{s1}- and β-casein found by RATTRAY and FOX (1993). However, cleavage site matches were not found in first 150 peptides from the extracts of smear cheese.

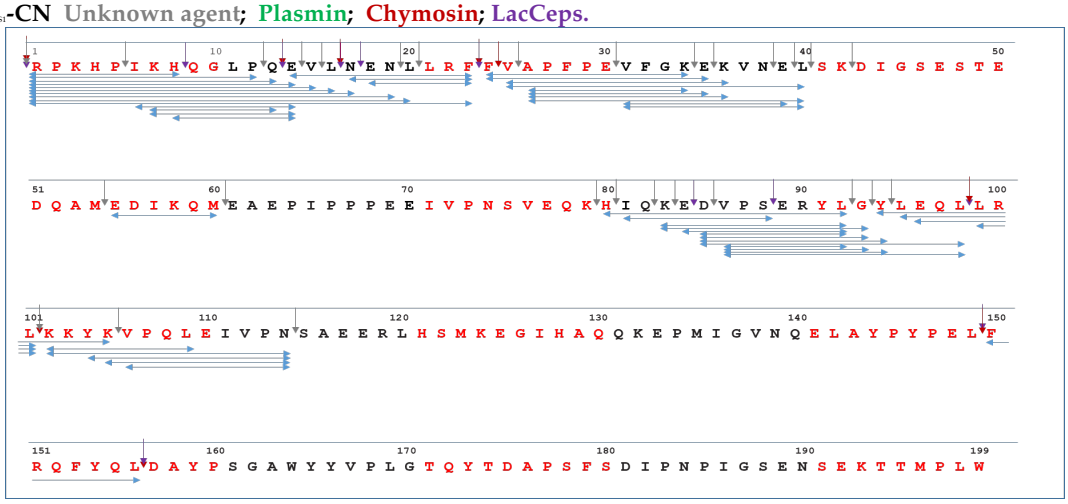
A total of 5176 α_{s1}-casein fragments were produced by hydrolysis during ripening. Peptides derived from α_{s1}-casein only at 0.41 mm were observed in regions 121-157 and 165-198. Those derived only at 9.02 mm and 2.05 mm and were found at regions 79-117. The region hydrolysed common to all depths, (0.41 mm, 9.02 mm and 2.05 mm) was 1-40 (Fig. 5 A). All these regions were highly susceptible to enzymatic hydrolysis for action of chymosin and lactocepins (ARDO *et al*., 2017) and proteinases from surface smear microbiota. The common fragment, α_{s1}-CN (f1-23) produced by the action of chymosin (FOX and McSWEENEY, 1996), was present at all depths. Other major chymosin-derived peptides, α_{s1}-CN (14-23), α_{s1}-CN (f25-36/38), α_{s1}-CN (f17-23/24), α_{s1}-CN (f 24-34) and α_{s1}-CN (f 25-34), were found at all depths. Peptides produced by action of lactocepins and/or chymosin at their N- or C- termini (ARDO *et al*., 2017) included α_{s1}-CN (f30-38), α_{s1}-CN (f31-37/38), α_{s1}-CN (f33-40), α_{s1}-CN (f154-164), α_{s1}-CN (f165-193), α_{s1}-CN (f169-189/190/197/198), α_{s1}-CN (f170-189/190/192/198) and α_{s1}-CN (f180-198/199). Lactocepin-derived peptide fragments found at all the depths were α_{s1}-CN (1-8/9/13/16/17), α_{s1}-CN (f7-13), α_{s1}-CN (f10-16), α_{s1}-CN (f122-139,142/143/144) and α_{s1}-CN (131-139/142/148). Most common peptides from α_{s1}-casein present only at surface were α_{s1}-CN (f 122-139/142/143), α_{s1}-CN (f 124-142/143/144), α_{s1}-CN (f 125-144) and α_{s1}-CN (f 150-155/156/157) and those present only at the center were α_{s1}-CN (f191/193-199). Peptides from α_{s1}-casein present at all the depths were α_{s1}-CN (f1-23), α_{s1}-CN (f14-23), α_{s1}-CN (f 24-34) and α_{s1}-CN (131-139/142/148).

A total of 4744 peptides were produced by hydrolysis of α_{s2}-casein during ripening. Peptides derived from α_{s2}-casein only at depth 0.410 mm were from region 68-98, those common to depths 0.410 mm and 9.02 mm were from the regions 98-114 and 175-207 and peptides common at depths 9.02 mm and 2.05 mm were found in regions 89-99, 102-115 and 181-207 (Fig. 5 B). Peptides produced at all the depths were mainly due to the action of plasmin, lactocepins (ARDO *et al*., 2017), chymosin (McSWEENEY *et al*., 1994) and mainly at the surface by proteinases from the smear microbiota. Fragments produced by the action of plasmin and lactocepins at their N-termini were α_{s2}-CN (f115-125) and α_{s2}-CN (f115-126). Action of plasmin and lactocepins (ARDO *et al*., 2017) on α_{s2}-casein helped to produce fragments α_{s2}-CN (f80-88), α_{s2}-CN (f150-161/162), α_{s2}-CN (f151-156/158/161/162/163) and α_{s2}-CN (f196-208), which were also found in smear ripened cheeses at all depths. Most common peptides from α_{s2}-casein present only at surface were α_{s2}-CN (f68-74/88/94), α_{s2}-CN (f71-94/98), α_{s2}-CN (f73-86/88), α_{s2}-CN (f75-88/94) and α_{s2}-CN (f87-95) and those present only at the center were, α_{s2}-CN (f176-181/182), α_{s2}-CN (f186-197), α_{s2}-CN (f188-199/202), α_{s2}-CN (f191-197/199/203) and α_{s2}-CN (f194-203). Peptides from α_{s2}-casein present at all the depths were α_{s2}-CN (f115-125), α_{s2}-CN (f151-156/158/161/162/163) and α_{s2}-CN (f196-208).

0.41mm α_{s1}-CN Unknown agent; **Plasmin**; **Chymosin**; **LacCeps.**



0.41mm α_{s1}-CN Unknown agent; **Plasmin**; **Chymosin**; **LacCeps.**



20.05 mm α_{s1}-CN Unknown agent; **Plasmin**; **Chymosin**; **LacCeps.**

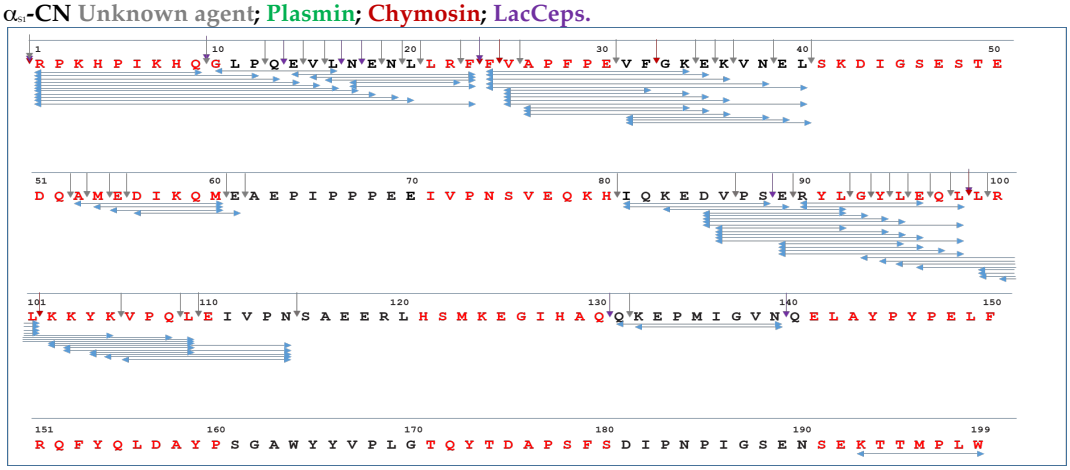
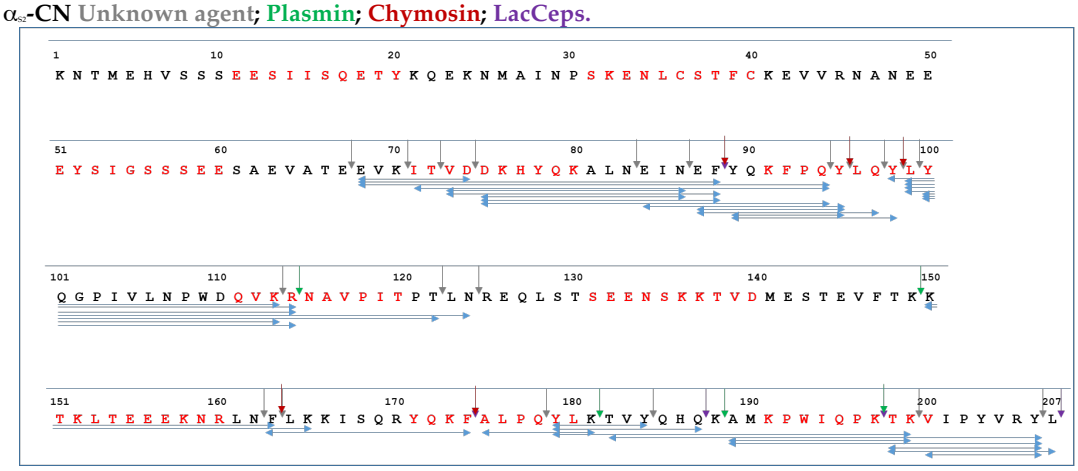
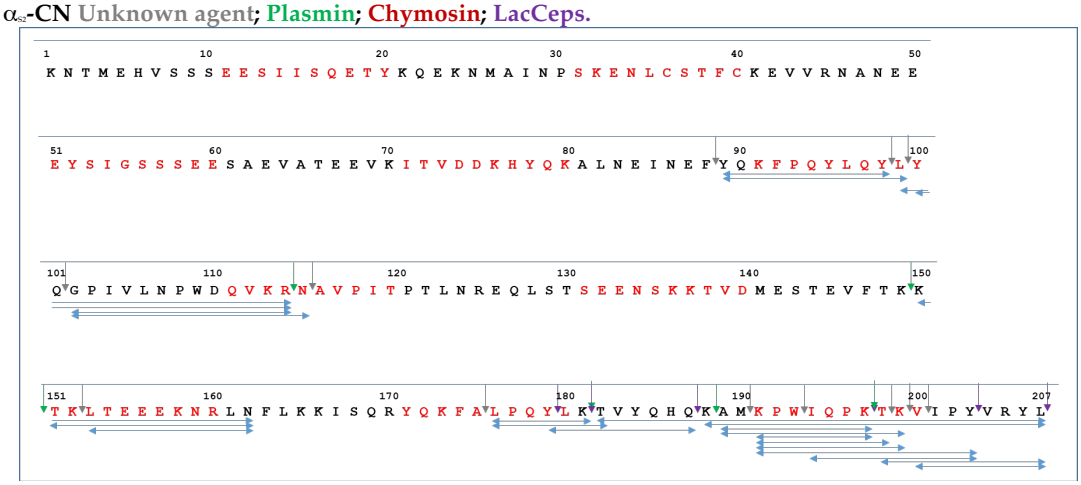


Figure 5 A. The primary structure of bovine α_{s1}-casein, showing the peptides in 10 mM citrate buffer pH 5.5-dispersed extracts of Gubbeen cheese slices taken at depth 0.41mm, 9.02 mm and 20.05 mm, together with known cleavage sites of by action of unknown agent, plasmin (Pla), chymosin (Chy), or Lactocepins (LacCeps).

0.41mm α_{s2}-CN Unknown agent; Plasmin; Chymosin; LacCeps.



9.02 mm α_{s2}-CN Unknown agent; Plasmin; Chymosin; LacCeps.



20.05 mm α_{s2}-CN Unknown agent; Plasmin; Chymosin; LacCeps.

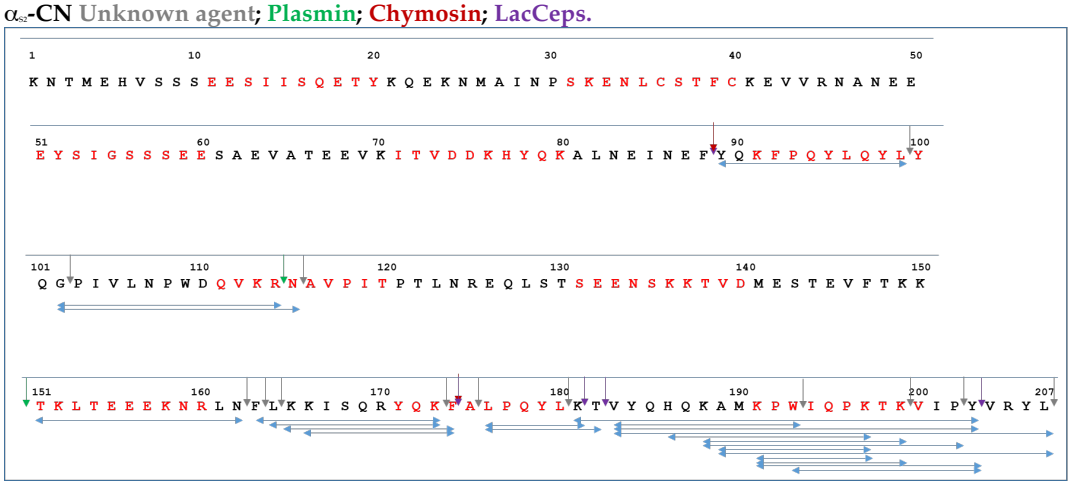
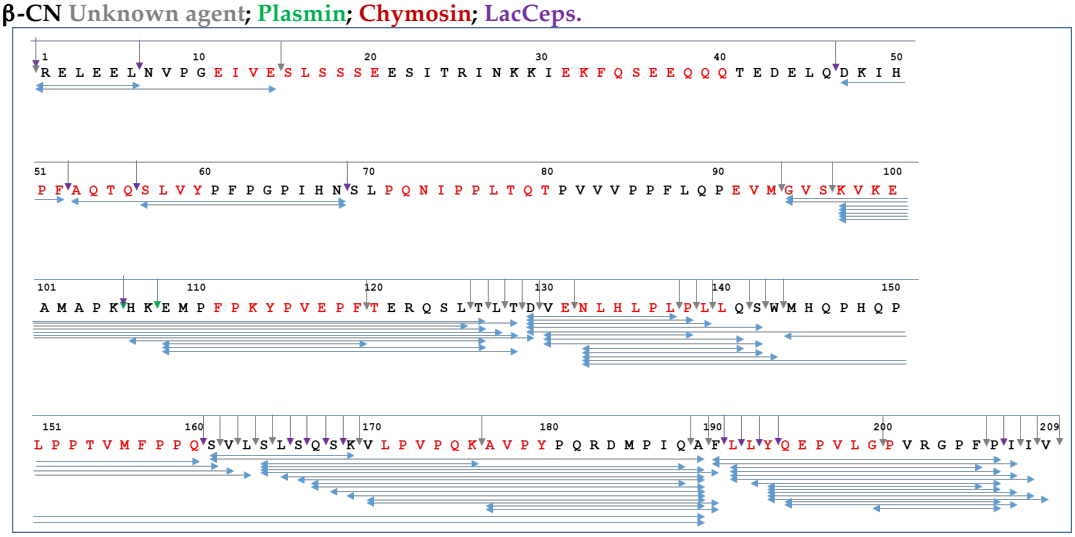
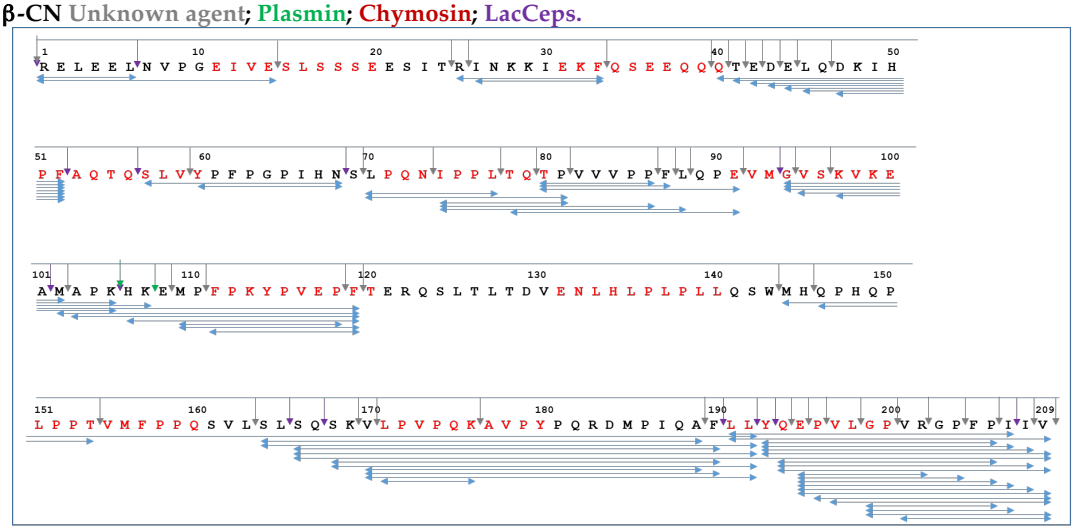


Figure 5 B. The primary structure of bovine α_{s2}-casein, showing the peptides in 10 mM citrate buffer pH 5.5-dispersed extracts of Gubbeen cheese slices taken at depth 0.41mm, 9.02 mm and 20.05 mm, together with known cleavage sites of by action of unknown agent, plasmin (Pla), chymosin (Chy), or Lactocepins (LacCeps).

0.41 mm β-CN Unknown agent; Plasmin; Chymosin; LacCeps.



9.02 mm β-CN Unknown agent; Plasmin; Chymosin; LacCeps.



20.05 mm β-CN Cheese: Unknown agent; Plasmin; Chymosin; LacCeps.

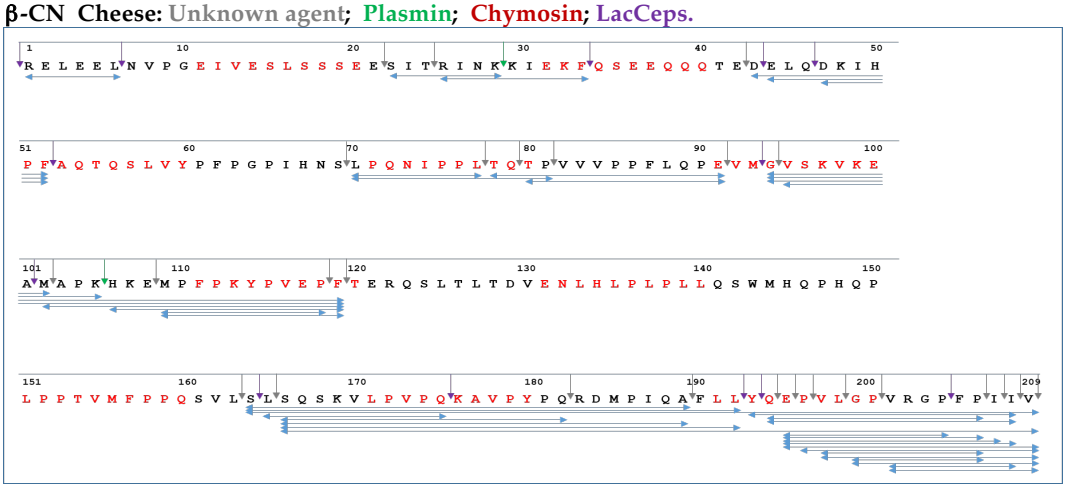
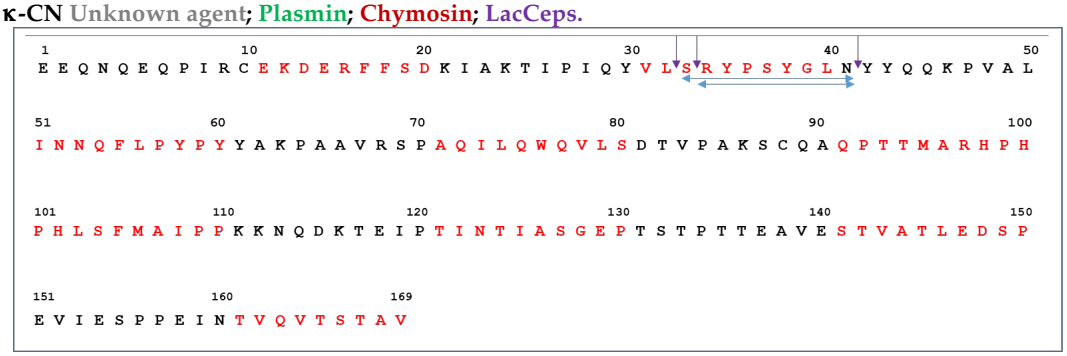
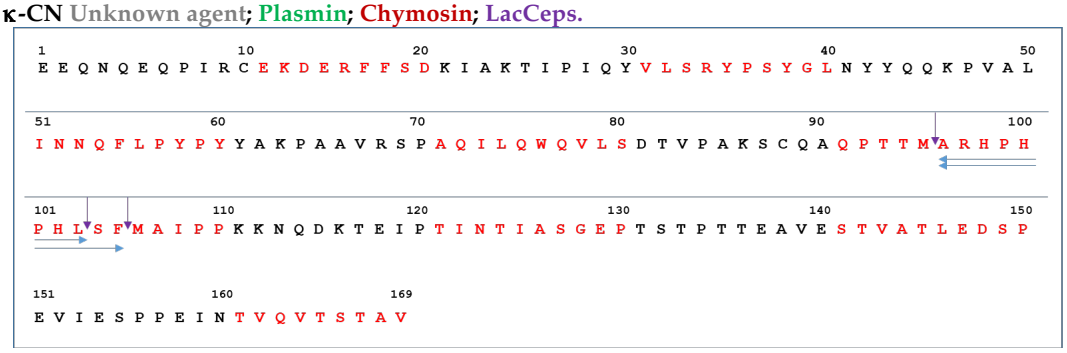


Figure 5 C. The primary structure of bovine β-casein, showing the peptides in 10 mM citrate buffer pH 5.5-dispersed extracts of Gubbeen cheese slices taken at depth 0.41mm, 9.02 mm and 20.05 mm, together with known cleavage sites of by action of unknown agent, plasmin (Pla), chymosin (Chy), or Lactocepins (LacCeps).

0.41 mm κ-CN Unknown agent; Plasmin; Chymosin; LacCeps.



9.02 mm κ-CN Unknown agent; Plasmin; Chymosin; LacCeps.



20.05 mm κ-CN Cheese: Unknown agent; Plasmin; Chymosin; LacCeps.

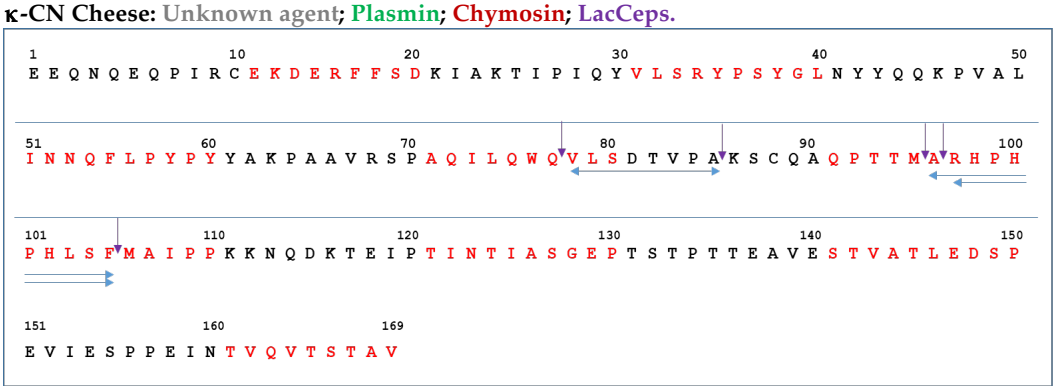


Figure 5 D. The primary structure of bovine κ-casein, showing the peptides in 10 mM citrate buffer pH 5.5-dispersed extracts of Gubbeen cheese slices taken at depth 0.41mm, 9.02 mm and 20.05 mm, together with known cleavage sites of by action of unknown agent, plasmin (Pla), chymosin (Chy), or Lactocepins (LacCeps).

A total of 5740 peptides were produced by hydrolysis of β-casein during ripening (Supplementary Data Table 4). Peptides derived from β-casein found only at depth 0.410 mm were produced from regions 130-161 and those produced only at depth 9.02 mm and 20.05 mm were from regions 39-52 and 69-119. Peptides commonly produced at all depths were found at region 1-6, 164-190 and 193-207 (Fig. 5 C). Peptide fragments derived from action of only plasmin on N- and -C termini were β-CN (f22-28), β-CN (f94/97-105), β-CN (f106-119) and β-CN (f129-137) with action of proteinases from the smear microbiota. Similarly, peptide fragments produced by the action of only lactocepins were β-CN (f1-

6/14), β-CN (f45/47-52), β-CN (f53/57-68), β-CN (f132-160), β-CN (f164/170/177190), β-CN (f165-175), β-CN(f166/176-182), β-CN(f166/168/192), β-CN(f191/193/194-207) and β-CN (f166/191/192-209). Most common peptides from b-casein present only at surface were β-CN (f 1-14), β-CN (f 53/57-68), β-CN (f 94-124/128), β-CN (f 95-125/126/127/128/129), β-CN (f 129/130/132-137/139/142), β-CN (f 132/142-161) and β-CN (f 144-162/163/189) and those present only at the center were, β-CN (f 22/23-28), β-CN (f 25-33), β-CN (f 70-77), β-CN (f 74-86), β-CN (f 78/80/84-91), β-CN (f 94-102/105), β-CN (f 95/95-119 and β-CN (f 201-204. Peptides from α_{s2}-casein present at all the depths were β-CN (f22-28), β-CN (f166/176-182), β-CN (f166/168/192) and β-CN (f166/191/192-209).

A small number of peptides from κ-casein was found at low concentrations during ripening (Fig. 5 D). Fragments produced from pH 4.6-soluble extract, by action of lactocepins at their N- or C- termini (ARDO *et al*., 2017) were κ-CN (f33-41), κ-CN (f78-85) and κ-CN (f96-103/105). κ-CN (f96-105) was produced by chymosin cleaving at its C-terminus (ARDO *et al*., 2017). More detailed study on enzyme specificities of the smear ripened cheese can be a future area of research.

4. CONCLUSION

The gross composition of smear ripened cheese was observed to be typical of the cheese variety. Overall % pH 4.6 SN/TN in all three batches G1, G2 and G3, indicated a normal proteolysis at the end of the ripening in the cheese. Extent of proteolysis was observed by urea-PAGE which showed extensive breakdown of α_{s1}- and β-casein essentially from surface to 1.23 mm, followed by normal patterns of proteolysis typical of bacterially ripened cheeses, towards the centre. Free amino acid analysis of surface layers of the cheese showed high levels of glutamic acid, glycine, alanine, tyrosine, and proline, whereas higher concentrations of threonine, lysine, phenylalanine and valine were found towards the inner layers of smear-ripened cheese. A total of 5614 peptides were identified during the study at depths 0.41mm, 9.02 mm and 20.05 mm.

Most common peptides from α_{s1}-casein present only at surface were α_{s1}-CN (f 122-139/142/143), α_{s1}-CN (f 124-142/143/144), α_{s1}-CN (f 125-144) and α_{s1}-CN (f 150-155/156/157) and those present only at the center were α_{s1}-CN (f191/193-199). Most common peptides from α_{s2}-casein present only at surface were α_{s2}-CN (f68-74/88/94), α_{s2}-CN (f71-94/98), α_{s2}-CN (f73-86/88), α_{s2}-CN (f75-88/94) and α_{s2}-CN (f87-95) and those present only at the center were, α_{s2}-CN (f176-181/182), α_{s2}-CN (f186-197), α_{s2}-CN (f188-199/202), α_{s2}-CN (f191-197/199/203) and α_{s2}-CN (f194-203). Most common peptides from b-casein present only at surface were β-CN (f 1-14), β-CN (f 53/57-68), β-CN (f 94-124/128), β-CN (f 95-125/126/127/128/129), β-CN (f 129/130/132-137/139/142), β-CN (f 132/142-161) and β-CN (f 144-162/163/189) and those present only at the center were, β-CN (f 22/23-28), β-CN (f 25-33), β-CN (f 70-77), β-CN (f 74-86), β-CN (f 78/80/84-91), β-CN (f 94-102/105), β-CN (f 95/95-119) and β-CN (f 201-204).

Common peptides from α_{s1}-casein at all the depths were α_{s1}-CN (f1-23), α_{s1}-CN (14-23), α_{s1}-CN (f 24-34) and α_{s1}-CN (131-139/142/148); from α_{s2}-casein at all the depths were α_{s2}-CN (f115-125), α_{s2}-CN (f151-156/158/161/162/163) and α_{s2}-CN (f196-208); β-casein at all the depths were β-CN (f22-28), β-CN(f166/176-182), β-CN (f166/168/192) and β-CN

(f166/191/192-209) and those from k-casein were κ-CN (f33-41), κ-CN (f78-85) and κ-CN (f96-103/105). κ-CN (f96-105).
Study of individual proteolytic enzymes and their effect on the caseins of smear ripened cheese and detailed study of microbiological aspects will be an interesting area for further research.

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PAPER

EFFECTS OF DIETARY LINSEED AND SYNTHETIC OR NATURAL ANTIOXIDANTS ON SHELF-LIFE OF PORK

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ABSTRACT

The effects of including extruded linseed in pig diets supplemented with either polyphenol-rich red grape skin extract (3 g kg^{-1}) or synthetic antioxidants (200 mg kg^{-1} α-tocopheryl acetate plus 0.21 mg kg^{-1} of selenium) on shelf-life of pork stored in modified atmosphere packaging (MAP) at different oxygen concentrations (0 and 70%) were evaluated. Linseed reduced n-6/n-3 fatty acid ratio in lipids of backfat and loin. Color parameters, pH, weight losse, oxidative stability (TBARS), did not differ between antioxidants neither in raw, nor in cooked, nor in stored muscle. High oxygen concentration in MAP increased TBARS and ΔE, yielding redder meat.

Keywords: antioxidant, extruded linseed, fatty acid composition, grape skin, modified atmosphere packaging, pork

1. INTRODUCTION

In recent years, consumers' awareness of the cause-effect relationship between dietary fat composition and health has prompted research into modify omega-6/omega-3 polyunsaturated fatty acids (PUFA) ratio in meat and meat products (CORINO *et al*., 2008; RILEY *et al*., 2000). It is known that in Western countries omega-6 fatty acids are the majority of PUFA in the food supply, whereas the consumption of omega-3 fatty acids is very low (SIMOPOULOS, 2002).
Among the factors that can affect the deposition of lipids and their fatty acid (FA) composition in pig meat, diet composition and slaughter weight play a pivotal role (LO FIEGO *et al*., 2010; WOOD *et al*., 2008). Thus pork can be a source of omega-3 PUFA whether pigs are fed with diets containing linseed or its by-products, feeds rich in α-linolenic acid (C18:3n-3, ALA) (CORINO *et al*., 2014; GUILLEVIC *et al*., 2009b; HOZ *et al*., 2003; MUSELLA *et al*., 2009).
On the other hand, the enrichment in PUFA can negatively affect the oxidative stability of pork. Lipid oxidation impairs the acceptability of meat modifying sensory attributes and nutritional quality (JAKOBSEN and BERTELSEN, 2000). To prevent the oxidative phenomena, synthetic antioxidants are usually added in pig diets, vitamin E, as α-tocopheryl acetate, being among the most common (DUNSHEA *et al*., 2005). Recently, consumers have shown an ever-increasing interest in natural antioxidants obtained from plant sources (HAAK *et al*., 2008; LORENZO *et al*., 2014). Winery by-products are rich in phenolic compounds whose antioxidant activity has been widely studied (BRENES *et al*., 2016). Further, anti-inflammatory, anti-carcinogenic, cardioprotective and vasodilatory properties have been ascribed to polyphenols (TEIXEIRA *et al*., 2014). In the Po Valley, where Italian pig production is concentrated, the cultivation of the grapevine is also widespread. Wine industry, like many other sectors of the Italian agri-food industry, produces a lot of waste products with consequent environmental problems. For instance, wastes represent almost one third of the total of the grapes used in wineries, posing serious storage and disposal problems (TEIXEIRA *et al*., 2014). The commitment to sustainable agriculture requires the reduction or elimination of these wastes. In light of all this, stakeholders of the pig industry have shown a strong interest in the possibility of utilize these by-products in the feeding of pigs. Nonetheless, the effect of grape skin extract in pig feeding on pork quality still needs to be elucidated (BRENES *et al*., 2016).
Usually fresh meat is commercialized packed in expanded polystyrene trays wrapped with stretchable cling films, characterized by high oxygen transmission rate, in order to maintain the typical bright color. In these conditions, fresh meat has a very short shelf-life due to microbial proliferation and a rapid brown discoloration (due to metmyoglobin formation) that normally takes place before unacceptable bacterial growth has occurred. In order to further increase the shelf-life of meats modified atmospheres packaging (MAP), usually characterized by variations in the content of N_2, CO_2 and O_2 in the package, is extensively used (SPANOS *et al*., 2016). Modified atmosphere packaging in an atmosphere high in oxygen (up to 80%) extends display life, prolonging the stability of the oxymyoglobin red pigments, but it promotes lipid oxidation (MCMILLIN, 2017).
The aims of this study were to investigate the effect of inclusion of extruded linseed in pig diets on carcass lipid fatty acid composition and to evaluate the protective effect of either supranutritional doses of vitamin E and selenium (Se) or grape skin extracts on the quality and shelf-life of pork packaged in modified atmospheres with or without oxygen in the inner gaseous atmosphere, during refrigerated storage.

2. MATERIALS AND METHODS

2.1. Animals and diets

All the experimental procedures were in accordance to the Italian legislation (D.Lgs 4 Marzo 2014 n. 26 art. 2 punto F).
Twelve castrated male Italian Large White pigs, evenly divided according to weight into three groups of four subjects each, were housed in 9 m^2 concrete floored pens. Starting from 79.4±7.4 kg body weight (BW) and till slaughtering, at 135.4±9.7 kg BW, the subjects were fed restricted for 70 days on 8.5% of metabolic weight ($BW^{0.75}$) either on a barley-soya bean meal feed (C group) or the same feed where 5% barley was substituted with 5% extruded linseed. Linseed diets were supplemented either with supranutritional levels of synthetic antioxidants (200 ppm of α-tocopheryl acetate and 0.21 ppm of Se (LE group) or with 3 g kg^{-1} feed of red grape skin extract (Enocianina Fornaciari, Reggio Emilia, Italy) (LGSE group), providing 29.8 ppm of polyphenols (expressed as gallic acid equivalent). Grape skin extract is a natural product used in the food industry as a supplement, nutraceutical or food coloring, included in finished products at concentrations ranging from 2 to 4g kg^{-1}, according to the manufacturer's suggestions, that we complied with.
The diets were isoenergetic and isoproteic and with the same lysine/digestible energy ratio. Water was always available through nipple drinkers and the diet was distributed in liquid form (water:feed ratio 3:1). Grape skin extract was diluted in the water of the diet.
Composition of experimental diets and their fatty acid proportions are shown in Table 1.

2.2. Slaughtering and sampling

The pigs were slaughtered on the same day in a commercial slaughterhouse, where the subjects were electrically stunned, in agreement with the Council Regulation (EC) No 1099/2009 on the protection of animals at the time of the killing.
After slaughtering, each carcass was graded using Fat-o-Meater, at level of 3/4 last rib, at 8 cm from the splitting line of the carcass (D.M. Mipaaf October 24, 2018 - GU n. 298, December 24, 2018) and dissected in commercial cuts. At dissection the whole left *Longissimus thoracis et lumborum* (LTL) muscle and a sample of subcutaneous adipose tissue at the last rib level, were removed from each carcass. The samples were stored at +4°C and sent to the laboratory for subsequent analyses.
At the lab, 24 h *post mortem*, individual LTL muscles were sliced and, on a subsample taken at the last rib, some physical parameters and oxidative stability were determined as described below. A second subsample of LTL muscle, destined to chemical analyses, was vacuum packed (Elegen, Scandiano, Reggio Emilia, Italy) and stored at -20°C. Eventually, six slices for each LTL muscle were packaged in modified atmosphere for the shelf-life study. Individual backfat samples were vacuum packed and stored at -20°C for the subsequent fatty acid composition determination.

2.3. Physical parameters of raw and cooked muscle

The values of pH were measured at 24 h *post mortem* using a portable Crison pH-meter equipped with a Xerolite electrode (Crison Instruments, Alella, Spain). The pH probe was calibrated using two buffers (pH 4.0 and 7.0).

Table 1. Composition of experimental diets (as fed basis).

		Diet[a]		
		C	**LE**	**LGSE**
Ingredients				
Extruded linseed	%	0.0	5.0	5.0
Barley	%	89.5	84.4	84.5
Solvent extracted soybean meal	%	7.0	7.0	7.0
L-Lysine	%	0.3	0.3	0.3
L-Threonine	%	0.1	0.1	0.1
Calcium carbonate	%	1.2	0.8	1.2
Dicalcium phosphate	%	1.0	1.0	1.0
Sodium chloride	%	0.4	0.4	0.4
Minerals and vitamins premix[b]	%	0.5	0.5	0.5
Vitamin E + selenium[c]	%	0.0	0.5	0.0
Red grape skin extract	%	0.0	0.0	0.3
Calculated nutrient composition[d]				
Digestible energy (DE)	Kcal/kg	3082	3151	3154
Calcium	%	0.78	0.76	0.80
Phosphorus	%	0.52	0.53	0.53
Digestible phosphorus	%	0.24	0.25	0.25
Lysine	%	0.85	0.87	0.87
Digestible lysine	%	0.73	0.75	0.75
Lysine/DE ratio	g/Mcal	2.75	2.75	2.75
Analyzed composition [e]				
Dry matter	%	90.5	90.6	90.7
Crude protein	% as fed	13.2	13.7	13.6
Crude fat	"	1.6	3.5	3.5
Crude fiber	"	5.0	5.0	4.9
ADF	"	7.0	6.8	7.0
NDF	"	20.6	19.6	20.8
ADL	"	1.3	1.2	1.4
Crude ash	"	5.2	5.1	5.0
FAs[f] composition (% of total FAs)				
C 14:0 (myristic)		0.59	0.26	0.32
C 16:0 (palmitic)		27.53	15.94	16.70
C 16:1 (palmitoleic)		0.08	0.02	0.02
C 18:0 (stearic)		1.30	2.86	2.79
C 18:1n-9 (oleic)		12.47	16.10	15.96
C 18:2n-6 (linoleic)		52.16	35.50	35.44
C 18:3n-3 (α-linolenic)		5.81	29.24	28.65
C 20:1 (eicosenoic)		0.06	0.04	0.11

[a]C, control (Extruded linseed, 0 g kg^{-1}); LE, (Extruded linseed, 50 g kg^{-1}, vitamin E (α-tocopheryl acetate), 200 mg and Se 0.21 mg kg^{-1}); LGSE, (Extruded linseed, 50 g kg^{-1}, red grape skin extract 3 g kg^{-1}). [b]The vitamins and minerals of the diet provided by premix (kg^{-1}): vitamin A, 15,000 UI; vitamin D3, 2000 IU; vitamin E (α-tocopheryl acetate), 50 mg; vitamin K, 2.5 mg; vitamin B1, 2.0 mg; vitamin B2, 5.0 mg; calcium D-pantothenate, 15.0 mg; niacin, 25.0 mg; vitamin B12, 0.036 mg; vitamin B6, 4.0 mg; folic acid, 1.0 mg; biotin, 0.15 mg; choline chloride, 346.0 mg; Zn (ZnO), 100.0 mg; Cu ($CuSO_4$), 15.0 mg; Mn (MnO), 25.0 mg; Fe ($FeSO_4$), 150.0 mg; I ($Ca(IO_3)_2$), 1.5 mg; Co ($CoCO_3$), 0.4 mg; Se (Na_2SeO_3), 0.1 mg. [c]Providing vitamin E (α-tocopheryl acetate) 200 mg and Se 0.21 mg kg^{-1} as fed, supported on $CaCO_3$. [d]According to Sauvant *et al.* (2004). [e]According to the Association of Official Analytical Chemists (1995). [f]Fatty acids

The sample surface color was determined using a Minolta CM-600d spectrophotometer (Konica Minolta Holdings, Inc, Osaka, Japan) with a window diameter of 8 mm and D65 as illuminant source. Before color measuring, the spectrophotometer was calibrated against a white plate supplied by the manufacturer. Color measurements complied the CIELAB color system, where the three fundamental coordinates are L*- "lightness", a*- "redness", b*- "yellowness" values. Further, Chroma (C), referred to as saturation index and color intensity, was calculated as: $(a^{*2}+b^{*2})^{0.5}$, and hue angle (h), spectral color, was calculated as follows: $\tan^{-1}$ (b*/a*). The average values were the mean of three measurements in different areas of the surface of the sample, avoiding the zones of visible fat. Drip loss was determined according to HONIKEL (1998), slightly modified. A slice of fresh muscle (about 100 g) was hooked and then suspended in an inflated bag, ensuring that the sample did not make contact with the bag. The weight loss percentage after 48 h of storage at 4°C was calculated. Cooking loss was calculated as weight difference between raw and cooked samples. In brief, slices of about 2.5 cm thickness, weighing approximately 100 g, placed in vacuum plastic bags, were put in water-bath till the core temperature reached 70°C. The internal temperature of samples was controlled during cooking with a temperature probe. The samples were weighed after cooling. Cooking losses were expressed as a percentage of the initial sample weight.
The Warner–Bratzler shear force (WBSF) was determined on the cooked samples. The samples were cut, parallel to the longitudinal orientation of the muscle fibres according to HONIKEL (1998) method, into six cylindrical cores (Ø 1.50 cm). Each core was sheared with a WBSF device attached to a Zwick Z50 kN Testing Machine (model BT1-FB050TN, Zwick Roell, Kennesaw, GA USA) with a 1kN load cell equipped with the V-shaped blade with a triangular hole of 60° at a speed of 250 mm/min. The peak force (average value of 6 measurements for each sample) was expressed as kg.

2.4. Chemical composition of muscle

After thawing, samples of LTL muscle were analyzed in duplicate to determine the moisture, ether extract with previous acid hydrolysis, and crude protein, according to the methods of the Association of Analytical Chemists (AOAC, 1995). Results were expressed as percentage of wet matter.

2.5. Fatty acid profile of backfat and muscle

Fatty acid (FA) profile of lipids in thawed samples of subcutaneous adipose tissue and LTL muscle was determined using a TRACE™GC Ultra (Thermo Electron Corporation, Rodano, Milano, Italy) equipped with a Flame Ionization Detector, a PTV injector, and a TR-FAME Column (Thermo Scientific, Rodano, Milano), 30 m long, 0.25 mm i.d., 0.2 μm film thickness. Total lipids were extracted from the samples of subcutaneous adipose tissue (IUPAC, 1979) and from LTL muscle (FOLCH *et al*., 1957). Then, an aliquot of 25 mg was subjected to methylation by means of a methanolic solution of potassium hydroxide (KOH 2N) according to FICARRA *et al*. (2010), using tridecanoic acid (C13:0) (Larodan Fine Chemicals AB, Malmö, Sweden) as internal standard. The injection of the fatty acid methyl ester sample (1 μL) was performed in split mode with a split flow of 10 mL/min, operating at a constant flow of 1 mL/min of helium as carrier gas. The temperature of injector and detector was kept at 240°C. The temperature program used for the analysis started from 140°C, was maintained for 2 min, then increased to 250°C, at a rate of 4°C min^{-1}, and kept at this temperature for 5 min. The peaks of the fatty acid methyl esters

(FAMEs) were recorded and integrated using Chrom-Card software (vers. 2.3.3, Thermo Electron Corporation, Rodano, Milano, Italy) and identified by comparison with the retention times of standard solutions with known quantities of various methyl esters (Supelco® 37 Component FAME mix, PUFA standard n.2, Animal Source, Supelco, Bellafonte, PA, USA and single FAMEs standard, Larodan Fine Chemicals AB, Malmö, Sweden). For quantification purposes, the response factor was calculated, and the method of the internal standard was used. The amount of each FAME in the sample was expressed as FAME relative percentage with respect to the total amount of FAMEs. Iodine value (IV) of backfat was calculated adopting the equation proposed by LO FIEGO *et al*. (2016): IV = 85.703 + [C14:0] x 2.740 - [C16:0] x 1.085 - [C18:0] x 0.710 + [C18:2n-6] x 0.986.

2.6. Oxidative stability of raw and cooked muscle

Oxidative stability was evaluated by the 2-thiobarbituric acid reactive substances (TBARS) measurements according to SIU and DRAPER (1978). In detail, each sample of muscle was minced and an aliquot of 2.5 g was homogenized in 12.5 mL of distilled water at 9500 rpm for 2 min, using an Ultra-Turrax tissue homogenizer (IKA, Germany), and then vortexed for 1 min at high speed. Samples were centrifuged for 20 min at 2000 rpm at 4°C with 12.5 mL of 10% trichloroacetic acid (TCA) (Sigma-Aldrich, Milan, Italy) and the supernatant decanted through a paper filter (Whatman 541). Four mL of clear filtrate were transferred into 15 mL pyrex screw cap test tubes and added of 1 mL of 0.06M 2-thiobarbituric acid (TBA). A distilled water-TCA-TBA reagent blank was prepared and treated like the samples. The samples were heated in a water bath at 80°C for 90 min and then cooled. Absorbance at 532 nm was measured against a blank sample on two replicates of each sample on a Jasco spectrophotometer (Model V550, UV/VIS, Tokyo, Japan) immediately after cooling. TBARS were expressed as mg of malondialdehyde (MDA) per kg of muscle using tetraethoxypropane (TEP) (Sigma-Aldrich, Milan, Italy) as a standard.

2.7. Packaging and Shelf-life study

From each loin, 6 slices (approximately 2-2.5 cm thick), designated for the evaluation of the shelf-life in two different modified atmospheres, were individually weighed and packed in a total of 72 high barrier trays lidded with a PET/EVOH/PE film (AERPACK System, kindly supplied by Coopbox Group, Italy). The whole package oxygen transmission rate (OTR) was < 0.1 cm^3 day^{-1} (air, 25°C). MAP was performed using a semiautomatic vacuum compensation thermosealing machine (Ca.Ve.Co, Italy). Two different gaseous mixtures were used: 70%N_2/30%CO_2 (N_2) and 70%O_2/30%CO_2 (O_2). Gas composition of the headspace was analysed before opening packages using a HWD-GC equipped with a concentric CRT I column (6ft x 1/4"; outer column: activated molecular sieve; inner column: porous polymer mixture) (Alltech Italia, S.r.l, Italy). Gascromatographic conditions: gas carrier helium (65 mL min^{-1}); temperature 55°C; analysis time 5 min. A septum was glued onto the surface of the lid and a 50 mL gas aliquot was withdrawn with a gastight syringe and injected in the gascromatograph. The calibration was performed by injecting separately pure gases as external standard (supplied by Sapio; pureness 99.9) and calculating the response factor for each one.
All samples packaged in modified atmosphere were stored in the dark at 3±1°C for a maximum of 8 days. Twenty-four trays (12 subjects x 2 different modified atmospheres) were removed from the refrigerators at day 4, 6 and 8 of storage, weighed to calculate weight losses and then submitted to color, pH and TBARS determination, as described

above (Sections 2.3 and 2.6). Color measurements were carried out after allowing a 30 min blooming, following pack opening. Moreover, overall color variation (ΔE) was calculated as $(\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{0.5}$ where ΔL^*, Δa^* and Δb^* are the difference between time 0 (at 24 h *post mortem*) and the values L^*, a^* and b^* respectively at 4 (ΔE_{0_4}), 6 (ΔE_{0_6}) and 8 (ΔE_{0_8}) days of refrigerated storage.

2.8. Statistical analysis

Statistical analysis was performed by means of analysis of variance using the GLM procedure of SAS (SAS Institute Inc., Cary, NC, USA). The statistical model for performance, carcass characteristics, qualitative characteristics and chemical composition of fresh LTL muscle, fatty acid composition of subcutaneous adipose tissue and LTL muscle, used dietary treatments (DT) (C, LE and LGSE) as independent variables.
The data from samples stored in MAP were statistically analyzed within storage day including in the model also the packaging atmosphere (P) (O_2 or N_2) and the interaction DTxP. The interaction was never statistically significant (P>0.05) and was thus eliminated from the model.
The two degrees of freedom of dietary treatments were *a priori* splitted into two orthogonal contrasts comparing, respectively, control group *vs* average of extruded linseed groups (C *vs* LE+LGSE/2) and antioxidant supplemented groups between them (LE *vs* LGSE).

3. RESULTS

3.1. Performance and carcass characteristics

No effect of dietary treatments (DT) was found on farm performances (average daily gain 0.8±0.1 kg, slaughter weight 135.4±9.7 kg) and carcass characteristics (carcass weight 113.7±8.3 kg, dressing percentage 83.9±1.2, backfat thickness 24.3±3.3 mm and lean meat content 54.3±2.1%) (data not reported in Tables). GUILLEVIC *et al*. (2009a) and HAAK *et al*. (2008), feeding linseed to finishing light weight pigs, could not detect either any difference in on farm and abattoir performances.

3.2. Fresh meat quality

The effect of DT on qualitative characteristics of meat is reported in Table 2.
According to CORINO *et al*. (2014), muscle pH is not affected by linseed feeding. Our results confirm the inferences of their meta-analysis. The control group showed a slightly higher b* and Chroma values (P <0.05), denoting a greater yellowness and color intensity than linseed groups. CORINO *et al*. (2008) did not find significant differences related to the values of b* in pig fed with linseed. While our Chroma values agree with data reported by JUÁREZ *et al*. (2001), but there was no obvious explanation for this trend, therefore it requires further investigation. No difference was found between LE and LGSE groups. Further, drip and cooking losses, oxidative stability in raw and cooked muscle, shear force and chemical composition (moisture, crude protein and lipid contents of LTL muscle) were affected neither by linseed nor by the antioxidants. CORINO *et al*. (2008) and HAAK *et al*. (2008) found that linseed inclusion did not influence these qualitative traits in meat of light pigs either. Moreover, BOLER *et al*. (2009) reported that vitamin E reduces lipid

oxidation but has no effect on any carcass characteristics and loin quality. Eventually, the lack of effects of linseed feeding on chemical parameters of the muscle (moisture, crude protein and lipid contents) confirms the findings of HOZ *et al*. (2003) on tenderloin.

Table 2. Qualitative characteristics and chemical composition of *Longissimus thoracis et lumborum* muscle from pigs fed with the experimental diets.

Items	Dietary treatment[a]			P-value		R-MSE[b]
	C (n=4)	LE (n=4)	LGSE (n=4)	C *vs* (LE+LGSE)/2	LE *vs* LGSE	
pH *post mortem* (24h)	5.58	5.53	5.59	0.679	0.236	0.067
L*	55.83	56.26	55.70	0.878	0.616	1.526
a*	1.97	0.74	0.56	0.127	0.847	1.284
b*	12.88	12.08	11.18	0.044	0.177	0.870
Chroma	13.13	12.15	11.20	0.049	0.232	1.049
Hue angle	81.98	86.75	87.17	0.142	0.908	5.047
Drip loss (%)	3.46	3.13	3.39	0.763	0.731	1.032
Cooking loss (%)	17.05	19.78	16.98	0.358	0.110	2.236
TBARS[c] (raw muscle)	0.045	0.069	0.091	0.134	0.396	0.034
TBARS[c] (cooked muscle)	0.386	0.405	0.279	0.698	0.341	0.178
Shear force (Kg)	4.99	4.87	4.85	0.669	0.957	0.471
Chemical composition (%)						
Moisture	72.30	71.96	72.04	0.544	0.896	0.785
Crude protein	23.39	23.16	23.44	0.845	0.575	0.688
Lipids	1.36	1.74	1.27	0.571	0.145	0.418

[a]C, control (Extruded linseed, 0 g kg^{-1}); LE, (Extruded linseed, 50 g kg^{-1}; vitamin E (α-tocopheryl acetate), 200 mg and Se 0.21 mg kg^{-1}); LGSE, (Extruded linseed, 50 g kg^{-1}; red grape skin extract 3 g kg^{-1}).

[b]Root Mean Square Error.

[c]TBARS (thiobarbituric acid reactive substances) expressed in mg of malondialdehyde (MDA) per kilogram of muscle.

3.3. Adipose tissue and intramuscular fatty acid composition

Table 3 shows lipid content and fatty acid composition of subcutaneous adipose tissue.
No differences attributable to extruded linseed dietary inclusion or type of antioxidant were found for lipid content of backfat. The proportion of total saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids was not affected by the inclusion of linseed in the diet, in agreement with MUSELLA *et al*. (2009) who detected no difference in the percentage of main FA classes in ham covering trimmed fat between control and linseed-fed pigs.
The total content of n-6 PUFA, although tendentially lower in linseed fed subjects, was not significantly influenced by dietary treatments ($P>0.05$) either. This is likely due to the limited number of experimental units. Overall, among n-6 PUFA and MUFA, only γ-linolenic (C18:3n-6) and heptadecenoic acids (C17:1) were affected by dietary linseed inclusion, which brought about a significant ($P<0.05$) reduction of their proportions. Conversely, n-3 PUFA increased significantly ($P<0.01$) with linseed dietary inclusion. This confirms the findings of RILEY *et al*. (2000). The increase of the proportion of the total n-3 PUFA is ascribable to the higher proportions ($P<0.01$) of α-linolenic (ALA, C18:3n-3) and eicosatrienoic (C20:3n-3) acids which trebled and docosapentaenoic acid (DPA, C22:5n-3)

that doubled, whereas eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) remained unchanged. GUILLEVIC *et al*. (2009b) could not find any correlation between ALA intake and the proportion of DHA in adipose tissues either. RILEY *et al*. (2000) hypothesized that the limited accumulation of longer chain PUFA in adipose tissues could be due to scant capacity for the synthesis of these products starting from dietary ALA.

Table 3. Lipid content (%) and fatty acid profile (% of total fatty acids) of backfat from pigs fed with the experimental diets.

Items	Dietary treatment[a]			P-value		R-MSE[b]
	C (n=4)	LE (n=4)	LGSE (n=4)	C *vs* LE+LGSE/2	LE *vs* LGSE	
Lipids	86.14	86.56	87.55	0.752	0.765	4.560
Fatty acids:						
C 10:0 (capric)	0.13	0.10	0.10	0.407	0.988	0.053
C 12:0 (lauric)	0.07	0.05	0.07	0.591	0.328	0.027
C 14:0 (myristic)	1.16	1.15	1.18	0.975	0.659	0.093
C 16:0 (palmitic)	24.66	24.59	24.39	0.735	0.731	0.785
C 17:0 (heptadecanoic)	0.35	0.32	0.30	0.338	0.673	0.055
C 18:0 (stearic)	15.32	14.95	15.47	0.843	0.437	0.896
C 20:0 (eicosanoic)	0.15	0.26	0.22	0.103	0.501	0.076
C 16:1 (palmitoleic)	1.62	1.51	1.57	0.553	0.674	0.208
C 17:1 (heptadecenoic)	0.32	0.26	0.24	0.016	0.517	0.040
C 18:1n-7 (vaccenic)	1.51	1.39	1.65	0.547	0.420	0.281
C 18:1n-9 (oleic)	39.47	38.08	39.30	0.956	0.220	2.040
C 20:1 (eicosenoic)	0.67	0.83	0.81	0.075	0.828	0.126
C 18:2n-6 (linoleic)	12.64	11.60	10.46	0.236	0.458	2.070
C 18:3n-3 (α-linolenic)	0.84	3.26	2.74	0.000	0.103	0.406
C 18:3n-6 (γ-linolenic)	0.09	0.05	0.07	0.026	0.152	0.022
C 20:2n-6 (eicosadienoic)	0.42	0.54	0.50	0.196	0.605	0.116
C 20:3n-3 (eicosatrienoic)	0.12	0.43	0.43	0.000	0.906	0.089
C 20:4n-6 (arachidonic)	0.30	0.25	0.26	0.323	0.859	0.070
C 20:5n-3 (eicosapentaenoic)	0.00	0.15	0.00	0.486	0.254	0.170
C 22:2n-6 (docosadienoic)	0.00	0.00	0.00	0.377	0.466	0.004
C 22:4n-6 (docosatetraenoic)	0.07	0.05	0.04	0.127	0.434	0.027
C 22:5n-3 (docosapentaenoic)	0.09	0.18	0.20	0.000	0.583	0.028
C 22:6n-3 (docosahexaenoic)	0.02	0.02	0.02	0.864	0.510	0.016
Total Saturated	41.83	41.40	41.72	0.719	0.717	1.185
Total Monounsaturated	43.58	42.06	43.57	0.587	0.363	2.224
Total Polyunsaturated	14.60	16.54	14.72	0.523	0.338	2.546
Total n-6	13.53	12.49	11.33	0.257	0.470	2.183
Total n-3	1.07	4.05	3.39	0.000	0.088	0.484
n-6/n-3 fatty acid ratio	12.64	3.09	3.37	0.000	0.622	0.780
Iodine Value[c]	63.72	62.98	61.79	0.410	0.520	2.511

[a]C, control (Extruded linseed, 0 g kg^{-1}); LE, (Extruded linseed, 50 g kg^{-1}; vitamin E (α-tocopheryl acetate), 200 mg and Se 0.21 mg kg^{-1}); LGSE, (Extruded linseed, 50 g kg^{-1}; red grape skin extract 3 g kg^{-1}).

[b]Root Mean Square Error.

[c]IV=85.703 + [C14:0] x 2.740 - [C16:0] × 1.085 - [C18:0] × 0.710 + [C18:2n-6] × 0.986

On the whole, these trends led to a significant (P<0.01) reduction in the n-6/n-3 PUFA ratio, as was previously observed also by MUSELLA *et al*. (2009) and RILEY *et al*. (2000). In our study, the n-6/n-3 PUFA ratio dropped from 12.6 in control pigs to less than 3.5 in linseed fed pigs. Thus, the inclusion of 5% extruded linseed in the finishing diet enabled to bring this ratio far below the threshold indicated by SIMOPOULOS (2008) to avoid adverse health consequences, without impairing technological parameters of subcutaneous adipose tissue. In fact, most Authors (e.g. LEBRET and MOUROT, 1998; LO FIEGO *et al*., 2005) indicate, as a guarantee of good preservation aptitude, contents of stearic acid (C18:0) and linoleic acid (C18:2n-6) higher than 12 and lower than 15%, respectively and an Iodine value minor than 70.
As showed in Table 3, and as expected, no difference was found between the linseed fed groups (LE *vs* LGSE) for any of the parameters taken into account.
Table 4 shows the fatty acid composition in LTL muscle. In general, the variations observed in FA compositions resembled what already seen in the backfat tissue. In fact, total SFA, MUFA and PUFA percentages did not differ among dietary treatments (P>0.05). Compared to C, linseed groups showed a higher content of lauric acid (C12:0; P<0.01) and lower in vaccenic acid (C18:1n-7; P<0.05). No hypothesis could be put forward to explain these variations that, though trivial, were statistically significant.
Total n-6 PUFA content, as in the backfat depot, although is tendentially higher in C group, was not affected by the dietary treatment and the only significant variation was shown by the docosatetraenoic acid (C22:4n-6; P<0.01), which resulted higher in the C group. The same trend was observed by RILEY *et al*. (2000). The total n-3 PUFA proportion, as seen in the backfat, was significantly higher (P<0.01) in the linseed groups. In detail, α-linolenic, eicosatrienoic, and eicosapentaenoic acids (P<0.01) more than tripled. Thus, EPA that did not change with diet in adipose tissue, increased in LTL muscle. This agrees with the results of CORINO *et al*. (2008), who observed that EPA is preferentially stored in the muscle rather than in the adipose tissue, and RILEY *et al*. (2000), who inferred that α-linolenic acid intake elicits eicosapentaenoic acid increments more in muscle than in adipose tissue. Hence, also in the muscle, the n-6/n-3 PUFA ratio was significantly reduced (P<0.01) to one-third in linseed fed pigs.
Not even in this tissue, except for the proportion of lauric acid, any variation was found between the two different dietary antioxidants, for any of the parameters taken into account.

3.4. Quality characteristics of meat stored in MAP

The analysis of the inner gaseous atmosphere composition of the experimental samples showed that the relative percentages of the gases have not changed throughout the storage time (data not shown). This result is not unexpected, because of the refrigerated (3±1°C) and short storage time length (8 days), the high barrier materials used, which strongly limits the gas transfers in and out the packages, and the bacteriostatic activity of CO_2 that, slowing the microbial growth, avoids oxygen consumption and as a consequence its decrease. In these conditions, all the modifications registered on the meat samples can be attributed only to the presence or absence of oxygen in the atmosphere surrounding the product and to meat composition.
The effects of dietary treatments and of gaseous mixtures in packaging during the 8 days of refrigerated storage on various physico-chemical characteristics of LTL muscle are shown in Table 5. The dietary treatments influenced most of the parameters studied.

Linseed groups exhibited lower weight losses; however, the reduction was significant ($P<0.05$) only on days 4 and 6.

Table 4. Fatty acid profile (% of total fatty acids) of *Longissimus thoracis et lumborum* muscle from pigs fed with the experimental diets.

Items	Dietary treatment[(a)]			P-value		R-MSE[(b)]
	C (n=4)	LE (n=4)	LGSE (n=4)	C *vs* LE+LGSE/2	LE *vs* LGSE	
C 10:0 (capric)	0.11	0.29	0.30	0.355	0.993	0.306
C 12:0 (lauric)	0.05	0.16	0.11	0.000	0.019	0.024
C 14:0 (myristic)	1.07	1.18	1.13	0.343	0.646	0.140
C 16:0 (palmitic)	23.11	23.85	23.48	0.442	0.656	1.117
C 17:0 (heptadecanoic)	0.12	0.14	0.21	0.303	0.185	0.076
C 18:0 (stearic)	13.41	13.50	13.10	0.780	0.381	0.616
C 20:0 (eicosanoic)	0.12	0.12	0.13	0.871	0.822	0.020
C 16:1 (palmitoleic)	2.81	2.99	2.73	0.801	0.261	0.304
C 17:1 (heptadecenoic)	0.07	0.15	0.18	0.062	0.560	0.075
C 18:1n-7 (vaccenic)	3.30	2.84	2.74	0.038	0.686	0.345
C 18:1n-9 (oleic)	39.39	39.79	40.68	0.612	0.646	2.639
C 20:1 (eicosenoic)	0.59	0.52	0.55	0.249	0.618	0.071
C 18:2n-6 (linoleic)	10.52	9.07	9.03	0.276	0.976	2.069
C 18:3n-3 (α-linolenic)	0.40	1.27	1.37	0.000	0.540	0.218
C 18:3n-6 (γ-linolenic)	0.08	0.09	0.13	0.350	0.352	0.057
C 20:2n-6 (eicosadienoic)	0.21	0.19	0.19	0.380	0.971	0.040
C 20:3n-3 (eicosatrienoic)	0.05	0.16	0.18	0.001	0.481	0.043
C 20:4n-6 (arachidonic)	3.53	2.49	2.50	0.093	0.991	0.897
C 20:5n-3 (eicosapentaenoic)	0.07	0.35	0.36	0.004	0.954	0.120
C 22:2n-6 (docosadienoic)	0.01	0.01	0.01	0.167	0.600	0.004
C 22:4n-6 (docosatetraenoic)	0.51	0.19	0.26	0.006	0.497	0.132
C 22:5n-3 (docosapentaenoic)	0.39	0.52	0.60	0.227	0.599	0.214
C 22:6n-3 (docosahexaenoic)	0.08	0.12	0.04	0.992	0.255	0.095
Total Saturated	38.00	39.23	38.46	0.364	0.469	1.452
Total Monounsaturated	46.16	46.29	46.88	0.811	0.776	2.824
Total Polyunsaturated	15.85	14.47	14.67	0.574	0.941	3.574
Total n-6	14.85	12.04	12.11	0.173	0.977	3.064
Total n-3	1.00	2.43	2.56	0.002	0.755	0.551
n-6/n-3 fatty acid ratio	15.33	4.99	4.76	0.000	0.827	1.438

[(a)]C, control (Extruded linseed, 0 g kg^{-1}); LE, (Extruded linseed, 50 g kg^{-1}; vitamin E (α-tocopheryl acetate), 200 mg and Se 0.21 mg kg^{-1}); LGSE, (Extruded linseed, 50 g kg^{-1}; red grape skin extract 3 g kg^{-1}).
[(b)]Root Mean Square Error.

The comparison between the antioxidants added to the linseed diets did not reveal significant differences ($P>0.05$) in this parameter.
In any time-lapse interval considered, the weight loss was unaffected by the packaging atmosphere.
The pH of LTL muscle ranged from 5.46 to 5.55 during the 8 days storage. These values are quite common in medium-heavy pigs. Dietary linseed inclusion did not affect the pH values, whereas grape skin extract addition yielded higher pH values than synthetic

antioxidant at day 6 (P< 0.01). However, in absence of a definite trend, this difference can be attributed to inter-animal variation. Our results conflict with the findings of LORENZO *et al*. (2014), who found that the addition of natural antioxidants, derived from grape seed extract, lowered the value of pH throughout storage. This difference is likely to be ascribable to the different origin of the grape extracts utilized (seed or skin) and, especially, to the fact that in the present work the extract was added to the diet of the pigs and not into the meat.

Table 5. Effect of the diet and packaging on the weight loss (%), pH, TBARS values and color variation (ΔE), measured on *Longissimus thoracis et lumborum* muscle, refrigerated 8 days (3 ± 1°C).

Items	Dietary treatment[a]			Packaging		P-value	P-value		R-MSE[b]
	C (n=24)	LE (n=24)	LGSE (n=24)	O_2 (n=36)	N_2 (n=36)	O_2 *vs* N_2	C *vs* (LE+ LGSE/2)	LE *vs* LGSE	
Day 4									
Weight loss %	3.67	2.70	2.84	2.86	3.28	0.233	0.021	0.737	0.834
pH	5.52	5.54	5.54	5.55	5.51	0.003	0.116	0.641	0.031
TBARS[c]	0.138	0.143	0.131	0.159	0.115	<0.001	0.932	0.186	0.016
ΔE0_4 [d]	3.58	3.83	3.94	5.30	2.27	0.001	0.724	0.911	1.978
Day 6									
Weight loss %	4.57	3.07	3.87	3.39	4.29	0.076	0.043	0.190	1.178
pH	5.46	5.46	5.50	5.47	5.49	0.059	0.083	0.008	0.026
TBARS[c]	0.078	0.032	0.044	0.103	0.001	<0.001	0.015	0.497	0.035
ΔE0_6[d]	3.81	4.23	3.30	5.12	2.44	<0.001	0.953	0.271	1.644
Day 8									
Weight loss %	5.13	4.22	4.78	4.44	4.98	0.314	0.272	0.396	1.286
pH	5.51	5.51	5.53	5.53	5.50	0.007	0.309	0.230	0.026
TBARS[c]	0.157	0.091	0.058	0.177	0.027	0.021	0.208	0.656	0.147
ΔE0_8[d]	3.88	4.17	3.75	5.33	2.53	0.001	0.922	0.641	1.761

[a]C, control (Extruded linseed, 0 g kg^{-1}); LE, (Extruded linseed, 50 g kg^{-1}; vitamin E (α-tocopheryl acetate), 200 mg and Se 0.21 mg kg^{-1}); LGSE, (Extruded linseed, 50 g kg^{-1}; red grape skin extract 3 g kg^{-1}).

[b]Root Mean Square Error;

[c]TBARS (Thiobarbituric acid reactive substances) expressed in mg of malondialdehyde per kilogram of muscle.

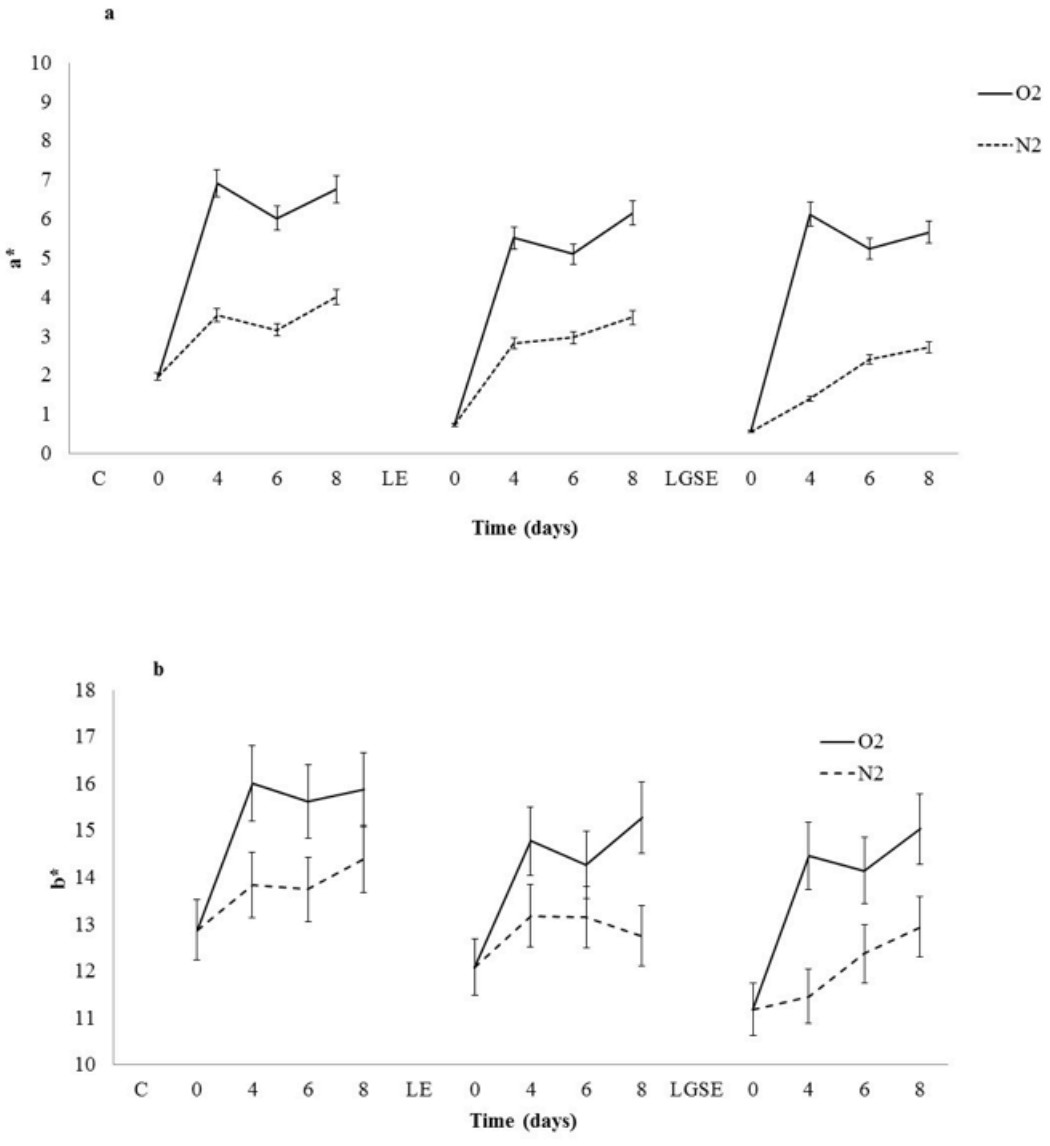
[d]$\Delta Ei\_j = \sqrt{(Lj - Li)^2 + (aj - ai)^2 + (bj - bi)^2}$

In 0_2 packaged samples, significant, though negligible, increases (P<0.01) in pH values were recorded at 4 and 8 days of storage. This agrees with MUHLISIN *et al*. (2014), who showed that, at increasing oxygen content in the gas mixture, pH of pork was higher than in samples stored at higher levels of nitrogen. However, VIANA *et al*. (2005) found that MAP did not exert a strong effect on pH of fresh pork loin.

The color evolution, expressed by ΔE, during refrigerated storage time, did not differ among dietary treatments. The ΔE values were all above the value of 2, which is considered the threshold to appreciate visual changes of the color (LORENZO *et al*., 2014). With regard to the gas mixtures, the ΔE in the samples stored in oxygen were significantly higher (P<0.01) in any time-lapse interval considered. Over storage, the ΔE values remained almost constant in the two gas mixtures, higher than 5 in O_2 and roughly half that in N_2.

Fig. 1 reports LTL color parameters evolution. In detail, as shown in Fig. 1a, the a* value, which is an index of redness, was consistently higher in high oxygen MAP over storage, regardless of the dietary treatment. The values observed in N_2 MAP are perceived as grey color, as the values ranged from 3.2 and 4.6 (DE SANTOS *et al.*, 2007). The same pattern was observed for the b* value (Fig. 1b).

Figure 1. *Longissimus thoracis et lumborum* muscle color parameters evolution: redness "a*" values (**a**) and yellowness "b*" values (**b**) in relation to storage time in MAP: oxygen (O_2) or nitrogen (N_2).
C, control group (Extruded linseed, 0 g kg^{-1}); LE group, (Extruded linseed, 50 g kg^{-1}; vitamin E (α-tocopheryl acetate), 200 mg and Se 0.21 mg kg^{-1}); LGSE group, (Extruded linseed, 50 g kg^{-1}; red grape skin extract 3.0 g kg^{-1})



As concerns the oxidative stability, in the time-lapses considered, TBARS values were unaffected by the dietary treatment on day 4 and 8 (Table 5). Only on day 6, meat from the control showed a greater oxidation ($P<0.05$) than in antioxidants groups. No difference was found between LE *vs* LGSE.
The different modified atmospheres affected the oxidative stability of the muscle, that was always lower in samples packaged in high O_2 MAP, which yielded to a significantly higher TBARS values on day 4 and 6 ($P<0.01$), and on day 8 ($P<0.05$) of storage. When oxygen is readily available, a substrate such as meat is more prone to oxidation (SMIDDY *et al*., 2002) and, in agreement with our results, SPANOS *et al*. (2016) observed that samples of LTL muscle stored in MAP oxygen concentration of 50% or higher showed a significantly lower oxidative stability compared to samples stored under 0% oxygen.
However, even the highest determined value of TBARS was lower than 0.18 mg MDA/kg meat, far below the threshold value of 1.0 mg MDA/kg muscle for organoleptic detection of rancidity as suggested by O'GRADY *et al*. (2008).
Considering that TBARS values up to 0.6 mg of MDA/kg of fresh meat are considered fresh (TARLADGIS *et al*., 1960), all our samples, regardless of the different MAPs, could be classified as fresh meat, through the 8 days of storage.

4. CONCLUSIONS

Our results confirm that 5% of dietary extruded linseed included in the pig finishing diet is a suitable means to increase n-3 PUFA content and reduce the n-6/n-3 PUFA ratio in pig tissues without affecting on live and slaughter performance and impairing technological characteristics of adipose depots. In general, under the point of view of human nutrition, it ameliorates the fatty acid profiles in both backfat and LTL muscle.
Also, qualitative characteristics and chemical composition of muscle are not affected by dietary linseed inclusion associated with either synthetic or natural antioxidants.
In this research linseed feeding, supplemented with supranutritional doses of antioxidants, does not impair oxidative stability compared to a standard diet and reduces weight losses during chilled storage.
As expected, high concentration of oxygen in MAP brings about an increase in oxidative products and yields redder meat, irrespective of the dietary treatment.
In linseed fed pigs, dietary red grape skin extract is as effective as synthetic antioxidant in maintaining quality characteristics of pork during storage, even in high oxygen MAP.

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PAPER

MULTILEGUME BAR PREPARED FROM EXTRUDED LEGUMES FLOUR TO ADDRESS PROTEIN ENERGY MALNUTRITION

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ABSTRACT

This study was planned to develop and characterize extruded multilegume savory bars as a protein supplementary nutrition. Legumes were extruded to prepare composite flour. Proportions of extruded flour were mixed with whey protein concentrate, honey and palm oil for preparation of protein bar. The product was evaluated for physico-chemical, minerals, calorific value, color, hardness, protein digestibility and sensorial characteristics. *In vitro* protein digestibility was found from 62.04 to74.98% and *in vivo* from 65.30 to 84.01%. Extrusion process and addition of whey protein concentrates significantly affected the nutritional and sensorial parameters of bars.

Keywords: legume protein, extrusion, bar, protein digestibility

1. INTRODUCTION

Malnutrition is an abnormal physiological condition triggered by imbalanced, inadequate or excessive consumption of nutrients (RIZWANA *et al*., 2015) while, proteins energy malnutrition (PEM) is a change of pathological conditions arises due to deficiency of protein calories (ERNEST *et al*., 2013). Developing economies are adversely affected by the malnutrition. Globally, there are more than 150 million children under the age of five years who are malnourished. The majority of these children are residing in just three countries of the South Asia *i.e.* India, Bangladesh and Pakistan where almost 54% of child deaths are linked to this menace (UNICEF, 2016). Unhealthy diet, ecological conditions and general living standard have a strong relation with diseases. According to Global Hunger Index (GHI), Pakistan is at 11th position from 118 countries with respect to malnourished population (22%), stunted growth (45%), wasting (10.5%) and mortality (8.1%) in children under 5 years of age (IFPRI, 2016). Likewise, according to National Nutrition Survey (2011), 58% of the population is facing the food security situation. Due to malnutrition, women and children are facing macro- and micro-nutrient deficiencies. About 31.5% of the children are underweight, 43.7% are stunted and 15.1% are suffering from wasting. Children (39-61%), pregnant women (38-69%) and non-pregnant women (26%-68%) are facing iron, zinc, vitamin A and D deficiencies (GOP-Pakistan, 2011).

PEM is one of the important public health issues in developing countries (VAN DER POLS-VIJLIBRIEF *et al*., 2014). Marasmus, kwashiorkor and marasmic-kwashiorkor are the primary reasons of PEM (ERNEST *et al*., 2013) that is associated with co-morbidities such as anaemia, tuberculosis diarrhea and malaria (le Roux *et al*., 2010) and these causes may lead to death. Several policies have been implemented to overcome the issue of PEM that involves different food based strategies such as dietary modifications, food enrichment and supplementation. School health programs are also initiated in various countries to mitigate this situation (ONIS, 2012).

Protein as a nutrient is considered a dietary component that evokes the widest array of complex scientific, economic and environmental issues, viewed as the most expensive but essential ingredient forming part of a healthy balanced diet (SCHÖNFELDT and HALL, 2012). Edible legumes belong to the family *Leguminosae* entitled as *Fabacae*. These are termed used for grain legumes which are generally grown up for their edible seeds. Legumes also called "a poor man's meat". Legumes are abundantly cultivated in subtropics and tropics areas of the world. They are the good alternative to animal protein for those people who have limited resources (ADEBOWALE and LAWAL, 2004). They possess amounts of amino acids such as leucine, lysine, aspartic acid, arginine and glutamic acid. They are vital sources for food proteins and also give rational essential amino acids when used with grains or other foods (SARWAR *et al*., 2013). It can be used with other food items to enhance the nutrition. They are also an excellent source of micronutrients as contain riboflavin, thiamin, niacin, selenium, folate, and pyridoxine (USDA, Agricultural Research Service 2012). They have good amounts of vitamin A, E and C (RAATZ; The Bean Institute 2010). Application of extrusion process on legumes modifies the physico-chemical parameters for improving functional properties in target applications (OSEN *et al*., 2015). Thermal extrusion has advantages as it helps to hinder anti-nutritional factors such as haemagglutinins, trypsin inhibitors, tannins, phytates which inhibit protein functionality and digestibility (ALONSO *et al*., 2000).

Since, legumes have good sources of protein and micronutrients and can be modified into an extruded product. They can be used to prepare protein rich bars, that can diversified the diet with natural approach to enhance the nutritional requirements and reduce the

malnutrition of poor regions by using as a cultural food. The present project was planned to prepare extruded multilegume savory bar in order to mitigate PEM. According to Institute of Medicine the recommended daily allowance of protein for adults older than 18 years is 0.8 g/kg/d and the youngsters (under 18 years of age) required 13-52 g of protein per day (NAP, 2005). The primary objective of this multilegume product was to provide the enough nutrients that fulfill the daily requirement of protein but will not cross the threshold level for children and adults in the absence of meat products to address PEM.

2. MATERIALS AND METHODS

2.1. Procurement of Materials

Chickpea (*Cicer arietinum* L), mung (*Vigna Radiata*), mash (*Vigna Mungo L*), soybean (*Glycine max*), whey protein concentrates (WPC 80), palm oil and honey were purchased from the markets of Faisalabad, Pakistan.

2.2. Preparation of extruded multilegume savory bars

Thermal extrusion has advantages to destroy the anti-nutritional factors such as haemagglutinins, trypsin inhibitors, tannins, phytates, and helps in production of bioactive peptides and improve protein functionality and digestibility. All legumes were soaked for 15 hrs and dried for thermal extrusion. Legumes were fed into a twin-screw extruder (DNDL 44, Bühler AG, Uzwil, Switzerland) using the method described by Tremaine and Schoenfuss (2014). Optimized extrusion conditions of feed flow rate (60 kg/hr), screw speed (250 rpm), feed moisture content (10%) and barrel exit temperature (160°C) were used for the preparation of extruded powder. Extrudates were pelletized and dried in vacuum oven. Drying continued at 40°C in oven for 26 hrs.
Chickpea flour and other legumes (mash, mung and soybean as composite flour) were mixed in proportions and prepared different treatments as described in table 1. Whey protein concentrates (3%); honey (3%) and palm oil (3%) were added in each sample for better mixing, sensorial and nutritional properties. Purpose of combining proteins from vegan and vegetarian diets is to provide sufficient amounts of some essential amino acids to make complete protein intake. Palm oil has good spreading properties, technically useful and economically beneficial as compared to animal fat as well. After mixing, sheeting was done, and cut into bars of 3.5 centimeter (cm) width, 2 cm height, and 9 cm in length. Each bar of approximately 50 g was packed individually in aluminum foil.
Using AACC 2000 methods, moisture content (method no; 44-15.02), crude protein (method no; 990.03), crude fat (method no; 30-10.01), crude fiber (method no; 962.09), ash (method no; 942.05) and NFE content were determined. Water activity of prepared bars was determined using a previously described AOAC method (AOAC, 2012; method no. 978.18). The color values for each treatment were determined through Color Meter (Color Test II, Neohuaus Neotec, Germany) by following the method described by Hunter (1987). Calorific values of bars were determined through Oxygen Bomb Calorimeter (IKA-WERKE, C2000 Basic, GMBH and CO. Germany) as described by Miller (1959). Hardness of bars was measured according to the method of Piga *et al*. (2005). Results were obtained on the basis of compression force (kg) used to press the bars.

Table 1. Treatment plan.

Treatment	Formulation
T_0	Chickpea flour/composite% as 100/0
T_1	Chickpea flour/composite% as 70/30
T_2	Chickpea flour/composite% as 55/45
T_3	Chickpea flour/composite% as 40/60
T_4	Chickpea flour/composite% as 25/75
T_5	Chickpea flour/composite% as 0/100

Composite flour contains mung, mash and soybean flours.

2.3. *In Vitro* study for protein digestibility

Using the method of Akeson and Stahmann (1964) (with some modifications), *in vitro* protein digestibility was determined. Aliquots of 250 mg of each sample were suspended in 15 mL of 0.1 mol equi/L HCl containing 1.5 mg/mL pepsin (Sigma®, St. Louis, MO, USA) and incubated for 3 hrs at 37°C in a water bath. Hydrolysis from pepsin was stopped after neutralization by adding 7.5 mL of 0.5 mol equi/L of NaOH, then pancreatic digestion started by the addition of 10 mL of 0.2 mol/L phosphate buffer (pH 8.0) containing 10 mg of pancreatin (Sigma®, St. Louis, MO, USA) with 1 mL of 0.005 mol/L sodium azide to hinder microbes growth and incubated at 37°C for 24 hrs. After hydrolysis with pancreatin, 1 mL of 10 g/100 mL of trichloroacetic acid was added and centrifuged at 550×g for 20 min. The supernatant was collected and the total protein content was calculated using Kjeldahl (on the basis of nitrogen content) using AOAC (2012) method.

$$\% \text{ Digestibility} = (Ns - Nb)/Ns \times 100$$

Ns = nitrogen content in the sample, Nb = nitrogen content in the blank.

2.4. *In Vivo* study for True Protein Digestibility (TPD)

Male Sprague-Dawley rats (350±12 g) of 9 weeks old were procured from Animal House, National Institute of Health, Islamabad, Pakistan and maintained under standard laboratory conditions at 28±2°C with constant light-dark cycle. Rats were fed on standardized chow for an acclimation period of 2 days and then 36 rats were divided into groups of 6 rats. Rats were fed for 10 days in which 2 days were for acclimation period. Rats were weighed on daily basis during study. After 4 days period, spilled food and feces were carefully collected and separated from each rat. The spilled food was dried for 72 hrs in air while collected feces were dried in oven overnight at 100°C, weighed, grinded and analyzed for nitrogen content. Weight of spilled food and uneaten food were minus from the total food supplied to rat to determine the nitrogen intake.
TPD was calculated as:

$$\text{TDP} = \frac{I - (F - F_k)}{I} \times 100$$

I= intake nitrogen, F= fecal nitrogen, and F_k=metabolic or endogenous fecal nitrogen.

2.5. Sensory Evaluation

Attributes like color, texture, folding ability, chewability, taste and overall acceptability of extruded multilegume savory bars were analyzed by a penal of judges using 9- Point Hedonic Scale system as described by Meilgaard et al. (2007). All experiments were conducted in triplicates and average values were considered as mean values. The significance of values was calculated statistically through mean using Analysis of variance (ANOVA) at probability of 0.05.

3. RESUKTS

3.1. Proximate composition of extruded flour

Extruded multilegume composite flours for each treatment were prepared by blending various amounts of mung, mash and soybean with chickpea and then analyzed for moisture, crude protein, crude fat, crude fiber, ash, NFE and mineral content. The mean values regarding proximate composition of composite flour is presented in Table 2.

Table 2. Proximate composition and mineral profile of flour of chickpea/mung, mash and soybean for savory bar development.

Chickpea flour/compo-site flour (%)	Moisture	Crude Protein[2]	Crude Fat	Crude Fiber[3]	Ash	NFE
T_0	3.98±0.18[1]	29.26±1.32	3.20±0.05	1.02±0.10	3.03±0.06	57.09±2.04
T_1	4.12±0.11	29.89±0.99	3.34±0.03*	1.35±0.13	3.09±0.09	54.17±1.98*
T_2	4.19±0.24	30.28±2.47	3.78±0.05*	1.98±0.10*	3.43±0.05	53.84±2.61*
T_3	4.06±0.11	30.49±3.97	3.86±0.03*	2.87±0.13*	3.51±0.09	50.20±1.39*
T_4	4.29±0.24*	30.58±2.64	4.94±0.05*	3.01±0.10*	4.28±0.05	49.84±2.09*
T_5	4.48±0.35*	31.43±3.29*	5.53±0.09**	3.30±0.08**	4.34±0.10*	47.50±1.87*
Mineral profile (mg/100g)	**Na**	**K**	**Ca**	**Fe**	**Zn**	
T_0	11.51±0.25	477.00±12.45	81.39±19.10	04.70±0.63	02.54±0.09	
T_1	13.65±0.29*	614.50±05.23**	113.85±2.62**	06.62±0.25*	03.01±0.09*	
T_2	14.72±0.21*	682.80±09.19**	129.70±5.69**	07.36±0.14*	03.30±0.03*	
T_3	15.86±0.36*	751.87±07.71**	146.55±2.70**	07.75±0.29*	03.45±0.09*	
T_4	16.87±0.49*	820.93±11.40**	155.40±2.22**	07.92±0.23*	03.75±0.08*	
T_5	18.65±0.39*	925.27±11.35**	188.25±4.50***	08.20±0.20*	03.94±0.09*	

[1]Mean values (on dry basis) ± standard deviation. Different superscripts (*) on values in columns show significance difference ($p< 0.05$) within treatment.

[2]Calculated using N x 6.25 for proteins

[3]Calculated by difference of 100 - (ash + proteins + fat +starch).

The moisture content was ranged from 3.98±0.18 to 4.48±0.35%, crude protein 29.26±1.32 to 31.43±3.29%, crude fat 3.20±0.05 to 5.53±0.09%, crude fiber 1.02±0.10 to 3.30±0.08%, ash 3.03±0.06 to 4.34±0.10% and NFE 47.50±1.87 to 57.09±2.04%. Significant ($p< 0.05$) difference in nutritional composition was observed with the increase of multilegume composite flours in formulations. Maximum Na content was observed in T_5 (18.65±0.39 mg/100g) and minimum (11.51±0.25 mg/100g) in T_0. K content of formulations was ranged from 477.00±12.45 to 925.27±11.35 mg/100g in which maximum content was observed in T_5 and minimum in T_0. Ca content of formulations was ranged from 81.39±19.10 to 188.25±4.50 mg/100g. The highest Ca content was observed in T_5 and the lowest Ca was noted in T_0. Maximum values for Fe were found in T_5 (08.20±0.20 mg/100g) and minimum values in T_0 (04.70±0.63 mg/100g). Zn content was found lowest in T_0 (02.54±0.09 mg/100g) and highest in T_5 (03.94±0.09 mg/100g). Significant ($p<0.05$) difference was found within treatments from control in mineral analysis.

3.2. Proximate composition of multilegume savory bars

The mean values regarding proximate composition of multilegume savory bars are shown in Table 3.

Table 3. Proximate composition of multilegume savory bars.

Savory bar legumes ratio	Moisture	Crude Protein[2]	Crude Fat	Crude Fiber[3]	Ash	NFE	Energy[4] (Calories/100 g)
T_0	3.99±0.21[1]	31.98±0.87	5.03±0.12	0.99±0.26	3.24±0.13	58.03±1.08	418.03±13.04
T_1	4.24±0.09	32.76±0.16	5.63±0.24	1.29±0.31	3.45±0.19	56.23±1.98	436.74±12.09*
T_2	4.35±0.19	33.01±0.34	5.98±0.76	1.92±0.41	3.69±0.23	53.12±2.42*	458.67±10.26*
T_3	4.40±0.20	33.56±0.23	6.73±0.53*	2.76±0.27*	3.93±0.17	51.89±2.32*	526.18±09.87*
T_4	4.43±0.29*	33.93±0.49*	7.09±0.49*	2.86±0.65*	4.56±0.32*	50.63±1.59*	530.17±15.76*
T_5	4.61±0.25*	34.23±0.95*	7.99±1.02*	3.19±0.51*	4.89±0.09*	48.53±0.99**	546.49±19.87**

Mineral profile (mg/100g)	Na	K	Ca	Fe	Zn
T_0	11.65±0.12	479.12±4.32	81.29±9.34	04.65±0.43	02.51±0.04
T_1	13.71±0.18*	618.32±5.76**	114.31±1.93*	06.68±0.18*	02.97±0.12*
T_2	14.78±0.31*	685.47±6.20**	130.82±4.32*	07.71±0.07*	03.23±0.31*
T_3	15.92±0.28*	763.38±8.24**	147.25±3.21*	07.82±0.15*	03.49±0.54*
T_4	16.93±0.17*	824.52±5.91**	156.32±1.99*	07.89±0.09*	03.87±0.42*
T_5	18.69±0.16**	927.36±6.32**	189.17±3.35**	08.40±0.87**	04.02±0.41*

[1]Mean values (on dry basis) ± standard deviation. Different superscripts (*) on values in columns show significance difference ($p< 0.05$) within treatment.

[2]Calculated using N x 6.25 for proteins.

[3]Calculated by difference of 100 - (ash + proteins + fat+ starch).

[4]Caloric values were determined bomb calorimeter.

In all treatments, the moisture content ranged from 3.99±0.21 to 4.61±0.25%, crude protein 31.98±0.87 to 34.23±0.95%, crude fat 5.03±0.12 to 7.99±1.02%, crude fiber 0.99±0.26 to 3.19±0.51%, ash 3.24±0.13 to 4.89±0.09% and NFE 48.53±0.99 to 58.03±1.08%. Significant ($p< 0.05$) difference was found in all treatments in comparison with control for moisture content, crude protein, crude fat, crude fiber, ash and NFE in bars prepared from multilegumes composite flour. Maximum Na content was observed in T_5 (18.69±0.16 mg/100g) and minimum in T_0 (11.65±0.12 mg/100g). K content was ranged from 479.12±4.32 to 927.36±6.32 mg/100g in which maximum content was observed in T_5 and minimum in T_0. Ca content was ranged from 81.29±9.34 to 189.17±3.35 mg/100g. Maximum value for Fe was found in T_5 (08.40±0.87 mg/100g) and minimum in T_0 (04.65±0.43 mg/100g). Zn content was found highest in T_5 (04.02±0.41 mg/100g) and lowest in T_0 (02.51±0.04 mg/100g). Significant ($p<0.05$) difference was found for mineral content in bars within treatments in each column. Maximum calorific value was noticed in T_5 (546.49±19.87 calories/100g) while lowest in T_0 (418.03±13.04 calories/100g).

3.3. Water activity

Non-significant ($P > 0.05$) difference was found for water activities in bar as all values were recorded around 0.50.

3.4. Color of extruded multilegume savory bars

Color reveals the first impression of a food product before consumed. It's the first score of a like and dislike for food commodity. The mean values for color score of extruded multilegume savory bar are shown in Table 4. Highest value (58.67±0.14) of *L* was found in T_2 while the lowest value (50.50±0.13) was noticed in T_0. Maximum color value of *a** was 7.85 in T_2 while the minimum was 5.30 in T_5 that shows coloring trend towards redness. Maximum color value for *b** was 19.4±0.09 in T_5 and lowest was 15.69±0.04 in T_3 that shows coloring trend toward yellowness. T_1, T_2, T_4 and T_5 were significantly ($P<0.05$) different from each other while T_0 and T_3 were non-significantly ($P>0.05$) different in color value of *L**. T_0, T_2, T_3, T_4 and T_5 were significantly ($P<0.05$) different from each other while T_1 was non-significantly ($P>0.05$) different in color value of a*. T_3 and T_5 show significant ($p<0.05$) difference than other treatment in color value of b*.

3.5. Hardness of extruded multilegume savory bars

Hardness is one of the quality attributes, which describe quality of food bars before testing. The mean values of hardness for bars have been listed in Table 4. Maximum force (kg) was noticed on T_5 (8.61±0.76) while minimum on T_0 (5.33±0.34). Highly significant values were observed for T_5, T_4 and T_3 as compared to others.

3.6. *In Vitro* and *in vivo* studies for protein digestibility

Protein digestibility values were calculated for each treatment and results regarding digestibility are shown in Figure 1 for both *in vitro* and *in vivo* studies. *In vitro* digestibility was observed between 62.04 to 74.98% while TPD *in vivo* values ranged from 65.30 to 84.01%. Maximum value for TDP was observed in T_5 (84.01±3.91) and lowest value in T_0 (65.30±3.43). All the treatments were significantly different ($P<0.05$) from control sample in both studies.

Table 4. Mean values of color and hardness (kg) of multilegume savory bar.

Treatments	Color		
	L*	a*	b*
T_0	[1]50.50±0.13	6.63*	17.8±0.07
T_1	54.81±0.09*	6.33	18.02±0.03
T_2	58.67±0.14*	7.85*	16.03±0.02
T_3	52.33±0.09	6.08*	15.69±0.04*
T_4	55.51±0.09*	5.40*	18.80±0.08
T_5	57.84±0.12*	5.30*	19.4±0.09*

Treatments	Hardness (kg)
T_0	[1]5.33±0.34
T_1	5.89±0.22*
T_2	6.17±0.43*
T_3	7.01±0.52**
T_4	7.99±0.56**
T_5	8.61±0.76***

[1]Mean values of triplicate representations + standard deviation, superscripts (*) show the significant difference ($p<0.05$) in same column.
L* represents the lightness ranging from darkness (0) to lightness (100).
a* represents redness varying from greenness (-a*) to redness (+a*).
b* represents the yellowness varying from blue (-b*) to yellow (+b*).

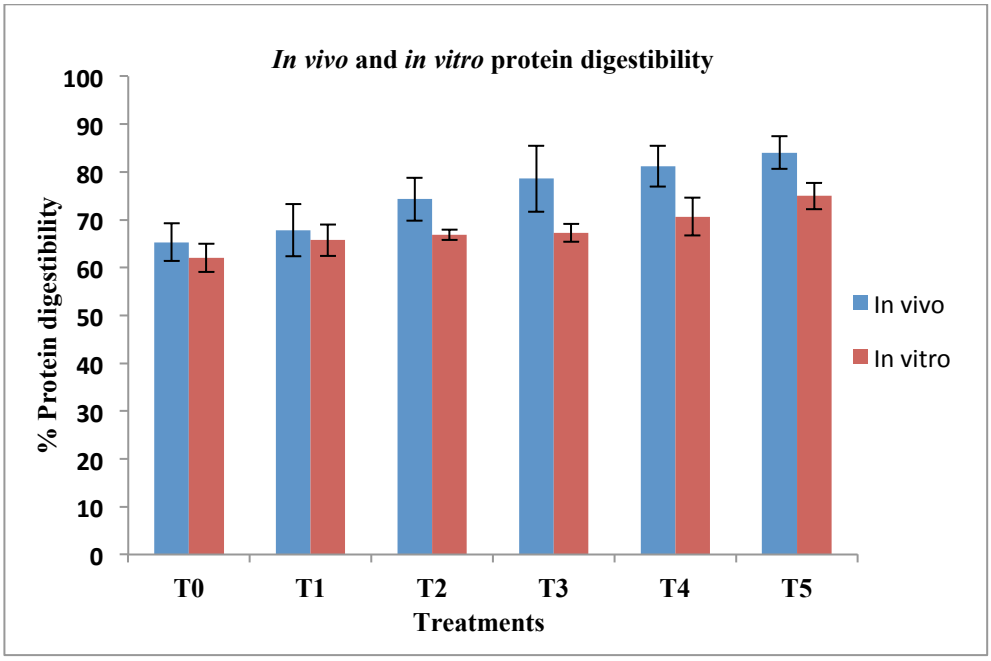
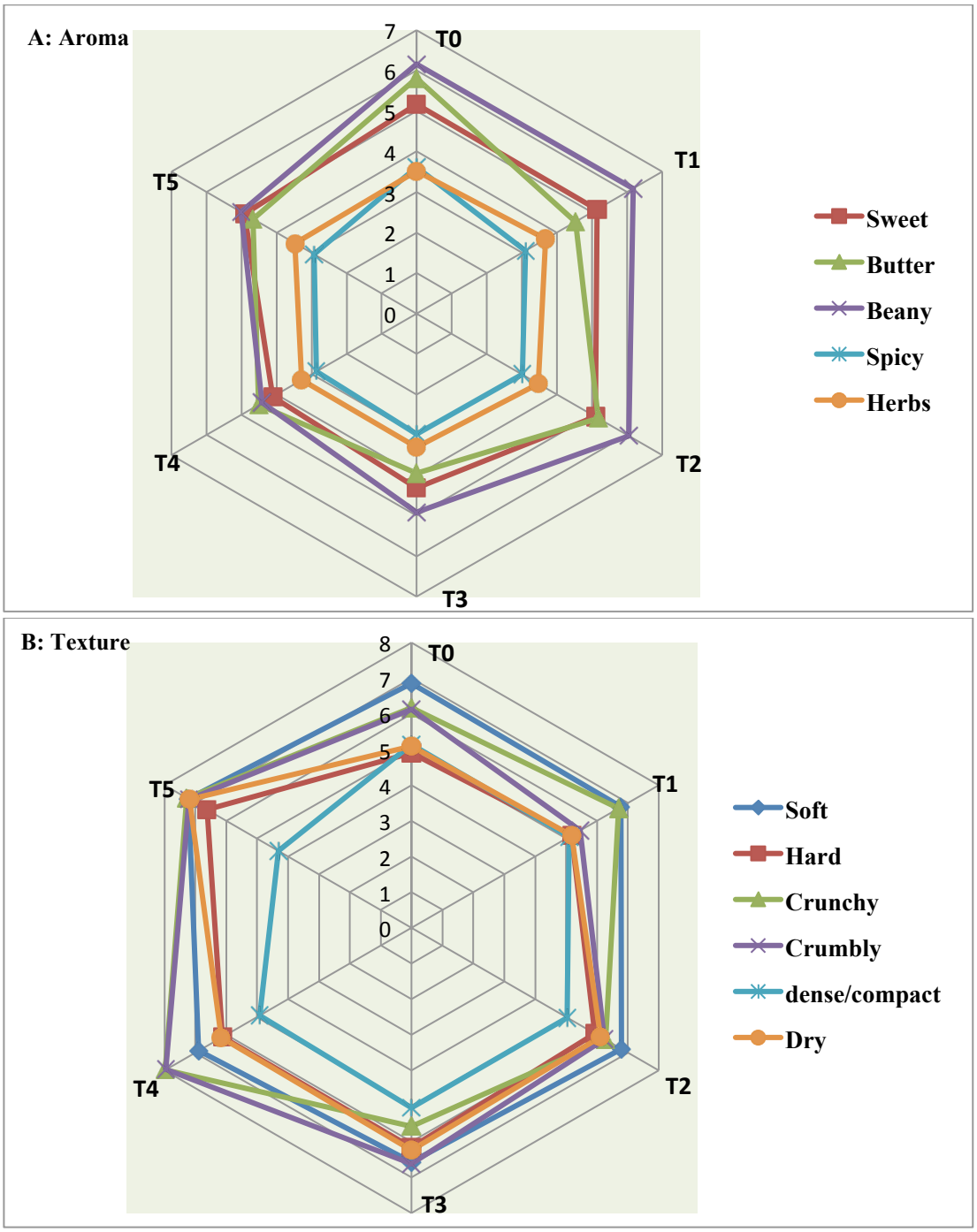


Figure 1. The *in vivo* and *in vitro* protein digestibility values (%) of different treatments. *In vivo* protein digestibility was evaluated using rats model and *in vitro* through pepsin and pancreatin.

3.7. Sensory evaluation of extruded multilegume savory bars

Values regarding aroma, texture and flavor analysis are shown Figure 2.



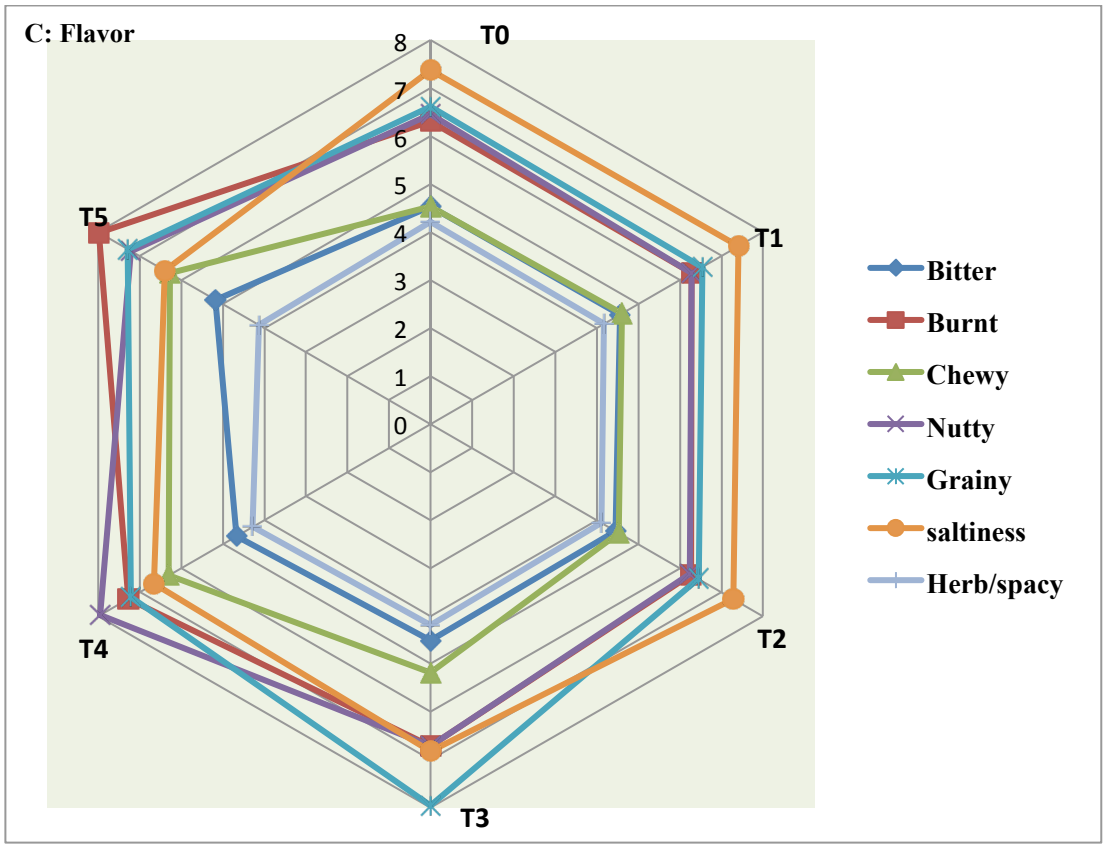


Figure 2. Mean values were obtained through 3 repetitions by 19 panelists each. (A= aroma, B= texture, C= flavor) Score range from 1 = dislike extremely to 9 = like extremely evaluated after 60 days of storage interval.

Texture observation of bar in T_3, T_4 and T_5 showed significant sweet, butter and bean aroma. Texture observation of extruded multilegume savory bars showed that all the treatments had rough texture; T_2 showed significant dryness in its texture, T_3, T_4 and T_5 showed crunchy and crumble texture. Bars made from T4 showed compact and dense texture whereas bars from T_5 were observed significantly soft in texture. Flavor analysis showed that T_3 has significant flavor of chewy, nutty and grainy, whereas flavor of bitter, burnt and saltish was found in T_5. Similarly, T_4 showed significant saltish flavor.

4. DISCUSSION

Legumes are good source of proteins and transformation of these into bars helps to mitigate the threatening situation of PEM. Addition of whey protein concentrate, honey and palm oil into bar was also added to improve nutritional and sensorial characteristics. Production of protein rich bars lower down PEM but also provide various minerals and vitamins to consumer in sufficient quantity to address various body functions. As legumes proportions were increased in treatments, values of protein, ash and energy increased. So, it is clear from results that addition of legumes in composite proportions helps to increase the nutritional status of bars. Nutritional value of proteins depends on the availability, digestibility and quantity of essential amino acids present in it. Extrusion helps in the inactivation of anti-nutritional factors and improves nutritional values. So, preparation of

bar through extrusion treatment is one of the best method to conserve protein constituents for mitigation of PEM.

ABDEL-GAWAD *et al*. (2016) prepared composite flours using legumes and wheat and observed protein content between 11.76 to 19.05%, fat content between 1.36 to 3.25%, crude fiber between 0.59 to 1.55% and ash content between 0.63 to 2.40%. All values are slightly different from current study that is due to use of whey protein concentrate and different legume species, as composition varies within species. JAHREIS *et al*. (2015) prepared legume flour and found Ca content (47-221 mg/100g), K content (1030 to 1760 mg/100g), Fe content (4.3 to 7.7 mg/100g) and Zn content (2.5 to 4.1 mg/100g); these observations are slightly different from present study. This might be due to addition of different legume species.

NADEEM *et al*. (2012) prepared date bars and showed moisture content (15.56 to 18.70%), protein content (7.41 to 14.96%), crude fat (5.55 to 8.37%), crude fibre (3.58 to 3.88%) and ash content (2.30 to 2.91%). As multilegume increases in the bar protein, crude fibre and ash content also increases and improves minerals profile such as Na, Ca, K, Fe, Zn and essential amino acids without disturbing the sensorial parameters (Bower and Whitten 2000). Added proteins are functioned to keep the ingredients of food bars intact, maximize the strength, set the structure and contribute to water holding capacity. Whey protein possesses viscosity, water holding properties and gel strength that contribute to bar firmness (ORTIZ *et al*., 2008). Fat content not only provide caloric values but also increases the palatability of bars. Additionally, fat possess to act as a binder with sweeteners in agglutination of the ingredient present in bar that helps to impart compactness and firmness to texture of food bars (ESCOBAR *et al*., 1996). Color is the first impression of a food product. It's the first score of a like and dislike food commodity. SREBERNICH *et al*. (2016) prepared cereal bar with different formulations and found L^* value (52.78 to 62.70), a^* values (8.39 to 11.93) and b^* values (23.53 to 27.90), slight difference was found in our findings in accordance to current findings; this might be due to different composition of bars. RAJABI (2017) prepared high protein extruded bars and found hardness values between 6.08 to 9.33 kg; these values are comparable to current findings. Increase in harness was observed with the change in composite flour (%) that is might be due to the cumulative effect of different flours.

ALMEIDA *et al*. (2015) prepared whey protein supplements and found their protein digestibility between 88.4 to 91.7% *in vitro* and these values are not comparable to current findings because in this study vegetable protein sources were used that have more anti-nutritional factors and can form more complex protein structure and may hinder protein digestibility (BUTTS *et al*., 2012). *In vivo* true protein digestibility increased as the extruded multilegumes proportions increased in bars. ERBERSDOBLER *et al*. (2017) stated that digestibility of protein is high around 89-96% in weaning foods based on beans and rice and these values have contradictions to current findings and this might be due to use of different legumes and processing conditions. NOSWORTHY *et al*. (2018) stated that extrusion of beans/legumes ameliorate the protein digestion as compared to other processing conditions. Changes in extrusion variables also affect the protein digestibility. With the increase in extrusion temperature, the protein digestibility values are increased as inactivation of protease enzymes occur rapidly as temperature increases. Increase in the shear forces increases the protein digestibility, this might be due to denaturation of protein increase with increasing screw speed (Bhattacharya and Hanna, 1985). In this study screw speed was 250 rpm and barrel exit temperature was 160°C, these conditions might be the reason of increase in protein digestibility of protein bars.

Extruded multilegume savory bar first time prepared, which has sensorial and nutritional qualities. Both nutritional and sensorial qualities of puffed cornmeal were enhanced when blended with milk protein isolates (ONWULATA, 2010), same is happened in current study. BANACH *et al*. (2016) prepared protein bar by using extruded milk protein concentrates that were more cohesive, softer and less textural changes were observed than bars prepared with the spray drying method. Hence, extrusion process modifies the physico-chemical parameters of ingredients not only the structural-function relationships of proteins and same findings are observed in the current work.

5. CONCLUSIONS

Compositional and sensorial characteristics of multilegume savory bars were assessed to consider the supplementation of protein from plant source (legumes) as alternate of meat protein. Proximate analyses showed that proportion of different legumes have significantly increased the nutritional values of savory bar. Significant increase in protein and minerals content were observed in bars and they have better protein digestibility in *vivo* and *vitro* studies conducted on rats. Protein from plant source can be an economical approach to produce these bars to mitigate PEM. So, these bars can be considered as well balanced fast food from nutritional point of view to handle the problems associated with PEM.

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PAPER

EFFECT OF DIFFERENT ILLUMINATION SOURCES ON COLOUR AND OXIDATIVE STABILITY OF SEASONED COPPA DI PARMA PGI

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ABSTRACT

The influence of different lighting durations, lamps and modified atmosphere packaging (MAP) on the colour and oxidative stability of lipids was studied in Coppa di Parma PGI. The samples were stored (4°C) in darkness or lighted by UV-free lamps. In trials 1 and 2, the samples were lighted 24 and 12 h/day, respectively, and were packaged in air. In trial 3, samples were packaged in MA (70% N_2/30% CO_2) and lighted 12 h/day. In air, illumination reduced oxidative stability, redness, colour saturation and increased the Hue angle. In MAP, the lighting conditions did not affect colour and oxidative stability. During storage the lipid oxidation increased. Overall, light negatively affected the studied parameters.

Keywords: colour, cured salami, lighting conditions, lipid stability, modified atmosphere packaging

1. INTRODUCTION

The colour of meat plays a pivotal role in determining the decision of the consumer to buy a particular product. Indeed, it is perceived as relating to the freshness, integrity and quality of the food. Thus, maintaining an attractive colour during storage is a key of success in the selling of meat. Discolouration in retail-fresh meats during display is ascribable to muscle pigment oxidation (oxymyoglobin to metmyoglobin). This is influenced by oxygen concentration, pH and secondary products of lipids peroxidation (PAPUC *et al*., 2017). In case of nitrites addition, as in the processing of Coppa di Parma PGI (Protected Geographical Indication), nitrosylmyoglobin formation occurs; this is an unstable pigment that, in presence of oxygen in cured meat, is oxidized to brownish nitrosylmetmyoglobin NOMMb (FOX, 1966): this evidence was confirmed in scientific literature, in which the discolouration of nitrosylmyoglobin has been linked to the combination of the presence of both oxygen in the headspace surrounding the product and light exposure during the display life (MØLLER and SKIBSTED, 2002; ZANARDI *et al*., 2002). Indeed, lipid oxidation, which takes place in intramuscular fat and/or membrane phospholipids, besides causing an unpleasant odour and flavour, brings about the loss of desirable colour, thereby reducing display life (BUCKLEY *et al*., 1995; PAPUC *et al*., 2017; RUIZ *et al*., 1999) and has deleterious effects on the organoleptic properties of meat and meat products as well as the digestibility of main nutrients (GARCÍA-LOMILLO *et al*., 2017; PATRAKOVA and GURINOVICH, 2015). Therefore, preventing or delaying both pigment and lipid oxidation enables the duration in which the meat maintains its bright-red colour to be extended. The oxidation of both lipids and pigments in meats are strictly related to similar processes (FAUSTMAN *et al*., 1989; PAPUC *et al*., 2017) and, in this regard, light may play a critical role. In fact, oxidative reactions can be initiated also by physical factors such as radiation and light (AMARAL *et al*., 2018). The effect of light on lipid oxidation has been shown in various foods, such as oils, butter, milk and meat (AURAND *et al*., 1977; CHAHINE and DEMAN, 1971; KIRITSAKIS and DUGAN, 1985; LUBY *et al*., 1986; WHANG and PENG, 1988); UV-light is more effective than visible light in inducing oxidation of lipids and pigments (ANDERSEN and SKIBSTED, 1991; BERTELSEN and BOEGH-SOERENSEN, 1986; ZHU and BREWER, 1998). Although the amount of radiation below 400 nm is small in fluorescent lamps used in display cabinets, it must be taken into account due to its deleterious effects on the display-life of meat (DJENANE *et al*., 2001). Meat products on retail shelves are provided in transparent packaging and with residual oxygen inside the packages; in association with the cabinet display light, these can cause discolouration of the packaged meat products (GIBIS and RIEBLINGER, 2011; MCMILLIN, 2008). To overcome this problem, in the last few decades the use of modified atmosphere packaging (MAP) for meat and sliced meat products is increasingly widespread as a tool to extend their shelf-life. However, the effect of lighting on the shelf-life of cured seasoned pork products has not been widely investigated. In particular, the Coppa di Parma PGI has never been studied under this profile. Coppa di Parma PGI is obtained from subjects of at least nine months of age weighing approximately 160 kg. After hand-salting, the muscular portion of the neck adhering to the cervical and the first two thoracic vertebrae is placed in a closed-ended beef gut casing and hand-tied with string. After two/three months of curing, Coppa is marketable. The product, cylindrical in shape, is 25-40 cm long and weighs at least 1.3 kg.

The aim of this work was to study the effects of display under different lighting times and lamps on the colour parameters and oxidative stability of Coppa di Parma PGI packaged in air as well as in a modified atmosphere.

2. MATERIALS AND METHODS

For the purposes of the study, a total of 18 Coppa di Parma PGI, obtained from a local retailer, were used. Three distinct trials were carried out. For each trial, three replications were performed. In each replication, 2 different Coppa were sampled and sliced.
In the first two trials, the slices of Coppa were air-packaged in trays made of a PET/EVOH/PE structure (oxygen permeability < 0.5 cm^3 m^{-2} $24h^{-1}$ bar^{-1}), lidded with a PET/PE film (oxygen permeability 80 cm^3 m^{-2} $24h^{-1}$ bar^{-1}). In the third trial, sliced Coppa was packaged with the same material (bottom and top) in a modified atmosphere (nominally 70/30 N_2/CO_2), using CAVECO equipment and working with the technique of vacuum compensation. The initial and final atmosphere composition was determined by a Handheld Gas Analyzer Checkpoint (Dansensor, Denmark).
In each replication, 26 trays were filled with 10 slices each of Coppa. Twenty-four trays were put in refrigerators at 4±1°C. Six trays were maintained in the dark, the other 18 were put into another refrigerator, divided into 3 sections separated by horizontal black screens. Each section (estimated area of 0.33 m^2) was illuminated with a specific lamp, whose characteristics are summarized in Table 1.

Table 1. Characteristics of the different lamps used.

Lamp identifier (code)	Colour temperature °K	Colour rendering	Wattage (W)	Luminous flux (lm)	Illuminance* (lx)
640 Basic Cool White (CW)	4000	62	18	1200	3640
827 Lumilux Interna Warm White (WW)	2700	80	18	1350	4090
76 Natura Neutral White (NW)	3500	75	18	750	2270

*The Illuminance (lx) values have been calculated simply dividing the nominal Luminous flux (lm) of each lamp by the exposed area (0.33 m^2) of the fridge shelves.

The distance between the lamp and the samples was about 40 cm. The samples contained in the remaining 2 trays were immediately assigned to the analyses (described below).
The samples were exposed either to continuous lighting (24/24 hours) (trial 1), or to a 12 hours on/12 hours off light cycle (trials 2 and 3). Eight trays (two for each lighting condition) were removed from the refrigerators after 24, 48, and 120 hours and then submitted to the analytical determinations.
On each Coppa, the lipid content was determined according to A.O.A.C. (1995) and the average lipid content was 29.3±5.5. Lipid oxidation was measured by the 2-thiobarbituric acid reactive substances (TBARS) method (SIU and DRAEPER, 1978). Each sample was minced, and an aliquot of 2.5 g was homogenised in 12.5mL of distilled water at 9500 rpm for 2 min, using an Ultra Turrax tissue homogenizer and then vortexed for 1 min at high speed. Samples were centrifuged for 20 min at 2000 rpm at 4°C with 12.5 mL of 10% trichloroacetic acid (TCA) and the supernatant decanted through a paper filter (Whatman 541). Four mL of clear filtrate were transferred into 15 mL pyrex screw cap test tubes and 1 mL of 0.06M 2-thiobarbituric acid (TBA) was added. A distilled water-TCA-TBA reagent

blank was prepared and treated in the same way as the samples. The samples were heated in a water bath at 80°C for 90 min and then cooled. Absorbance was measured at 532 nm in spectrophotometer (Jasco model V550, UV/VIS, Tokyo, Japan). Results were expressed as mg of malondialdehyde (MDA)/kg.

The surface colour of Coppa slices was determined using a CM-600d spectrophotometer (Konica Minolta Holdings, Inc., Osaka, Japan) with a window diameter of 8 mm and D65 as the source of illumination. Before colour measuring, carried out on both lean and fat tissue, the spectrophotometer was calibrated against a white plate supplied by the manufacturer. Colour measurements complied with the CIE colour convention (CIE, 1986), where the three fundamental outputs are L^*- "lightness", a^*- "redness", b^*- "yellowness" values. Chroma (C^*), also referred to as saturation index and colour intensity, was calculated as: $[(a^{*2}+b^{*2})]^{0.5}$ and Hue angle (h^*) calculated as follows: $\tan^{-1}(b^*/a^*)$. Overall colour change (ΔE) was calculated as $[(\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{0.5}]$ where ΔL^*, Δa^* and Δb^* are the difference between time 0 and the values L^*, a^* and b^*, respectively, at 24 ($\Delta E1$), 48 ($\Delta E2$) and 120 ($\Delta E3$) hours. The average values for both fat and lean were the mean of 25 determinations in each fraction.

The data were submitted for analysis of variance, with the lamps and exposure times as independent variables (SAS, 1996). In addition, interaction effect between exposure time and lamp was evaluated; this was statistically significant for none of the examined parameters ($P>0.05$) and was thus removed from the model.

3. RESULTS AND DISCUSSION

3.1. Trial 1

In this trial, the samples were packaged in air and illuminated 24h/d (Table 2). L^* values did not vary with lighting conditions, either in the lean fraction or in the fat. However, the values of most of the other colour parameters differed between the samples kept in the dark and those exposed to the lamps. In fact, in the lean component, light exposure led to a significant reduction of both a^* ($P<0.01$) and C^* ($P<0.05$) values and an increase in the h^* value ($P<0.01$), whereas the b^* value was unaffected. Very similar trends were reported for fresh pork studied by ZHU and BREWER (1998). CIERACH and NIEDŹWIEDŹ (2014) reported a decrease of 4-7 units of a^* values in *Semitendinosus* muscle of beef after few days of light exposure using a lamp of 3000 K and 3 lightning intensity (500, 1000 and 1500 lux), demonstrating that intensity of 500 lx has less influence on the beef colour changes. In the scientific literature on this topic, light intensity has been chosen as a parameter to be standardized, by adjusting the lamp to product distance (BÖHNER *et al*., 2014; BÖHNER and RIEBLINGER, 2016; HAILE *et al*., 2013; SØRHEIM *et al*., 2017). In our work a fixed lamp to product distance has been chosen, in order to simulate the actual lighting condition of packaged Coppa in the retail display. It is evident that, despite the lighting intensity values, the simultaneous presence of oxygen (20.9%) and the continuous lighting over 120 hours deeply affect the colour of the product, without no appreciable difference among the three lamps used.

Display time, too, had no effect on the L^* value, as reported by other Authors (CIERACH and NIEDŹWIEDŹ, 2014); however, all the other colour characteristics in the lean fraction changed significantly during the 120 hours display period (Table 2). The a^* value decreased over time ($P<0.01$), while the b^* value increased, significantly ($P<0.05$) only at 48 hours of storage.

Table 2. Effect of lighting and display time on colour parameters and TBARS values (mg MDA/kg) in air packaged Coppa di Parma PGI with 24 hours lighting/day.

Trial 1	Lighting /Lamp				Time of display (h)			R-MSE (df 66)
	Darkness	CW	WW	NW	24	48	120	
Lean:								
*L**	44.40	45.90	44.78	45.36	43.65	45.87	45.80	5.02
*a**	16.58^{A}	12.06^{B}	12.81^{B}	12.41^{B}	14.69^{A}	13.99^{A}	11.72^{B}	1.69
*b**	16.62	17.76	17.05	17.26	16.16^{b}	18.32^{a}	17.03^{ab}	3.24
*C**	23.76^{a}	21.65^{b}	21.64^{b}	21.51^{b}	22.15^{AB}	23.38^{A}	20.90^{B}	2.86
*h**	44.50^{B}	55.31^{A}	52.04^{A}	53.62^{A}	47.37^{Bb}	51.80^{ABa}	54.93^{Aab}	6.16
Fat:								
*L**	63.90	65.17	63.26	62.32	63.58	63.71	63.69	4.41
*a**	8.17^{A}	4.52^{B}	5.76^{B}	5.47^{B}	6.23	6.27	5.44	2.06
*b**	12.96^{b}	13.94^{ab}	14.30^{a}	14.03^{ab}	13.21^{b}	14.31^{a}	13.89^{ab}	1.87
*C**	15.53	14.87	15.63	15.28	14.93	15.85	15.20	2.38
*h**	59.32^{B}	73.63^{Aa}	69.56^{Ab}	70.48^{Aab}	66.68^{b}	67.75^{ab}	70.31^{a}	5.74
TBARS	0.288^{Bb}	1.414^{Aa}	0.741^{ABb}	0.717^{ABb}	0.323^{B}	0.413^{B}	1.635^{A}	0.91

^{a,b}: $P<0.05$; ^{A,B}: $P< 0.01$. CW: 640 Basic Cool White lamp; WW: 827 Lumilux Interna Warm White lamp; NW: 76 Natura Neutral White lamp.

The opposite trends of *a** and *b** values brought about a significant increase of the *h** value during storage ($P<0.01$). The value of *C** decreased significantly ($P<0.01$) between 48 and 120 hours. Changes in *a**, *b**, *C** and *h** values, and hue angle (*h**) indicated that the lean fraction of the samples tended to be less red and more grey as storage time increased, probably due to a progressive loss of nitrosylmyoglobin and the consequent increase of NOMMb, according with the findings of other Authors (BÖHNER *et al*., 2014; HAILE *et al*., 2013).

As shown in Fig. 1, 24 hours under the Basic Cool White (CW) led to an *a** value lower ($P<0.05$) than under the other lamps, whereas at 48 and 120 hours *a** values were similar regardless the lamps.

Also with regard to the fat fraction (Table 2), the light lowered the *a** value ($P<0.01$), which did not differ among lamps; instead, it increased the values of *b** ($P<0.05$) and *h** ($P<0.01$) without affecting the *C** value. Indeed, storage time led to an increase of *b** and *h** values ($P<0.05$), as observed in the lean fraction. Overall, the lipid fraction of the sliced Coppa tended to yellow, as a consequence of both light exposure, oxygen in contact with the product and storage time, which promote the fat fraction oxidation.

Samples under the CW lamp showed TBARS values significantly higher than those kept in the dark ($P<0.01$) or illuminated by the other two lamps ($P<0.05$).

At 24 and 48 hours, TBARS values were similar, while they increased significantly at 120 hours ($P<0.01$).

The evolution of TBARS values during display, depending on the different lighting conditions, is shown in Fig. 2.

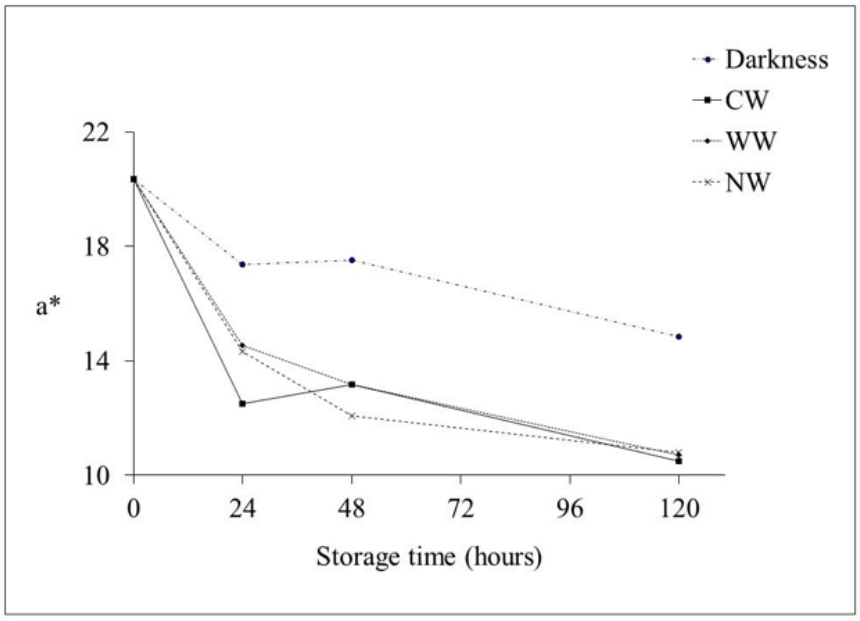

Figure 1. Values of CIE *a** (redness) in Coppa di Parma PGI packaged in air, displayed under continuous lighting.
CW: 640 Basic Cool White lamp; WW: 827 Lumilux Interna Warm White lamp; NW: 76 Natura Neutral White lamp.

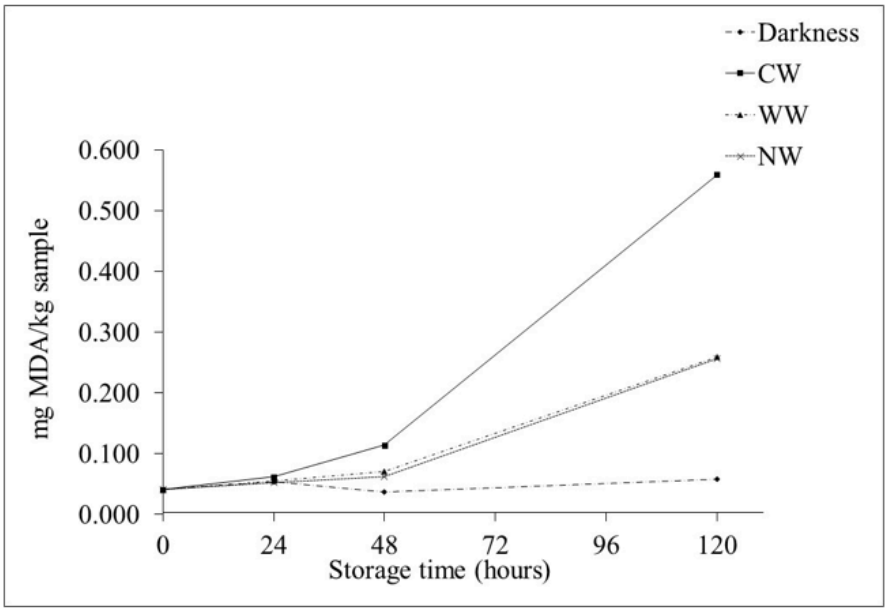

Figure 2. TBARS values (mg MDA/kg) in Coppa di Parma PGI packaged in air, displayed under continuous lighting.
CW: 640 Basic Cool White lamp; WW: 827 Lumilux Interna Warm White lamp; NW: 76 Natura Neutral White lamp.

Lipid oxidation did not develop in the dark. During storage, TBARS values increased in all the lighted samples. At 120 hours, the samples under the CW lamp gave values higher ($P<0.01$) than those under the other two lamps. These results may be explained considering the different emission spectra of the three lamps tested in this work (data from technical data sheet by OSRAM). Cool White (CW) has a strong emission in the short wavelengths (blue and green zone of the spectrum – from 430 to 560 nm); instead, Warm White (WW) and Natura Neutral White (NW) lamps are characterized by an emission in the blue zone significantly lower respect to CW; NW also shows a good emission in the red zone (630-700 nm). Some Authors underlined the importance of the lamps' emission spectra and the correlated energy. BÖHNER and RIEBLINGER (2016) demonstrated that shorter wavelengths and higher irradiance provoke increased oxygen absorption with concomitant colour changes of Bologna sausages. The continuous lighting promotes also lipid oxidation, but low energy emitting lamps will affect to a lesser extent this product degradation, as demonstrated in Figure 2, where the TBARS values in Coppa stored under NW and WW lamps are lower than those determined on the samples stored under CW lamp.

3.2. Trial 2

In this trial, the slices of the Coppa were also packaged in air, but using a 12 hours on/12 hours off light cycle. As observed in trial 1, the L^* value (Table 3) did not vary in any tissue, either with the type of illumination or with the storage time. For the other colour parameters studied, the trends observed did not differ markedly from the first trial, though only a^* and h^* values varied significantly.

Table 3. Effect of lighting and display time on colour parameters and TBARS values (mg MDA/kg) in air packaged Coppa di Parma PGI with 12 hours lighting/day.

Trial 2	Lighting /Lamp				Time of display (h)			R-MSE (df 66)
	Darkness	CW	WW	NW	24	48	120	
Lean:								
L^*	40.63	42.46	41.61	42.00	40.74	41.35	42.93	3.86
a^*	18.23^{A}	14.72^{B}	15.73^{B}	15.04^{B}	17.67^{Aa}	15.83^{ABb}	14.29^{B}	2.81
b^*	14.95	15.86	15.55	15.38	14.99	15.05	16.27	3.80
C^*	23.77	21.93	22.38	21.75	23.38	22.10	21.89	4.19
h^*	38.56^{Bb}	46.60^{A}	43.67^{ABa}	44.58^{A}	39.55^{B}	42.23^{B}	48.28^{A}	5.88
Fat:								
L^*	61.70	62.34	60.16	59.74	59.77	61.86	61.32	6.73
a^*	9.39^{Aa}	6.32^{B}	7.45^{ABb}	7.98^{AB}	8.97^{A}	7.78^{AB}	6.60^{B}	2.50
b^*	12.85	14.07	13.89	14.33	13.20	14.56	13.59	0.83
C^*	16.23	15.80	16.13	16.72	16.34	16.88	15.44	2.82
h^*	54.13^{B}	66.78^{A}	63.52^{A}	62.31^{A}	57.29^{Bb}	62.66^{ABa}	65.12^{A}	7.95
TBARS	0.340^{A}	1.044^{B}	0.790^{AB}	0.658^{AB}	0.340^{B}	0.519^{B}	1.258^{A}	0.73

^{a,b}: $P<0.05$; ^{A,B}: $P< 0.01$. CW: 640 Basic Cool White lamp; WW: 827 Lumilux Interna Warm White lamp; NW: 76 Natura Neutral White lamp.

The statistical data processing demonstrates that illuminated samples showed a lower a^* value ($P<0.01$) and a higher h^* value ($P<0.05$) in the lean fraction; whereas, only h^* changed in the fat fraction ($P<0.01$), and no significant differences were found among the lamps.
At increased storage time, a^* values decreased, significantly at 120 hours ($P<0.01$), both in lean and fat components, with a corresponding increase ($P<0.01$) of the h^* values in both tissues.
In Fig. 3, the a^* value evolution on the surface of Coppa slices exposed to different light sources or stored in the dark is reported. The colour of samples stored in the dark changes, even if slightly, probably because of high partial pressure of oxygen in the headspace of the packages. If the samples stored under light are considered, it is possible to observe a different influence of the lamps used. Samples stored under CW lamp shows a more pronounced decrease of the a^* value between 48 and 120 hours of discontinuous lighting in comparison with the results obtained with WW and NW lamps. Discontinuous lighting also affects the colour of Coppa slices, but highlights the influence of different emitting lamps on the product colour fading.

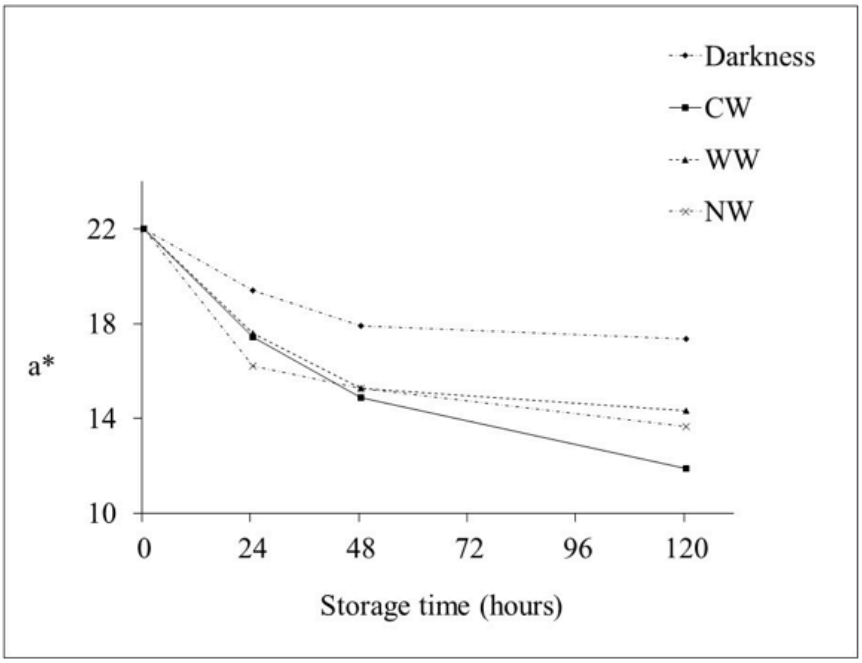

Figure 3. Values of CIE a^* (redness) in Coppa di Parma PGI packaged in air, displayed under 12 h/d lighting.
CW: 640 Basic Cool White lamp; WW: 827 Lumilux Interna Warm White lamp; NW: 76 Natura Neutral White lamp.

TBARS values showed the same path observed in trial 1. Thus, the samples kept in the dark provided the lowest values, but only the CW lamp caused significantly higher values ($P<0.01$). Moreover, as in trial 1, TBARS increased with time, significantly at 120 hours ($P<0.01$).
TBARS evolution during storage under different lighting conditions is shown in Fig. 4: darkness preserved samples from oxidation, which developed more markedly in the

illuminated samples, showing the highest value under the CW lamp. These data are consistent with those obtained for the colour changes.

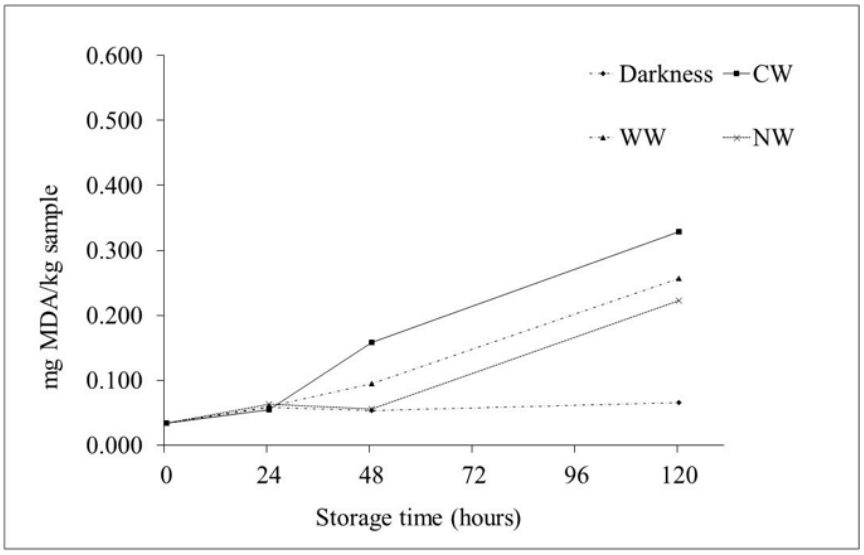

Figure 4. TBARS values (mg MDA/kg) in Coppa di Parma PGI packaged in air, displayed under 12h/d lighting.
CW: 640 Basic Cool White lamp; WW: 827 Lumilux Interna Warm White lamp; NW: 76 Natura Neutral White lamp.

3.3. Trial 3

In Table 4 are shown the data concerning the samples packaged in modified atmosphere (MAP) exposed to continuous lighting 12h/d. As regards the effect of the type of lighting, the results showed no statistically significant variation ($P>0.05$) of the values of the parameters taken into account, neither in the lean nor the fat fraction. As concerns the a^* value, our results agree with the findings of MARTÍNEZ *et al*. (2007) on fresh pork sausages and of DJENANE *et al*. (2001; 2003) on fresh beef steaks, and partially with the results of HAILE *et al*. (2013) on colour stability of cooked ham. The effect of residual oxygen and light exposure on the quality of cured boiled sausages (BÖHNER *et al*., 2014) and of Norwegian salami (SØRHEIM *et al*., 2017) has been studied. In our work the residual oxygen inside the MA packages was not measured, so we can only hypothesize a complete oxygen depletion during the packaging process, which makes irrelevant the lighting of the product concerning the colour fading. Another consideration is that the cured products examined in the cited works differ from Coppa, and it is well known that the type of meat and the production process may influence the specific sensitivity against oxygen and light.
If storage times are considered, the few significant differences observed in the lean fraction showed an erratic path, difficult to explain. These same considerations also apply to the evolution of a^* value in the samples displayed under different lightings; as Fig. 5 clearly shows, MAP maintained the red colour, regardless of light exposure and source; though it must be noted that the a^* values at 0 hours were lower in this trial.

Table 4. Effect of lighting and display time on colour parameters and TBARS values (mg MDA/kg) in Coppa di Parma PGI packaged in a modified atmosphere with 12 hours lighting/day.

Trial 3	Lighting /Lamp				Time of display (h)			R-MSE (df 66)
	Darkness	CW	WW	NW	24	48	120	
Lean:								
*L**	41.24	40.92	43.00	41.48	41.48	41.99	41.51	3.36
*a**	13.45	13.84	13.03	13.94	13.64^{ab}	14.05^{a}	13.00^{b}	1.60
*b**	12.30	12.05	12.50	12.44	12.12	12.48	12.38	1.37
*C**	18.43	18.52	18.18	18.81	18.37^{ab}	18.92^{a}	18.17^{b}	1.13
*h**	42.63	41.46	44.29	42.07	41.87	42.04	43.93	5.66
Fat:								
*L**	56.35	57.37	57.09	56.22	56.04	57.19	57.04	5.61
*a**	6.63	5.95	6.17	6.93	6.97^{a}	6.78^{ab}	5.51^{b}	2.32
*b**	10.39	10.99	11.06	11.01	10.82	10.85	10.92	1.13
*C**	12.68	12.87	13.07	13.40	13.23	13.15	12.64	1.92
*h**	59.83	64.02	63.64	60.66	59.83^{b}	60.90^{ab}	65.38^{a}	7.82
TBARS	0.312	0.306	0.283	0.277	0.254^{B}	0.271^{B}	0.358^{A}	0.07

^{a,b}: P<0.05; $^{A, B}$: P< 0.01. CW: 640 Basic Cool White lamp; WW: 827 Lumilux Interna Warm White lamp; NW: 76 Natura Neutral White lamp.

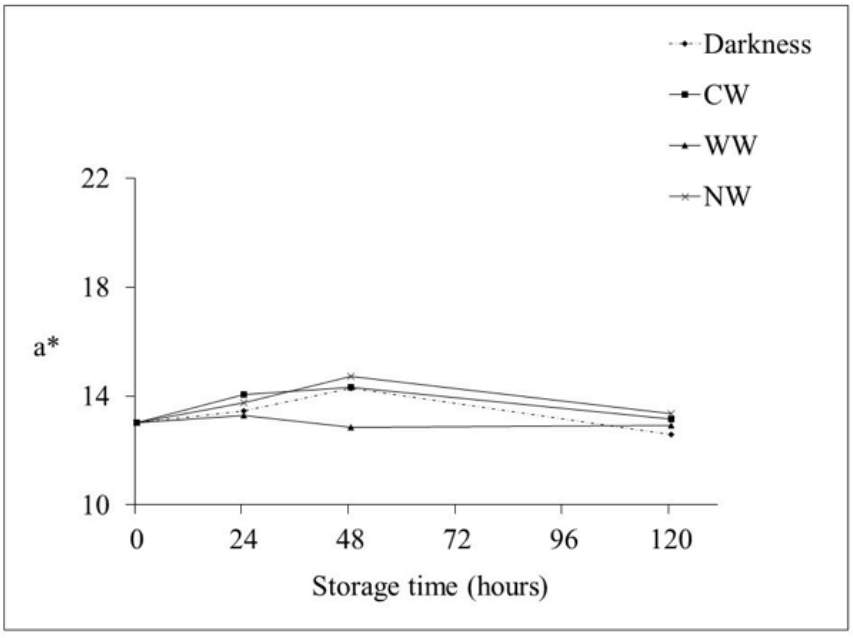

Figure 5. Values of CIE *a** (redness) in Coppa di Parma PGI packaged in a modified atmosphere, displayed under 12 h/d lighting (1c).
CW: 640 Basic Cool White lamp; WW: 827 Lumilux Interna Warm White lamp; NW: 76 Natura Neutral White lamp.

At increasing storage time, a significant decrease at 120 hours ($P<0.05$) of the a^* value and a corresponding significant increase ($P<0.05$) of the h^* value took place in the adipose fraction.
The TBARS values did not differ between the samples maintained in the dark and those exposed to light. Also in this case, as well as the colour evolution, eliminating oxygen in contact with Coppa means protect the product against lipid oxidation, even in presence of a luminous source emitting high energy wavelengths, such as CW lamp (refer to Fig. 6). Our findings are consistent with the results of MARTÍNEZ *et al*. (2007) on fresh pork sausages, with DJENANE *et al*. (2001; 2003) on fresh beef steaks and with GIMENEZ *et al*. (2004; 2005) on gilt-head sea bream and salmon fillets.
As in the two previous trials, after 120 h of storage, TBARS values increased significantly ($P<0.01$), though changes were rather slight. Our results conflict with those of MØLLER *et al*. (2000) who could not detect any difference among TBARS values in samples lighted or kept in the dark. The Authors stated that this could be due to the low fat content of their hams, much lower than the one found in our samples of Coppa, which was well above 29%.

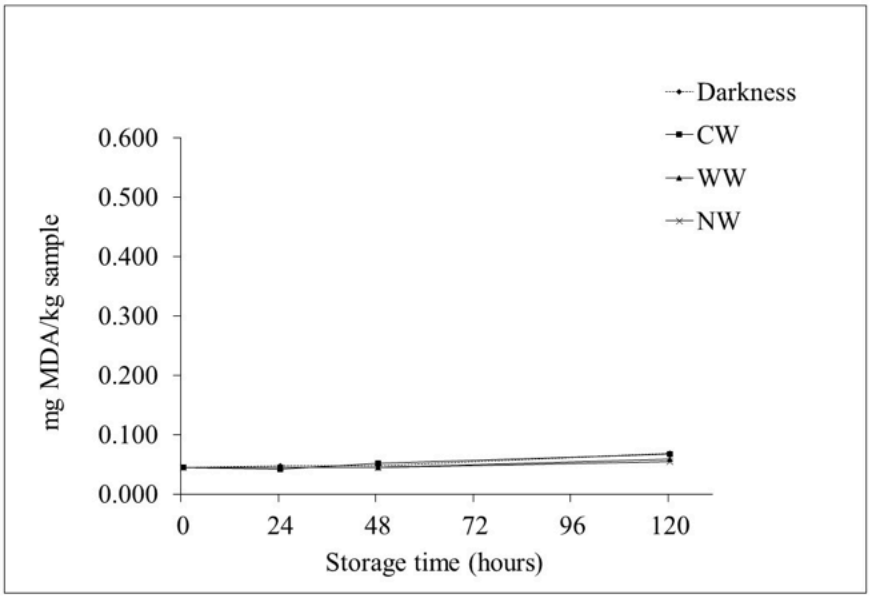

Figure 6. TBARS values (mg MDA/kg) in Coppa di Parma PGI packaged in a modified atmosphere, displayed under 12h/d lighting.
CW: 640 Basic Cool White lamp; WW: 827 Lumilux Interna Warm White lamp; NW: 76 Natura Neutral White lamp.

Table 5 shows changes in the value ΔE that expresses, in the CIE L^*, a^*, b^*, the quantification of the colour difference.
As regards the type of lighting, it can be observed that at 24 hours (ΔE1) the CW lamp brought about a colour variation greater than the other lighting conditions, which was significant only when compared with the dark ($P<0.01$). Regarding the ΔE2 values, they were greater than ΔE1, as expected, and were significantly lower in the samples kept in the dark ($P<0.05$). At 120 hours (ΔE3) the colour difference was still greater and although

the samples kept into the dark gave the lowest value, no statistically significant variation among lighting conditions was found (P>0.05).

Table 5. Effect of lighting and display time on colour change (ΔE) in the lean of Coppa di Parma PGI.

	Lighting /Lamp				Treatment			R-MSE (df 66)
	Darkness	CW	WW	NW	Trial 1	Trial 2	Trial 3	
ΔE1	5.28^{B}	7.63^{A}	6.21^{AB}	6.38^{AB}	7.73^{A}	6.37^{AB}	5.02^{B}	2.60
ΔE2	5.68^{b}	8.80^{a}	8.76^{a}	8.15^{a}	9.64^{A}	8.76^{A}	5.14^{B}	3.58
ΔE3	7.73	9.54	9.10	9.08	11.59^{A}	9.98^{A}	5.01^{B}	3.69

^{a,b}: P<0.05; $^{A, B}$: P< 0.01. CW: 640 Basic Cool White lamp; WW: 827 Lumilux Interna Warm White lamp; NW: 76 Natura Neutral White lamp. ΔE1, ΔE2 and ΔE3 are calculated colour differences between time 0 and 24, 48, 120 hours, respectively

With regard to the colour difference among the trials, data showed that the presence of oxygen caused a significant increase of ΔE2 and ΔE3 values (P<0.01), while at 24 hours (ΔE1) only permanent lighting gave colour changes higher (P<0.01) than in MAP stored samples. No visual evaluations have been performed, in order to establish if the colour changes can be detected by consumers, but it was demonstrated that if ΔE>2 a small difference is observed, and when ΔE>5 the changes are well distinguished (FLEISHMAN *et al*., 1998; LINDSTRÖM, 2008).

4. CONCLUSIONS

From our results it may be concluded that the lighting with UV-free lamps negatively affects colour characteristics of Coppa di Parma PGI sliced and then packaged in air. Further, illumination causes a significant increase of lipid oxidation and the Basic Cool White lamp appears to be somewhat more detrimental. When the product is packaged in a modified atmosphere, colour and lipid stability are not affected by light exposure or source.
One hundred and twenty hours of storage led mainly to a loss of redness and to a significant increase of lipid oxidation.

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PAPER

INFLUENCE OF RELATIVE HUMIDITY ON DRIED CA^{++}-ALGINATE FILMS AND COMPOSITES MADE WITH SOY AND PECTIN

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ABSTRACT

Dried Ca-alginate films were manufactured±pectin or soy protein isolate (SPI) as well dried un-gelled (no Ca^{2+}) pure alginate films: conditioned at either 57% or 100% relative humidity (RH). With the exception of the un-gelled alginate films, all films conditioned at 100% RH were more transparent than at 57% RH. High RH films resulted in higher % elongation at break, distance and work to puncture values than their corresponding films conditioned at 57% RH. ATR-FTIR scans showed several peak shifts when the film forming solutions were gelled with Ca^{2+} and when the 'wet' films were dried.

Keywords: alginate, film, pectin, relative humidity, soy protein isolate, tensile strength

1. INTRODUCTION

Global production of packaging materials is increasing and was estimated to exceed 180 million tons per year (CUTTER, 2006; NURA, 2018). Food packaging accounts for a large portion of this material and is estimated to attribute to roughly 70% of the $100 billion packaging market in the U.S. alone (CUTTER, 2006). Much of this packaging material is made from synthetic plastics. However, mounting environmental concerns about plastic use and the rising cost of petroleum has led to an increased interest in natural food packaging material such as polysaccharides, proteins and/or lipids (RHIM, 2004; CUTTER, 2006; DA SILVA *et al.*, 2009; JANJARASSKUL and KROCHTA, 2010). However, edible packaging materials are not normally meant to entirely replace conventional packaging. Rather, the efficiency of food preservation can be improved by using primary edible packing together with less nonedible packaging. A good example is replacing plastic or cellulose casings used for frankfurters with manufactured co-extruded (in line) collagen casings, alginate or their combination (BARBUT, 2015). The frankfurters are then packaged in a strong oxygen barrier plastic pouch, which can be used as a barrier during cooking and/or distribution.

A popular example of a polysaccharide that has been used in edible films is alginate. It is used as food wraps (e.g., spring rolls) and for small diameter sausage casings. One of the more desirable properties of alginate is its ability to very quickly form cold-set gels in the presence of calcium (OUWERX *et al.*, 1998; MØRCH *et al.*, 2006). Alginates are derived from marine brown algae (*Phaeophyceae*) and also produced by soil bacteria (STEPHEN *et al.*, 2006). Chemically, alginate is made up of (1→4) β-D-mannuronic (M) and α-L-guluronic (G) acid. Regions in alginate made up of solely M or G residues are referred to as M or G blocks and these areas are interspersed with MG alternating blocks (FANG *et al.*, 2007; LEE *et al.*, 2018).

Several factors influence the use of edible films in food applications including: availability, cost, functional attributes, mechanical properties, optical quality, barrier properties, structural resistance to water and sensory acceptability (DA SILVA *et al.*, 2009; JANJARASSKUL and KROCHTA, 2010). Typically protein and polysaccharide films are fairly good oxygen barriers at low to medium RH and have good mechanical properties but, due to their hydrophilic nature, are poor water vapour barriers (RHIM and NG, 2007). Today, several large and medium size sausage manufacturers are using co-extrusion technology. Unofficial estimates put this number at 30% of the US market. This allows sausage manufacturers to move from a batch to a continuous process and thus increase production volumes (BARBUT, 2015). Additionally, fewer people handle the product and thus the risk of microbial contamination of fresh sausages is reduced (ANONYMOUS, 2012). Some manufacturers are also using cook in the bag technology, and therefore further reduce the risk of re-contamination (after cooking) with pathogens such as *Listeria*.

However, there are several challenges of casings manufactured from solely alginate or collagen. Researchers have reported that alginate casings are prone to calcium migration (e.g., casings dissolve over time), while collagen casings may shrink more during frying (VISSER, 2012). One solution is producing composite co-extruded casings/films, which take advantage of synergistic interactions between individual components. The industry has already adopted this approach by adding ingredients such as starch and cellulose to co-extruded alginate casings, as well as producing co-extruded alginate-collagen hybrid casings. Although these composite alginate casings are used commercially, there is little or no scientific literature regarding the properties of such 'wet' films. In fact, most of the literature reports on 'dry' alginate films for use after they have been cast. For example,

researchers have created composite 'dry' alginate films with proteins such as, whey, soy, and whey (SHIH, 1994; VILLAGOMEZ-ZAVALA *et al.*, 2008; WANG *et al.*, 2010), or polysaccharides such as pectin, kappa-carrageenan, pullulan, sago starch, and carboxy-methyl cellulose (XU *et al.*, 2003; TONG *et al.*, 2008; DA SILVA *et al.*, 2009; FAZILAH *et al.*, 2011; GOHIL, 2011; BIERHALZ *et al.*, 2012; PAŞCALĂU *et al.*, 2012; XIAO *et al.*, 2012; GALUS and LENART, 2013). In certain cases, synergism was seen when 'dry' composite films made from 50:50 alginate and low methoxyl pectin showed higher tensile strength and elongation than either pure alginate or pectin films (GALUS and LENART, 2013).
HARPER *et al.* (2013) started publishing on the 'wet' film area (90-95% water content), which recently became popular with the use of co-extruded sausage casings. They examined how various protein (whey, soy, and gelatin) and carbohydrate (pectin, cellulose, starch, carrageenan, and gellan gum) additives influence the mechanical and microstructural properties of composite films/casings. However, so far very few comparisons have been made between the physical properties of 'wet' and dried alginate films. Understanding the role water plays in these films is important for future development of this area. Therefore, the objective of this work was to explore how drying 'wet' alginate and alginate composite films affect these physical properties. Additionally, the effect of rehydration of the dried films (i.e., exposing the dried films to high humidity) on their physical properties has been studied. For this work, composite alginate films were manufactured with either soy protein isolate (SPI) or low-methoxylated pectin and compared to pure alginate films.

2. MATERIALS AND METHODS

2.1. Film preparation

The 'wet' alginate films were produced according to the methods described by Harper et al. (2013). Briefly, 5% (w/w) alginate (GRINDSTED® Alginate FD 6965, Danisco USA Inc., Rochester, NY, USA) solution was prepared. Later, 1.5 g portions were rolled onto a plastic covered stainless steel board using a stainless steel roller with a recess of 0.34 mm. The roller, with the film on it, was placed into a 5% (w/w) $CaCl_2$ (Fisher Scientific, Fair Lawn, NJ, USA) bath for 1 min to gel the film.
Soy protein isolate (SPI; Soy Protein Supro 515, protein 90%, supplied by Hela Spice Canada, Inc., Uxbridge, ON, Canada) was dissolved in 23°C double deionized water to form a 1% wt. protein solution. A 0.25% wt. pectin solution was made by dissolving low methoxyl pectin (LMP; GENU® pectin type LM-104 AS-Z, CP Kelco, Lille Skensved, Denmark) into 70°C double deionized water. 5% wt. alginate was also mixed by hand (total mixing time of 15 min) into both the protein and pectin solutions. Additionally, a control sample of 5% wt. alginate with no protein or pectin was made. To remove some of the air incorporated into the solutions during mixing, the solutions were degassed by using a vacuum packager (Multivac Canada Inc., Woodbridge, ON, Canada) at 7.3 kPa for 25, 50 and 75 sec. The solutions were allowed to hydrate for a minimum of 2 hr at 23°C prior to film formation. The 'wet' films were produced using a stainless-steel roller and then placed into the calcium bath. Alginate films were also produced without calcium as a gelling agent. In this case, alginate solution was rolled between two sheets of plastic on the stainless-steel board. The top layer of plastic was carefully peeled away from the film, leaving the film on the bottom sheet of plastic for drying.

2.2. Drying and conditioning of the films

The 'wet' alginate and alginate composite films were pinned to a plastic covered smooth piece of wood (to prevent curling) and left to dry overnight at room conditions (~23°C and ~52% RH). The alginate films without calcium were simply left on the bottom sheet of plastic to dry. The following day the films were removed from the plastic and placed in jars with either 57% RH (NaBr controlled) or 100% RH, and left to condition overnight. Prior to conditioning, the films that would later be used for tensile testing were cut into 75 mm X 25 mm strips using a razor blade, and their thickness measured. Three thickness measurements (top, centre and bottom) were taken on each film using a digital micrometer (Testing Machines Inc., Islandia, NY, USA) and the average thickness of each film was used for the tensile stress calculations.

2.3. Mechanical properties

The puncture and tensile properties of the films were evaluated using a texture analyzer (TA.XT Icon. Texture Technologies Corp., Hamilton, MA/Stable Micro Systems, Godalming, Surrey, UK). The films were kept in their RH respective jars right until the testing. For the puncture test a 5 mm ball probe was used to puncture the uncut films. The trigger force was set at 5 g and the test speed was 10 mm/s. From the generated force-distance graph, the force (N), distance (mm) and work (N mm) to puncture the films were determined. Tensile testing was conducted according to the ASTM-D882 standard tensile testing procedure (ASTM, 2010). The grippers were spaced 50 mm apart and the test distance was set at 75 mm. The trigger force for testing was 8 g and the test speed was 2 mm/s. The tensile strength (MPa), % elongation at break and the Young's modulus of the films were determined from the generated stress-strain graphs.

2.4. Optical properties

The visible light transmission (400-780 nm) of the films was measured using a single beam spectrophotometer (USB 2000, Ocean Optics Inc., Dunedin, FL, USA). The integration time was 25 ms with 2 scans to average and a boxcar width of 4. In total eighteen films per treatment were scanned (six per trial). The average light transmission of the eighteen films was calculated for each of the treatments.

2.5. ATR-FTIR analysis

Spectra were acquired using an FTIR spectrometer (IRPrestige-21, Shimadzu Corporation, Tokyo, Japan) equipped with a total attenuated reflection press (MIRacle™, Pike Technologies, Madison, WI, USA). Scans were taken of the alginate and alginate-low methoxyl pectin film forming solutions, gelled 'wet' films prior to drying and the gelled dried films conditioned at 57% RH. Additionally, the alginate and low methoxyl pectin powders used to make the films were scanned. All of the powders were conditioned at 57% RH overnight. For consistency, all of the films were scanned with the side of the film that was touching the roller, during gelling in the calcium bath, face up. All samples were tested at 23°C. The data were collected from 600-4000 cm^{-1} with an average of 30 scans per sample and a resolution of 4 cm^{-1}. The second derivatives of the original spectrums were

taken using Grams-32 spectral analysis software (Galactic Industries Corp., Salem, NH, USA) to determine the wavenumber of the peaks. Sampling was performed in triplicate.

2.6. Statistical analysis

The experimental design was a completely randomized block with three independent trials. For both the puncture and tensile tests six films per treatment were tested in each of the three trials. The average of the six measurements was taken for each treatment in each trial and used for statistical analysis (SAS Version 9.2, SAS Inst., Cary, NC, USA). A General Linear Model was used for analysis of variance (ANOVA) and a Tukey's multiple comparison analysis (P-value ≤ 0.05) was used to detect statistical significance between film type means.

3. RESULTS AND DISCUSSION

3.1. Mechanical properties of formed films

The purpose of this work was to compare the physical properties of dried alginate and alginate composite films to their 'wet' film counterparts. In our previous work, when low methoxyl pectin (LMP) or soy protein isolate (SPI) were added to 'wet' alginate films, the composite films exhibited different mechanical properties from the pure 'wet' alginate films (HARPER *et al.*, 2013; HARPER *et al.*, 2015). For this reason, pectin and SPI were chosen as ingredients for the alginate composite films manufactured in this study. In order to be able to directly compare the dried films in this study to the 'wet' films produced in earlier work, the films were manufactured in the same way. In the present study, the films were dried and then conditioned at 57% and 100% relative humidity in order to also evaluate how rehydration influences the films' physical properties. Overall, all of the dried films (conditioned at 57% or 100% RH) showed higher puncture force and tensile strength but lower puncture distance and elongation values compared to their corresponding 'wet' films. For example, the percent elongation at break was 140% for the 'wet' alginate-pectin films (HARPER *et al.*, 2015) and 4.6% and 12.3% for the dried alginate-pectin films conditioned at 57% and 100% RH, respectively (Table 1). The control alginate film gelled with Ca had an elongation value of 88% while after drying and conditioned it to 57% or 100% RH the values were only 5.8% and 12.0%, respectively. Therefore, it is important to emphasize that the rehydrated dried films (i.e., 100% RH films) did not have the same mechanical properties as their original 'wet' films, thus demonstrating that drying caused irreversible changes in the alginate film structure.

All the dried and reconditioned films could be tested after conditioning at 57% and 100% RH, with the exception of the alginate films that were not gelled with calcium. After conditioning at 100% RH, the un-gelled alginate films were very sticky and unable to hold their shape for testing. For this reason, no mechanical or optical tests were performed on these films. As expected, differences in puncture force were seen between the films conditioned at 57% and 100% RH (Fig. 1). Interestingly, the trend was not consistent for all treatments. In the case of the gelled alginate and alginate-pectin films, the films conditioned at 100% RH required less force to puncture than those conditioned at 57% RH, while the opposite was true for the alginate-SPI films. REMUNAN-LOPEZ and BODMEIER (1997) also explored the differences in puncture strength between 'dry' and 'wet' alginate films. They found that 'wet' films had lower puncture strength than 'dry'

films, which they attributed to the plasticizing effect of water. A similar trend was expected in this work. It should be noted that the 'wet' films in their study were immersed in water as opposed to exposed to humid air, which may have influenced the uptake of water by the films and thus their resulting mechanical properties.

Table 1. Mechanical properties of dried calcium-alginate (ALG) films with and without low methoxyl pectin (LMP) or soy protein isolate (SPI) conditioned at 57% and 100% relative humidity.

Treatment	Distance to Puncture (mm)	Work to Puncture (N mm)	Tensile Strength (MPa)	Elongation at Break (%)	Young's Modulus (MPa)	Thickness (um)
ALG (no Ca) 57	3.2±0.3^{c}	7.3±0.4^{c}	90.9±32.8^{a}	3.7±1.3^{b}	2729.2±298.4^{a}	0.007±0.002^{c}
ALG 57	6.6±0.5^{b}	29.4±1.9^{b}	30.0±5.2^{b}	5.8±1.3^{b}	867.2±294.0^{b}	0.018±0.002^{b}
ALG 100	11.3±0.5^{a}	39.5±4.3^{a}	9.9±0.3^{b}	12.0±2.9^{a}	179.7±15.5^{c}	0.020±0.001^{b}
ALG LMP 57	5.7±0.3^{b}	27.8±3.5^{b}	38.8±5.7^{b}	4.6±0.6^{b}	1190.9±228.1^{b}	0.018±0.002^{b}
ALG LMP 100	11.9±0.1^{a}	41.0±1.8^{a}	9.9±1.5^{b}	12.3±2.4^{a}	178.7±45.2^{c}	0.018±0.002^{b}
ALG SPI 57	2.7±0.4^{c}	4.8±1.1^{c}	24.5±3.5^{b}	3.2±0.3^{b}	989.6±265.6^{b}	0.035±0.002^{a}
ALG SPI 100	11.6±0.3^{a}	31.6±2.4^{b}	4.4±0.1^{b}	16.1±3.2^{a}	64.5±4.4^{c}	0.034±0.001^{a}

Means±standard deviation, $^{a-c}$ show significant differences (P<0.05) between means (n=18).

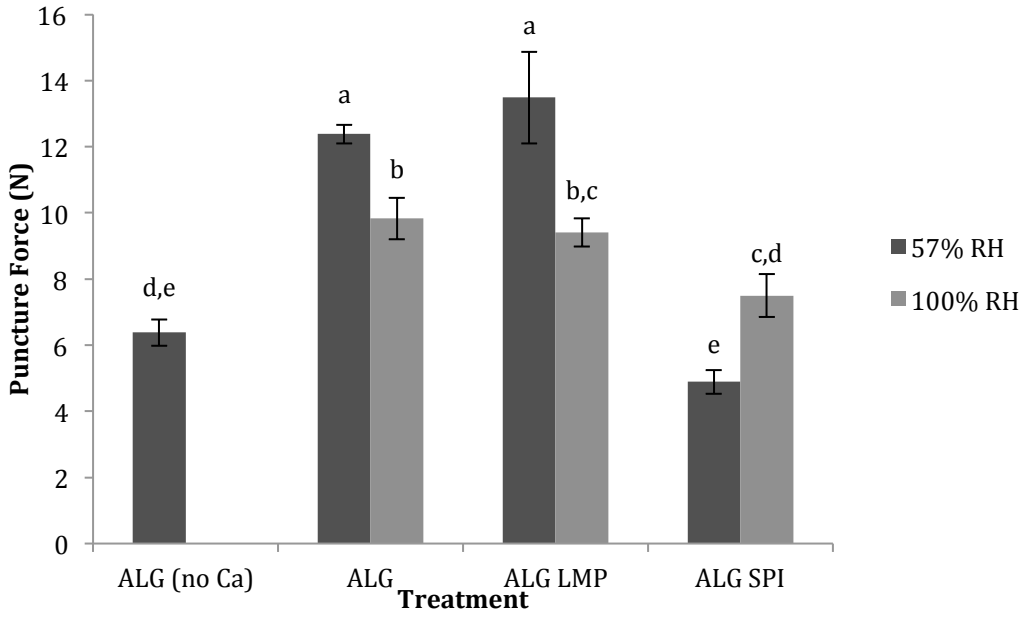

Figure 1. Puncture force of dried calcium-alginate (ALG) films with and without low methoxyl pectin (LMP) or soy protein isolate (SPI) conditioned at 57% and 100% relative humidity (RH). Note: ALG with no Ca does not form a film and hence could not be evaluated.
$^{a-e}$ show significant differences (P<0.05) between means (n=18).

The puncture force trends between treatments are similar to what we have previously reported for the 'wet' alginate films (HARPER *et al.*, 2015). In both the dried (57 & 100% RH) and 'wet' alginate films, it took significantly less force to puncture the alginate-SPI films (3.8N) than the pure alginate films (6.3N). This may be a result of the soy proteins interrupting the alginate film network. No differences in puncture force existed between the alginate-pectin (5.8N) and pure alginate films (5.3N) for the 'wet' or dried films (HARPER *et al.*, 2015). As expected, the un-gelled pure alginate films had significantly lower puncture force than the gelled pure alginate films. Similarly, it has been reported that 'dry' alginate films gelled with calcium have increased tensile strength over their un-gelled counterparts (FAZILAH *et al.*, 2011).

Differences in the puncture distance were also observed between the dried films conditioned at 57% and 100% RH (Table 1). In all cases, the films conditioned at 100% RH showed a greater distance to puncture than the films conditioned at 57% RH. Again, this is likely due to the plasticizing effect of water in the 100% RH conditioned films. Although no differences were observed between the three 100% RH treatments, the alginate and alginate-pectin films conditioned at 57% RH showed significantly higher puncture distance values than the alginate-SPI and un-gelled alginate films conditioned at 57% RH. Similar trends were observed for 'wet' alginate films where the pure 'wet' alginate films had significantly higher puncture distance values (17mm) than 'wet' alginate-SPI films (13mm) (HARPER *et al.*, 2013), but not significantly different values from the 'wet' alginate-pectin films (17mm) (HARPER *et al.*, 2015). The work to puncture results followed a similar pattern as the puncture distance, with the films conditioned at 100% RH having significantly higher work values than those conditioned at 57% RH (Table 1). Both the alginate-SPI and un-gelled alginate films conditioned at 57% RH required less work to puncture than the pure alginate films conditioned at 57% RH. No difference in the work to puncture existed between the alginate-pectin and pure alginate films conditioned at either relative humidity.

Tensile testing is another method used to measure the strength and elasticity of biopolymer films. Fewer differences were seen in the tensile strengths of the dried films gelled with calcium and then reconditioned at either 57% or 100% RH (Table 1). Our previous work indicated that, 'wet' alginate pectin films had significantly higher tensile strength than the pure 'wet' alginate films. This was also the case here when the films conditioned at 57% RH were compared. In general, it was expected that films conditioned at 57% RH would have higher tensile strength than those conditioned at 100% RH. Other work has shown that increasing the relative humidity of 'dry' calcium-alginate films decreases their tensile strength (OLIVAS and BARBOSA-CÁNOVAS, 2008). However, the authors also reported that this effect was less pronounced when plasticizers such as glycerol, sorbitol, fructose or PEG-8000 were absent from the film formulations. They argued that the non-plasticized films had a reduced capacity to absorb water and thus their mechanical properties were less influenced by changes in relative humidity. In the current work, plasticizers were not used. This may explain why more differences between the 57% and 100% RH films tensile strengths were not observed.

The tensile strengths of the Ca-alginate films in the present study were lower than some of the values reported in the literature for 'dry' alginate films. Tensile strengths of 85.9, 64.7, and 24.1 MPa were reported for dried glycerol-plasticized calcium-alginate films conditioned at 50%, 56%, and 98% RH, respectively (RHIM, 2004; OLIVAS and BARBOSA-CÁNOVAS, 2008). In each of these cases, the alginate films were dried prior to being immersed into $CaCl_2$. Alternatively, the films in the present study were gelled with $CaCl_2$ prior to drying, which may account for some of the differences observed between the

studies. The percent elongation at break (% EAB) values in the present study (Table 1) were similar to those reported for other 'dry' alginate and alginate composite films (RHIM, 2004; OLIVAS and BARBOSA-CÁNOVAS, 2008; FAZILAH *et al.*, 2011; GOHIL, 2011; GALUS and LENART, 2013).
Similar to the puncture distance, the % EAB was significantly higher for films conditioned at 100% RH than films conditioned at 57% RH (Table 1). Unlike the puncture distance, no significant differences were found between the various films conditioned at 57% RH. Contrarily, GALUS and LENART (2013) reported higher elasticity values for blended 'dry' alginate-pectin films than pure alginate or pure pectin 'dry' films. GOHIL (2011) also reported that adding up to 60% low methoxyl pectin improved the elasticity of 'dry' alginate films. Additionally our previous work on 'wet' alginate films showed that alginate-pectin films had significantly higher % EAB than pure alginate films (HARPER *et al.*, 2015). Therefore, it appears that any benefit low methoxyl pectin imparted on the elasticity of 'wet' alginate films was lost when the films were dried. On the other hand, adding soy protein isolate to the alginate films did not influence the % EAB of either the 'wet' (HARPER *et al.*, 2013) or dried (57% 100% RH) films.
The Young's modulus is a measurement of the film's stiffness. Similar to the tensile strength, the un-gelled alginate film had the highest Young's modulus of all of the films (Table 1). No differences in modulus were seen between the gelled alginate, alginate-pectin and alginate-SPI films conditioned at the same relative humidity, but in all three cases the films conditioned at 57% RH had higher stiffness values than those conditioned at 100% RH. The alginate-SPI films were significantly thicker than the alginate-pectin and gelled alginate films while the un-gelled alginate films were the thinnest (Table 1). Since the un-gelled films were rolled between two sheets of plastic (with a roller), and not submerged in a $CaCl_2$ bath, these films were thinner than the gelled films prior to drying and thus, it is no surprise that the dried un-gelled films were thinner than the dried gelled films.
All the dried films (conditioned at both RH) in the present study had higher puncture force and tensile strength values than their corresponding 'wet' films produced in earlier work. For instance, the 'wet' alginate-SPI films had a tensile strength of 1.6 MPa (HARPER *et al.*, 2013), while the dried alginate-SPI films had tensile strengths of 24.5 and 4.4 MPa for the films conditioned at 57 and 100% RH, respectively. On the other hand, all the 'wet' films had higher puncture distance and % EAB than their corresponding dried (57 & 100% RH) films. The % EAB values in the present study ranged from ~3 to 16% whereas those previously reported for 'wet' alginate films were ~80 to 140%. Therefore, it can be concluded that drying the films improved their puncture and tensile strength but decreased their elasticity. As mentioned before, even after rehydration the films did not have the same properties as the original 'wet' films.

3.2. Films optical properties

With the exception of the un-gelled alginate films, all of the films conditioned at 100% RH were more transparent than the films conditioned at 57% RH (Fig. 2). Visually, the alginate-pectin, alginate-SPI and pure alginate films conditioned at 57% RH were a whitish colour. On the other hand, the pure alginate and alginate-pectin films conditioned at 100% RH were clear and transparent (upper lines in Fig. 2), while the alginate-SPI films conditioned at 100% RH had a slight yellowish-brown tint to them. It is believed that the whitish colour of the films conditioned at 57% RH was a result of salt crystals on the surface of the film. This hypothesis was supported by SEM images which showed very

small salt crystals on the surface of the alginate-SPI, alginate-pectin and pure alginate films conditioned at 57% RH (images not shown). It would also explain why the un-gelled alginate films conditioned at 57% RH were much more transparent than all of the other films conditioned at 57% RH. At both relative humidity conditions, the alginate-SPI films appeared to be least transparent of the films, although a greater difference was seen for the films conditioned at 100% RH. The alginate-pectin films conditioned at 57% RH also appeared to be slightly less transparent than the pure alginate films. This is in agreement with other researchers who have reported 'dry' alginate films to be more transparent than 'dry' pectin films (PARRIS *et al.*, 1995; DA SILVA *et al.*, 2009; GALUS and LENART, 2013).

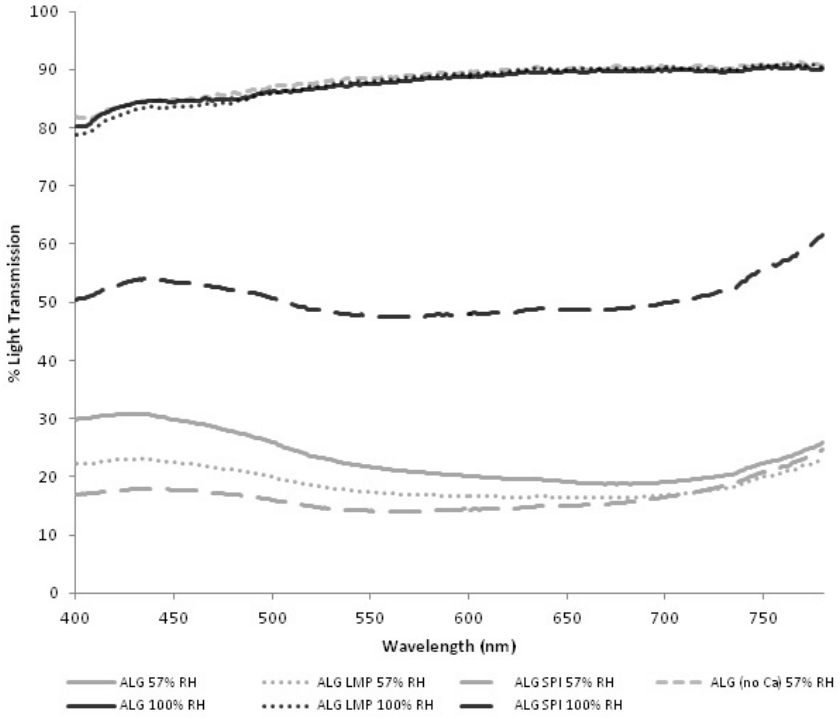

Figure 2. Visible light transmission (n=18) of dried calcium-alginate (ALG) films with and without low methoxyl pectin (LMP) or soy protein isolate (SPI) conditioned at 57% and 100% relative humidity.

3.3. FTIR

The ATR-FTIR spectra of the alginate, and alginate-pectin film forming solutions as well as the 'wet' and dried (57% RH) films are shown in Fig. 3. In addition, the alginate and pectin dry powders that were used to make the films were scanned (Fig. 3).
Table 2 summarizes the main vibrational peaks of the various spectra in the fingerprint region (1750-800 cm^{-1}). Assignments of the peaks was based on literature (SARTORI *et al.*, 1997; KACURAKOVA *et al.*, 2000; PEREIRA *et al.*, 2009; PAPAGEORGIOU *et al.*, 2010). Calcium is known to interact with the carboxylate groups in the alginate. The two absorbance bands associated with the symmetric and asymmetric stretching vibration of the COO^- groups of alginate occurred at 1426-1414 cm^{-1} and 1597-1591 cm^{-1} for the films and film forming solutions. This is in agreement with other studies that reported these two

peaks to occur in the 1422-1404 cm^{-1} and 1622-1596 cm^{-1} ranges (SARMENTO *et al.*, 2006; MOHAMADNIA *et al.* 2007; JAYA *et al.*, 2009; PAPAGEORGIOU *et al.*, 2010; PAŞCALĂU *et al.*, 2012). It should be noted that there were actually two peaks detected in the 1600 cm^{-1} region in the present study; however, the peak at 1635-1631 cm^{-1} has been attributed to the water in the films as it was not present in the alginate powder spectra (Fig. 3).

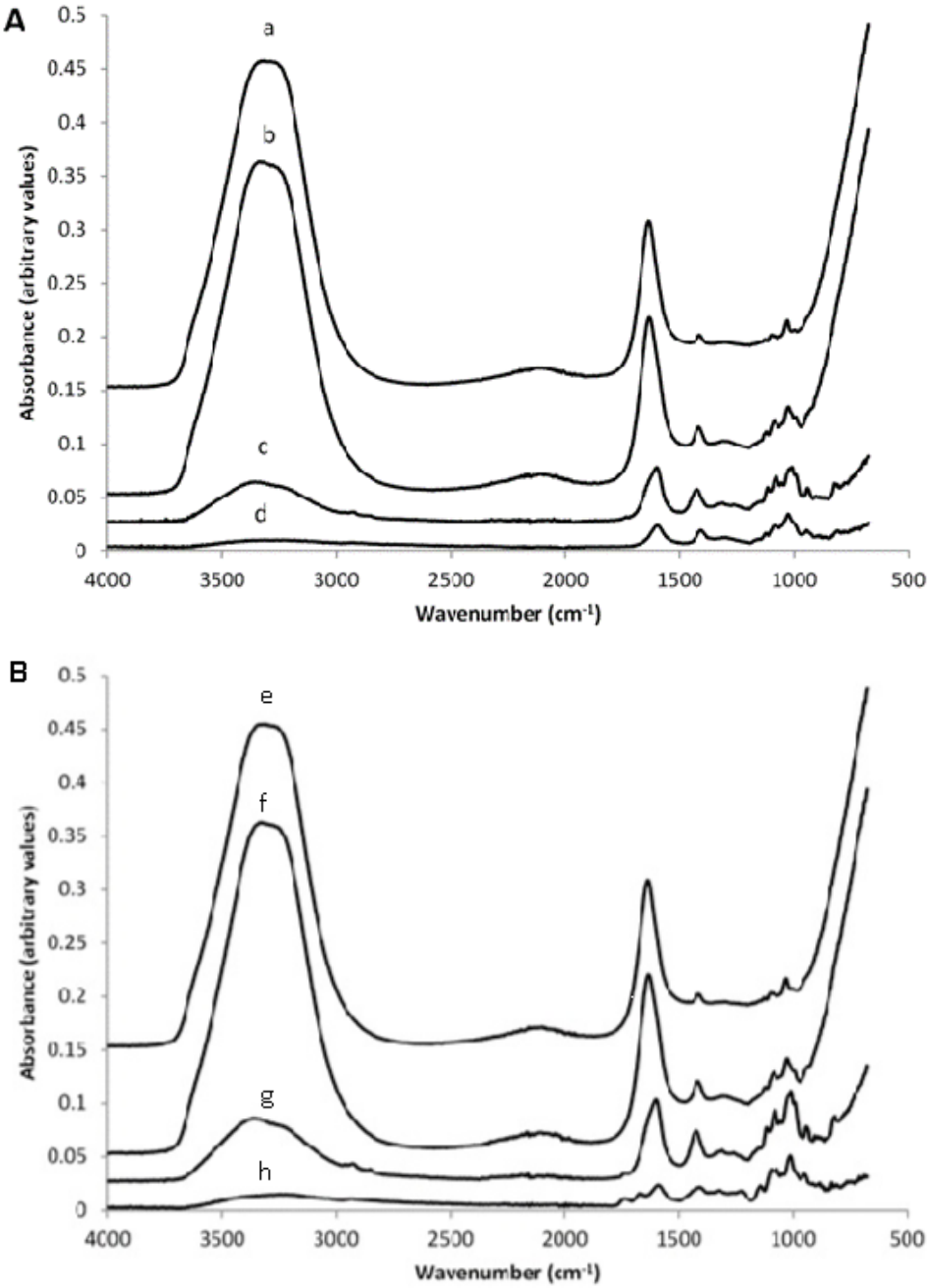

Figure 3. ATR-FTIR spectra in the 4000-675 cm^{-1} range of alginate (A), and alginate-low methoxyl pectin (B). Film forming solutions (a, e); 'wet' films (b, f); 'dry' films (c, g); and alginate (d), and low methoxyl pectin (h) powders.

Table 2. Assignment of the main vibrational peaks for the ATR-FTIR spectra in the 1750-800 cm^{-1} range.

Vibration[a]	Alginate				Alginate-Low Methoxyl Pectin			
	Film Forming Solution	'Wet' Film	'Dry' Film	Alginate Powder	Film Forming Solution	'Wet' Film	'Dry' Film	Pectin Powder
C=O stretching								1743
-								1676
-	1635	1632	1633s		1633	1632	1631s	
Asymmetric COO^- stretching	1597s	1591s	1596	1594	1596s	1594s	1595	1589
Symmetric COO^- stretching	1414	1418	1426	1410	1414	1418	1422	1441s 1408 1326
C-O stretching			1312	1318			1312	
S=O stretching								1224
C-O, C-C & C-O-C stretching	1126 1102 1032	1125 1085 1028	1117 1080 1028	1124 1088 1027	1126 1101 1032	1124 1086 1028	1117 1081 1028	1141 1100 1072 1047
-	999	992	1010 989	995s	1000	993	1010 989	1013
C-O stretching	950	945	941	944	949	944	939	947
C-O of 3,6-anhydrogalactose								886
C-O-SO_3 on C4 of galactose								830
Mannuronic acid residues?			824	811			822	

[a]SARTORI *et al*., 1997; KACURAKOVA *et al*., 2000; PEREIRA *et al*., 2009; PAPAGEORGIOU *et al*., 2010.
s - shoulder peak.

For both treatments, the peak associated with the symmetric stretching vibration of the COO^- shifted to a higher wavenumber (1414-1415 cm^{-1} to 1418-1420 cm^{-1}) when the film forming solution was gelled with calcium. PAŞCALĂU *et al.* (2012) reported a similar shift when 'dry' alginate-kappa-carrageenan films were gelled with calcium. SARTORI *et al.* (1997) also observed an increase in wavenumber of the symmetric stretching vibration of the COO^- when sodium alginate was gelled with calcium. They stated that a peak shift should be expected as the environment around the carboxyl group changes when Na^+ ions are replaced by Ca^{2+} ions, since the two ions have different charge densities, radii and atomic weights. A second shift of the peak associated with the symmetric stretching vibration of COO^- to higher energy (wavenumber) was observed when the gelled 'wet' films were dried. In this case, the peak shifted from 1418-1420 cm^{-1} to 1422-1426 cm^{-1}. Shifts in the peak associated with the asymmetric stretching vibration of the COO^- on the alginate were less defined.

Several other peak shifts occurred when the alginate and composite solutions were gelled with calcium into 'wet' films. These included shifts from 1102-1101 cm^{-1} to 1086-1085 cm^{-1}, 1032 cm^{-1} to 1028 cm^{-1}, and 1000-999 cm^{-1} to 993-992 cm^{-1}. SARTORI *et al*. (1997) also reported shifts towards lower wavenumbers for several peaks in the 1150-1000 cm^{-1} region when sodium alginate was gelled with calcium. They suggested that the shift to lower frequencies was caused by weakening in the C-C and C-O bonds, likely due to these

bonds being shared with calcium ions. They also found a new peak at 1010 cm^{-1} when sodium alginate was gelled with calcium. While this peak was not observed in the 'wet' films in the current work, it was present in the spectra of the dried films. Other peaks that were only observed in the dried film spectrum include the peaks at 1312 cm^{-1} and 824-820 cm^{-1}. It is suspected that these peaks were masked by the large percentage of water (~95%) in the 'wet' films. Drying the 'wet' films also caused shifts towards lower frequencies of several peaks. These included shifts from 1125-1124 cm^{-1} to 1117 cm^{-1}, 1086-1085 cm^{-1} to 1081-1080 cm^{-1}, and 993-992 cm^{-1} to 989 cm^{-1}.
Overall, there were no differences observed between the alginate, and alginate-pectin treatments in either the film or the film forming solutions' spectrum. It was expected that a peak around 1760-1740 cm^{-1} would be observed in the alginate-pectin treatments due to the esterified carboxyl groups of the pectin (ISMAIL *et al.*, 2012). It is thought that because the amount of pectin added to the alginate was low (0.25%), the intensity of these peaks was too low to be detected in the analysis of the composite film and film forming solution spectra.

4. CONCLUSIONS

The results demonstrate that water plays a critical role in determining the mechanical properties of alginate films. Dried alginate films conditioned at 57% RH had different mechanical and optical properties than their corresponding dried alginate films conditioned at 100% RH. While drying gelled 'wet' alginate films appeared to increase their puncture and tensile strength, it also decreased their elasticity. Rehydrated dried films did not have the same properties as the original 'wet' films, suggesting that drying caused irreversible changes in the alginate film structure.
Dried alginate-pectin films did not show significantly different puncture or tensile properties compared to pure Ca-alginate films at either 57% or 100% RH. This was contrary to some of our earlier work on 'wet' films which showed 'wet' alginate-pectin films to have higher tensile stress, % EAB, and puncture work than pure 'wet' Ca-alginate films (HARPER *et al.*, 2015). Therefore, any benefits low methoxyl pectin imparted on 'wet' alginate films, appeared to be lost once the films were dried. On the other hand, adding SPI to both the dried and reconditioned films (57% & 100% RH), as well to 'wet' alginate films, decreased their puncture force and work but did not affect the films' tensile properties. Adding low methoxyl pectin did not cause any detectable differences in the FTIR spectra of the films or film forming solutions in the 1750-800 cm^{-1} region. However, for both treatments, peak shifts were detected when the film forming solution was gelled with Ca^{2+}. These results suggest that inferences on the behaviour of dried alginate composite films cannot necessarily be drawn from the results of corresponding 'wet' alginate composite film, and this is important for further development of such composite films.

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PAPER

EFFECT OF COCONUT TESTA FLOUR ON COOKIE CHARACTERISTICS

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ABSTRACT

A study was conducted to assess the effect of coconut testa flour (CTF) on quality characteristics of cookies by varying the proportion of CTF in wheat flour from 10 to 60% (w/w). Cookies samples prepared according to a standard recipe were evaluated by proximate composition, hardness, amylose content, and shelf-life stability. A 30 members sensory panel was employed to determine the critical limit of CTF fortification for acceptable quality cookies. Results showed that CTF substitution up to 30% was possible without affecting the overall acceptability of cookies. Keeping quality of the cookies remains within the acceptable range throughout three-month storage period.

Keywords: coconut testa flour, cookies, functional properties, storability, wheat flour

1. INTRODUCTION

Proper use of agriculture byproducts is an integral part of the sustainable agriculture development. The trend in the world today is to convert byproducts into useful products through the manipulation of microorganisms or recycle them as much as possible. Coconut testa, for instance, is a byproduct generated on a daily basis by coconut processing industries such as desiccated coconut, virgin coconut oil and milk powder. Previous studies showed that coconut testa constitutes approximately 18% (w/w, wet basis) of the total weight of the whole coconut endosperm (MARIKKAR and MADURAPPERUMA, 2012). By using the statistics provided by PERERA *et al* (2014), it can be estimated that around 30,000 kg of coconut testa could be generated out of 100,000 nuts of Sri Lankan tall variety. As the removed testa is either wasted or under-utilized by the processing factories without any value addition, it is timely to undertake research studies to use them in food applications. This might have various direct and indirect environmental and economic impacts reducing the disposal costs and increasing the added value of the final products, giving emphasis to explore their health benefits. Recently, ADEKOLA *et al* (2017) suggested that testa of coconut can be used to produce a flour to supplement wheat flour to prepare foods such as cookies with low-glycemic index.

According to KLUNKLIN and SAVAGE (2018), cookies are hardly regarded as healthy snack because of their high levels of rapidly digestible carbohydrate, high fat content, low levels of fiber and only modest amounts of protein. Nevertheless, the nutritional value of these product is considered low due to the nutritional composition of refined wheat flour composition. In this background, in recent years, a large variety of vegetable flour products derived from legumes, oilseed, tubers, corn, rice, etc., have been evaluated in cookies formulation in the attempt to produce nutritionally fiber and/or protein enriched products (ZUCCO *et al.*, 2011; CHUNG *et al.*, 2014; PACIULLI *et al.*, 2018; DIAZ *et al.*, 2019).

As of date, supplementation of wheat flour with defatted coconut testa flour in cookie formulation has been scantily investigated. Hence, in this study, the effect of partial substitution of wheat flour by coconut testa flour on the proximate composition, storage stability and consumer acceptance of cookies has been investigated.

2. MATERIALS AND METHODS

2.1. Materials

Wheat flour, coconut testa flour, salted butter, icing sugar, fresh milk, potable water and soybean oil, baking powder, distilled water were used for cookie dough preparation. Samples of commercial wheat flour were obtained from Serendib Flour Mills (Pvt) Ltd and samples of partially-defatted coconut testa flour (CTF) of Sri Lankan commercial variety were supplied as a generous gift by the coconut research institute, Lunuwila, Sri Lanka. Other ingredients namely, table salt, fresh milk, soybean oil, and baking powder were obtained from Puttlam Salt Ltd, Ambewela Milk Ltd, Lanka Soy (Pvt) Ltd and Cargills Food City (Pvt) Ltd, respectively.

2.2. Chemicals

Agar culture media, potato dextrose agar were obtained from Himedia (Pennsylvania, USA); petroleum ether, concentrated sulfuric acid, potato starch, sodium hydroxide , hydrochloric acid, boric acid were purchased from Merck (Darmstadt, Germany); di-methyl sulfoxide, 3,5-dinitrosalicylic acid, sodium potassium tatrate, bromocresol, methyl red, ethanol, methanol, iodine reagent, deionized water, sodium chloride, monosodium phosphate and disodium phosphate were obtained from Fluka (Buchs, Switzerland).

2.3. Functional properties of flours

2.3.1 Water and oil absorption capacity

The water and oil absorption capacities were determined by the method of SOSULSKI *et al.* (1976). Flour samples (one gram, in triplicate) were mixed with 10 ml distilled water and 10 ml of soybean oil separately in 2 centrifuge tubes. Samples were kept at room temperature for 30 min and centrifuged with Eppendorf 8510R centrifuge (Eppendorf, Germany) at 3000 rpm for 30min. The volume of free water and free oil were read directly from the centrifuge tube. The water absorption and oil absorption were examined as percent water-bound per gram of flour and percent oil-bound per gram of flour, respectively.

2.3.2 Bulk density

Bulk density of flour sample was examined using the method described by JONES *et al.* (2000) with slight modifications. Flour samples (ten grams, in triplicate) were measured in a 50 mL measuring cylinder. The cylinder was tapped on a wooden plank until no visible decrease in volume was noticed and volume of flour sample was recorded. The bulk density was calculated using the following formula:
Bulk Density (g/cc) = Weight of sample / Volume of sample

2.3.3 Swelling capacity

The method of OKAKA and POTTER (1977) with some modifications was used for determining the swelling capacity. Flour samples (one gram, in triplicate) were filled up to 10 ml mark in a 100 ml graduated cylinder was added with water to adjust total volume to 50 ml. The top of the cylinder was tightly covered and mixed by inverting the cylinder. The suspension was inverted again after 2 min and allowed to stand for further 30 min. The volume occupied by the sample was taken after 30 min.

2.3.4 Emulsion activity

The emulsion activity and stability were followed using the method of YASUMATSU *et al.* (1972). Emulsion was prepared in calibrated centrifuged tube by adding flour samples (one gram, in triplicate) into 10 ml distilled water and 10ml of soybean oil. The emulsion was centrifuged at 3000 rpm for 5 min. The emulsion activity was estimated using following equation:

$$\text{Emulsion activity } (\%) = (\text{Height of emulsion layer} / \text{ Total height of mixture}) \times 100$$

2.3.5 Least gelation concentration

Least gelation concentration was evaluated using the method of COFFMAN and GARCIA (1977) with slight modification. Flour samples [2 to 30% (w/v), in triplicate] were mixed with 5 ml distilled water and heated at 90°C for 1 h in a hot-water bath. After allowing it to cool under tap water, it was kept at 4 °C for 2 hrs. The least gelation concentration was determined at the concentration when the sample from inverted tube did not slip.

2.4. Composite flour preparation

Composite flour samples were prepared according to the following mixing ratio: T_0 100% WF; T_1 WF supplemented with 10% CTF; T_2 WF supplemented with 20% CTF; T_3 WF supplemented with 30% CTF; T_4 WF supplemented with 40% CTF; T_5 WF supplemented with 50% CTF; T_6 WF supplemented with 60% CTF.

2.5. Cookies preparation

Formulation of cookies was carried out according to a method described in AACC with slight modification (SCIARINI *et al.*, 2013). Cookies were baked in three batches, each with 60 cookies. Initially, salted butter (30g) was creamed with icing sugar (40 g), and baking powder (2 g) for 3 min at low speed in a mixer (the bowl will be scraped every minute). Water was added thereafter in varying proportions to make the required consistency of dough. Mixing was continued using Kenwood mixer (Model KM200, Korea) for 2 min at a high speed. Finally, 100g of flour was added and mixed for 2 min at low speed while scraping the bowl at every 30 sec. The dough was allowed to rest for 10 min before rolling-on and cutting (62 mm diameter, 5.5 mm thick). The cut dough pieces were then baked for 25 min in an oven (BIOBASE forced air drying oven, China) pre-heated at 150 °C. The cooled cookies were then left at 25 °C for 30 min and packed in sealed plastic bags until further analysis.

2.6. Sensory evaluation

This is a ranking test, which is aimed to rank cookie samples according to the preference of a thirty-member semi-trained panelists. Panelists were asked to rank seven coded samples according to the intensity of sensory attributes namely color, smell, taste, texture and overall acceptability of biscuits. A seven-point Hedonic scale (1: dislike very much; 2: dislike; 3: dislike slightly; 4: neither like nor dislike; 5: like slightly; 6: like; 7: like very much) was used to evaluate degree of liking for each sensory attribute.

2.7. Proximate compositional analysis

For sampling under each treatment category, 7 cookies were selected randomly from each of the three batches. Moisture, crude fat, crude protein and ash contents of coconut testa flour were determined according to methods described in AOAC (2005) manual. The carbohydrate content of the flour was calculated by difference [100- (crude protein + crude fat + ash + moisture+ crude fiber)].

2.8. Determination of amylose content

Amylose content was determined according to the iodimetric method described by JULIANO *et al.* (1968) with slight modification. Iodine reagent was prepared dissolving 1.0 g of iodine and 10 g potassium iodide in water and the volume was made up to 500 ml. A series of standard amylose solutions was prepared dissolving appropriate amounts of amylose in 10 ml of 1.0 N solution of sodium hydroxide and made up to100 ml with water. Powdered cookie sample of 100 mg each from T_0, T_1, T_2, and T_3 was weighed accurately, and dissolved in 1.0 mL 95% ethanol and 10 mL of 1.0 N sodium hydroxide and heated for 10 min in boiling water bath. The solution was made up to 100 ml in a volumetric flask. An aliquot (2.5 ml) of this solution was then added with 20 ml distilled water and three drops of phenolphthalein. Subsequently, 0.1 N hydrochloric was added dropwise until the pink color just disappear. An aliquot (1 ml) of iodine reagent was added into this and the volume was made up to 50 ml. The resulting color was left for 20 min to fully develop before the absorbance reading was monitored at 620 nm with a Perkin-Elmer Lambda 3B double beam UV/Visible spectrophotometer. Iodine solution of same concentration as above but without amylose was used as reference cell. Concentration of amylose in individual cookie sample was calculated using the standard curve.

2.9. Hardness test

For sampling under each treatment category, three cookies were selected randomly from each of the three batches. Hardness of all samples was carried out using Brookfield CT3 texture analyzer using the following test criteria was applied in this test. Test Type: TPA; Target: 4.0mm; Hold Time: 0s; Trigger Load: 15.0g; Test Speed: 0.50mm/s; Return Speed: 0.5mm/s. The value for hardness was derived from the automatic calculation from texture profile analysis curve.

2.10. Shelf life studies

For storability test, biscuit samples containing 5 cookies were packed under hygienic condition in clean packages and sealed immediately to protect product quality. Low-density polyethylene (LDPE) bags were used for packaging. During shelf life study, sample bags were kept at room temperature (27°C and RH 60%) until all analyses were performed. The shelf life of cookies was evaluated based on moisture content and the microbial count (total plate count and yeast and mould count) at once in every two weeks' time continuously for three months.

2.11. Microbiological analysis

Enumeration of aerobic colony count was done by incubating microorganisms in nutrient agar (NA) medium under 37°C for 48 hours; the yeast and mould count was done by incubating in potato dextrose agar (PDA) medium with 0.01% Chloramphenicol held at room temperature (SLS 516:1991).

2.12. Statistical analysis

Samples were selected randomly from three batches. All measurements were carried out in triplicate data (n=3). All the results were presented in the form of mean ± standard

deviation (SD). Data were statistically analyzed by one-way analysis of variance (ANOVA) with using Tukey's Test of MINITAB (version 15) statistical package at 0.05 probability level. Results of sensory evaluation results were analyzed using non-parametric Friedman rank sum test using the Minitab software package version 14.00 (SAS 1998).

3. RESULTS AND DISCUSSION

3.1. Functional properties of flours

The functional properties of cereal and non-cereal flours are known to influence the quality attributes of finished bakery products. A comparative analysis of the physicochemical and functional properties of wheat and coconut testa flours would therefore be beneficial. According to the data presented in Table 1, both of these displayed significant ($p<0.05$) differences in all of the properties studied. The swelling capacity, which is related to the irregular positioning of polymer in granules and small size of starch granules (WONG and LELIEVRE, 1982) was higher for CTF (30±0.01) than WF (20±0.01). SURESH and SAMSHER (2013) stated that varietal differences, particle size differences, and methods of processing are other factors that may influence the swelling capacity of flour. In an earlier report, CHANDRA *et al.* (2015) noticed that the swelling capacity of composite flours increased with increasing level of incorporation of rice, green gram and potato flour into wheat flour. From the results of this study, it can be predicted that a composite flour made by mixing WF with CTF would gain a higher swelling capacity with reference to the control.

According to Table 1, the bulk density of CTF (0.66±0.01) was slightly higher than that of WF (0.49±0.01). The bulk density (g/cm^3) of flour is defined as the density measured without the influence of any compression (CHANDRA *et al.*, 2015). According to LAM *et al.* (2008), bulk density of flour samples depends on particle size, density of individual particle, moisture as well as surface characteristics, which are generally influenced by the method of preparation. Undoubtedly, the preparation methods of CTF and WF were not similar as one which was cereal based while the other was non-cereal-based. According to SURESH and SAMSHER (2013), the high bulk density of flour suggests its suitability for multiple uses in food preparations. Water absorption capacity of WF and CTF were 65±0.0% and 75±0.0%, respectively. BERTON *et al.* (2002) stated that water absorption capacity is associated with protein and starch contents of flour. However, the particle size reduction and high content of fiber would increase the water absorption capacity of flour (SURESH and SAMSHER, 2013; CHANDRASHEKER and DESIKACHAR, 1984). Our preliminary investigation too confirmed that CTF was a rich source of protein and crude fiber, which were relatively higher than those of wheat flour (Table 1).

As the capacity of protein to enhance the formation and stabilization of emulsions is important for many applications in food products, information on emulsion activity of flour is beneficial (CAUVAIN and YOUNG, 2006). In the present study, emulsion activity of CTF was lower (25±0.00) than WF (50±0.00). According to previous researchers, the emulsion activity of flour would increase with higher amounts of soluble proteins (GABRA and KAUR, 2014; YASUMATSU *et al.*, 1972) and reduce with fiber content (YASUMATSU *et al.*, 1972). The higher crude fiber content as noticed before in CTF could be a probable reason for lower emulsion activity displayed by CTF. When considering least gelation concentration of the two flour samples, WF showed lower amount (8%,

w/v) while CTF displayed a higher value (18%, w/v). This means that WF would form gel quickly at lower concentration than CTF. In an earlier report, AKINTAYO *et al*. (1999) stated that lowest gelation concentration increased with the gelation of protein.

Table 1. Comparative functional and nutritional properties of coconut testa flour (CTF) and wheat flour (WF).

Functional/Nutritional properties	Flour	
	CTF	**WF**
Swelling capacity (ml)	30^{b}±0.01	20^{a}±0.01
Water absorption capacity (%)	75^{b}±0.00	65^{a}±0.00
Oil absorption capacity (%)	52.7^{a}±2.52	58.50^{b}±1.32
Emulsion activity (%)	25^{a}±0.00	50^{b}±0.00
Least gelation concentration(%,w/v)	18^{b}±0.00	8^{a}±0.00
Bulk density(g/cc)	0.66^{b}±0.01	0.49^{a}±0.01
Moisture (%)	2.27±0.42*	14.0±0.03†
Ash (%)	4.50±0.14*	1.82±0.29 †
Protein (%)	23.82±0.99*	11.68±0.11 †
Fat (%)	10.17±1.84*	1.91±0.09 †
Total carbohydrate (by difference)	59.24*	70.59 †

Each data in the table represents mean of triplicate analysis. Means in the same raw bearing different superscripts are significantly different ($p<0.05$) from each other. *Marasinghe *et al.* (2019) and †Majzoobi *et al.* (2011).

The oil absorption capacity is an indicator of the suitability of the flour in facilitating enhancement of flavor and mouth feel in food preparations. The oil absorption capacity was higher for WF (58.50±1.32) than CTF (52.7±2.52). According to CHANDRA *et al.* (2015), the presence of high fat content in flours might affect adversely their oil absorption capacity. In this study, CTF having a lower oil absorption capacity was reasonable because our earlier investigation showed that the fat content of CTF was relatively higher (10.17 %) than those of WF (Table 1) (MAIZOOBI *et al*., 2011). SURESH and SAMSHER (2013) commented that amino acid composition, protein conformation, surface polarity and hydrophobicity are other factors that may exert influence on the oil-binding capacity of flour.

3.2. Sensory attribute evaluation

Flour is the main ingredient in any biscuit formulations. There have been a number of attempts to improve the nutritional qualities of biscuits by partially substituting WF with non-wheat flour ingredients (KLUNKLIN and SAVAGE, 2018). Replacing WF with other types of flour would most likely affect the nutritional values as well as the organoleptic characteristics of biscuits (HOODA and JOOD, 2005). In order to determine the critical limit of fortification of CTF in biscuit formulation, a proper method of sensory evaluation

and statistics are necessary. According to Table 2, the preference of the panelists for color attribute sharply dropped after substitution of 10% CTF due to unappealing off-white grainy appearance; but the score values for T_2 and above were tended to increase due to brown color development caused by higher amounts of CTF. Apart from Maillard reaction, the mild brown color of CTF could also be a contributory factor for brown color development in biscuits. This has caused the score values of colour to go up with further substitution. There was, however, no significant difference between the control and the different levels of CTF substitution with respect to smell characteristics (Table 2). Other attributes such as taste, texture, and overall acceptability were more sensitive to the changing content of CTF in the formulation. With regard to these three sensory attributes, there was no significant difference between the control and the CTF substituted biscuit samples up to the level of 30% substitution (Table 2). According to the panelists, a slight bitter taste and undesirable mouth-feel were noticed in biscuit samples exceeding 30% CTF level. Therefore, CTF substitution up to 30% would be maximum permissible limit for good quality cookies. Hence, the proximate compositional analyses and storage studies were performed only for biscuit samples made out of composite flour containing 0, 10, 20 and 30% CTF supplementation.

Table 2. Results of Friedman test along with sum of ranks of sensory attributes of different treatments.

Treatments	Color	Smell	Taste	Texture	Overall acceptability
T_0	158.0^{e}	128.0^{a}	152.0^{c}	161.0^{c}	166.5^{c}
T_1	86.5^{a}	126.5^{a}	153.5^{c}	156.5^{c}	151.0^{c}
T_2	103.5^{b}	107.5^{a}	136.5^{c}	150.0^{c}	148.0^{c}
T_3	110.0^{b}	112.0^{a}	141.5^{c}	139.5^{c}	132.5^{bc}
T_4	123.0^{c}	116.5^{a}	101.0^{b}	105.5^{b}	111.5^{b}
T_5	126.5^{c}	116.5^{a}	78.5^{a}	67.5^{a}	77.0^{a}
T_6	132.0^{d}	133.0^{a}	77.0^{a}	60.0^{a}	53.5^{a}
CL	***	ns	***	***	***

Means in the same column bearing different superscripts are significantly different from each other. Abbreviations: CL, confidence level; ns, not significant; ***$p<0.0001$. T_0, biscuit formulation by wheat flour; T_1, biscuit formulation by wheat flour mixed with 10% coconut testa flour; T_2, biscuit formulation by wheat flour mixed with 20% coconut testa flour; T_3, biscuit formulation by wheat mixed with 30% coconut testa flour; T_4, biscuit formulation by wheat flour mixed with 40% coconut testa flour; T_5, biscuit formulation by wheat flour mixed with 50% coconut testa flour; T_6, biscuit formulation by wheat flour mixed with 60% coconut testa flour.

3.3. Proximate composition of biscuits

Proximate compositions of cookies samples displaying good overall acceptability were compared with those of control sample as shown in Table 3. Generally, cookies available in the market are generally prepared from wheat flour, which lacks dietary fibre contents and good quality protein due to deficiency in lysine. Our preliminary investigation confirmed that composite flour preparation using CTF would be beneficial because CTF

was found to be rich in protein (Table 1) as well as crude fiber (Table 3). In a previous study, APPAIAH *et al.* (2014) also indicated that testa of copra was a rich source of protein and crude fiber; it contained about 9.3% of protein and 11.6 % of crude fiber. When compared with the control, cookies prepared with composite flour showed significant increase ($p<0.001$) on the moisture, protein and crude fiber contents, but with regard to ash and fat contents of cookies, there was no significant ($p>0.05$) difference among all substitution levels (Table 3). This indicated that substitution of WF with CTF did not have any direct impact on both ash and fat contents of the finished products. As previously noticed in Table 1, water absorption capacity of CTF was higher than WF due to high fiber content of CTF. This necessitated an extra amount of water to be added to maintain better consistency of the dough at higher level of substitution of CTF. With regard to total carbohydrate content of cookies, there was a significant ($p<0.001$) decline along with the rising substitution level of CTF.

Table 3. Proximate composition of cookie samples made out of different treatments (g/ 100 g dry matter basis).

Treatment	Moisture	Ash	Protein	Fat	Crude Fiber	Other Carbohydrates (by difference)
T_0	0.79^{a}±0.04	1.11^{a}±0.15	5.59^{a}±0.50	14.28^{a}±0.41	7.90^{a}±0.14	70.32^{d}± 0.12
T_1	0.95^{b}±0.04	1.17^{a}±0.23	7.43^{b}±0.15	14.59^{a}±0.23	8.95^{b}±0.07	66.91^{c}±0.58
T_2	1.24^{c}±0.02	1.33^{a}±0.00	9.63^{c}±0.18	14.99^{a}±0.47	11.25^{c}±0.5	61.56^{b}±0.75
T_3	1.48^{d}±0.02	1.50^{a}±0.23	11.79^{d}±0.60	15.54^{a}±0.16	15.85^{d}±0.1	53.84^{a}±0.33
CL	***	ns	***	ns	***	***

Each data in the table represents mean of triplicate analysis. Means in the same column bearing different letters are significantly different from each other. Abbreviations: ns, not significant; ***($p<0.0001$). For other abbreviations, see Table 2.

3.4. Amylose content and hardness

The data presented in Table 4 compares the amylose content of CTF substituted cookie samples with that of the control sample. Amylose being a linear polymer contribute important characteristics to starch present in cereals, and hence it can affect the physicochemical properties of the finished products (MAJZOOBI *et al*., 2011). The control showed the highest amylose content (27.4 %), which fell within the range of amylose content of wheat flour of different cultivars (20 to 30 %) reported in other studies (MAJZOOBI *et al*., 2011). The amylose contents of T_1, T_2, and T_3 were tended to decline with the decrease of wheat flour component in the composite. In terms of texture, cookies made out of 100% wheat flour was seemed to be the hardest (14.86 N) and the hardness of T_1, T_2, and T_3 were found to decline, with the cookie sample T_3 which represented CTF substituted cookies at 30% level displaying the lowest value of 8.27 N. Generally, the hardness of cookie is strongly influenced by the development of gluten network whereby gluten promotes the network development by attracting water molecules from the mixture. BARAK *et al.* (2013) previously pointed out that WF having a higher gluten-

content would influence the hardness of cookies. The composite flour made out of CTF substitution would contain lesser amount of gluten, which leads to the decline in the harness of the cookies.

Table 4. Variation of amylose content and harness of cookies[1].

Treatment	Amylose content (%)	Hardness (N)
T_0	27.40^{c}±0.79	14.86^{d}±0.01
T_1	22.77^{b}±0.50	13.42^{c}±0.02
T_2	16.50^{a}±0.75	11.63^{b}±0.01
T_3	15.10^{a}±0.26	8.27^{a}±0.01

[1]Each data in the table represents mean of triplicate analysis. Means in the same column bearing different superscripts are significantly different ($p<0.05$) from each other. For abbreviations, see Table 2.

3.5. Shelf-life stability

Moisture and microbiological stability are important attributes to test the shelf-life stability of products. When considering keeping quality of food materials, the initial moisture content, storage temperature, microbiological quality and packaging materials are important determining factors. In this study, keeping quality of cookies samples are tested in terms of moisture content and microbial growth along the storage period of three months. Generally, moisture absorption would not only influence microbiological stability but also textural properties such as hardness. CAUVAIN and YOUNG (2006) stated that moisture content of freshly produced cookies should not exceed the limit of 5% while other standards specified that the moisture content of cookies should be less than 6% during storage period (SLS 251:1991; BIS 1974). According to data presented in Table 5, moisture content of samples significantly increased with storage period but none of them exceeded the upper limit of moisture content throughout the three months of storage period.

Table 5. Changes in the moisture content of cookie samples with time.

Treatment	Time (in weeks)						
	W0	W2	W4	W6	W8	W10	W12
T_0	0.79^{a}±0.04	0.88^{a}±0.04	1.04^{b}±0.06	1.07^{b}±0.05	1.88^{c}±0.05	2.17^{d}±0.04	2.98^{e}±0.09
T_1	0.94^{a}±0.04	1.22^{b}±0.03	1.35^{c}±0.04	1.49^{c}±0.01	2.07^{d}±0.1	2.59^{e}±0.04	3.20^{f}±0.03
T_2	1.25^{a}±0.03	1.40^{b}±0.02	1.57^{b}±0.01	1.87^{c}±0.05	2.43^{d}±0.08	3.11^{e}±0.09	3.41^{f}±0.07
T_3	1.47^{a}±0.03	1.65^{b}±0.02	1.60^{b}±0.03	2.22^{c}±0.16	2.74^{d}±0.06	3.34^{e}±0.08	3.97^{f}±0.05

Each value in the table represents the mean of triplicate analyses. Means in the same row bearing different superscripts are significantly ($p<0.05$) different from each other. Abbreviations: W0, week0; W2, week2; W4, week4; W6, week6; W8, week8; W10, week10; W12, week12. For abbreviations, see Table 2.

The observed gain in moisture content might be due to high water permeability of LDPE as well as the high-humidity conditions of the storage. Previously, SIRIPATRAWAN (2009) stated that there was a moisture migration between the atmosphere and the packaged low-moisture food product such as rice crackers through packaging material during storage until the food reaches equilibrium with the environment. He noticed that the moisture barrier performance of packaging materials was dependent on permeability coefficient; rice crackers packaged in polypropylene pouches had higher shelf-life values than those in polyethylene pouches because polypropylene had lower permeability coefficient.

In this study, the changes in total plate count and yeast and mold count through the three-month storage period were compared with initial amount as shown in Table 6. According to WHO standard (WHO, 1994), total plate count and yeast and mould count of biscuit samples should not exceed 2.0×10^5 cfu g-1 and 1.0×10^4 cfu g-1, respectively. Although both microbial counts of all samples were found to increase with storage period, none of them exceeded the upper limit along the three-month storage period. This clearly showed that microbiological quality of cookies remains within the safe limit throughout the three-month storage period.

Table 6. Changes in total plate count, yeast and mold count of cookies samples with time.

Treatment	Time (in weeks)			
	W0	W4	W8	W12
	Total plate count (CFU/g)			
T_0	$(1.30^{a}\pm0.07)\times10^3$	$(1.42^{a}\pm0.09)\times10^3$	$(2.08^{b}\pm0.03)\times10^3$	$(2.53^{c}\pm0.05)\times10^3$
T_1	$(1.47^{a}\pm0.04)\times10^3$	$(1.57^{a}\pm0.05)\times10^3$	$(2.18^{b}\pm0.03)\times10^3$	$(2.97^{c}\pm0.06)\times10^3$
T_2	$(1.57^{a}\pm0.05)\times10^3$	$(1.70^{a}\pm0.02)\times10^3$	$(2.46^{b}\pm0.04)\times10^3$	$(4.46^{c}\pm0.15)\times10^3$
T_3	$(1.67^{a}\pm0.05)\times10^3$	$(2.05^{b}\pm0.04)\times10^3$	$(2.97^{c}\pm0.04)\times10^3$	$(5.53^{d}\pm0.15)\times10^3$
	Yeast and mould count (CFU/g)			
T_0	$(2.23^{a}\pm0.2)\times10^2$	$(2.67^{ab}\pm0.15)\times10^2$	$(3.13^{b}\pm0.12)\times10^2$	$(6.27^{c}\pm0.25)\times10^2$
T_1	$(2.30^{a}\pm0.26)\times10^2$	$(3.03^{b}\pm0.15)\times10^2$	$(3.50^{b}\pm0.10)\times10^2$	$(7.50^{c}\pm0.30)\times10^2$
T_2	$(2.70^{a}\pm0.10)\times10^2$	$(3.40^{b}\pm0.10)\times10^2$	$(4.30^{c}\pm0.41)\times10^2$	$(8.30^{d}\pm0.25)\times10^2$
T_3	$(3.03^{a}\pm0.15)\times10^2$	$(4.03^{b}\pm0.20)\times10^2$	$(5.23^{c}\pm0.20)\times10^2$	$(9.10^{d}\pm0.10)\times10^2$

Each value in the table represents the mean of triplicate analyses. Means in the same row bearing different superscripts are significantly ($p<0.05$) different from each other. Abbreviations: W0, week0; W4, week4; W8, week8; W12, week12; For other abbreviations see Table 2.

4. CONCLUSIONS

This study attempted to evaluate the suitability of CTF as a partial substitute for WF in cookies formulation. Sensory evaluation showed that CTF substitution up to 30% was possible without affecting the overall acceptability of cookies. According to proximate analysis, cookies partially substituted with CTF could have enhanced levels of protein and crude fiber contents. The effect of substation on hardness of cookies was positively

correlated with amylose content of composite flour; this could also be due to lowering of gluten network formation, which usually brings hardness to cookies. Shelf-life studies showed that moisture content and microbiological quality parameters of cookies would remain within the safe limit throughout the three-month storage period. The findings of this study could eventually help enhance the economic value of coconut testa generated as byproduct by the coconut processing industries in Sri Lanka.

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PAPER

CONSUMERS' WILLINGNESS TO CONSUME SUSTAINABLE AND LOCAL WINE IN ITALY

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ABSTRACT

Wine production is a relevant sector of the Italian economy and recently consumer demand has drastically changed due to their orientation towards new attributes of quality wines. Sustainability aspects are credence attributes that have an effect on consumers' perceived quality. Moreover, local production of wine is seen as a sign of quality. However, much is still to be studied about drivers that can push consumers towards new quality attributes of wine such as sustainability and local production. Profiling consumers could be a first step to better understand consumers' decisions on local and sustainable wine. Thus, the paper has the purpose of understanding the Italian consumers' willingness to consume sustainable and local wine. Data has been collected from a sample of Italian consumers (N=1,099) using a web-based survey. A binary logistic regression model, selected on the basis of the model AIC was used. The findings showed the large majority of the interviewed are willing to consume local and sustainable wine.

Keywords: Italian consumer behavior, local wine, logistic regression, sustainable wine, willingness to consume

1. INTRODUCTION

Consumer demand toward food products has drastically changed in recent years in most developed countries. Consumers take more interest in aspects like quality, origin and organic production and apparently less interest in 'strictly' environmental concerns that are more "altruistic" (with benefits for the society rather than the individual) (DEFRANCESCO *et al*., 2017; LÄHTEENMÄKI, 2013; PERITO *et al*., 2019). In particular, consumers seem more interested in environmental aspects associated with organic production, that have more direct benefits on health than other environmental issues (for example, the use in the production process of less water, fuel, workers' rights, etc.,) (DEFRANCESCO *et al*., 2017; LÄHTEENMÄKI, 2013).

Though in recent decades sustainable food consumption has been extensively studied (VERAIN *et al*., 2012), it is no easy matter to assess the consumer preferences for environmental concerns, combined with other aspects (e.g., brand, origin, price, frequency of consumption, etc.). VAN DAM and VAN TRIJP (2011) pointed out sustainability as an abstract verbal construct with no objective meaning. According to SZOLNOKI (2013), it is still very difficult to define the term sustainability because not only each country, but also each entrepreneur and every individual has a different understanding of sustainability, especially in the wine sector. However, even if it is not feasible to have a unanimously accepted definition of sustainability, a general consensus is focused on a consumption pattern that covers a wide variety of aspects (e.g. environment, health and safety of the food products, rural and local development, …) (VERAIN *et al*., 2012).

Taking in to consideration the wine sector, consumer perception of sustainable wines seems generally associated to the terms such as organic and local (ZUCCA *et al*., 2009; CBI, 2016). The sustainable wine is traditionally considered as superior quality wine, due to the production without pesticides and fertilizers (considered harmful to health) in the vineyard (BERGHOEF and DODDS, 2013) and consumers are not willing to trade off this specific quality of a wine for only environmental/social features (LOCKSHIN and CORSI, 2012). Moreover, consumers pay attention to different issues, such as price, origin, and production process (GALLENTI *et al*., 2019). They seem interested in environmentally friendly matters, but only with organic labels (SOGARI *et al*., 2013; GRANKVIST and BIEL, 2001; MAGNUSSON *et al*., 2003; DEFRANCESCO *et al*., 2017) and geographical indication labels (BONCINELLI *et al*., 2019; SÁENZ-NAVAJAS *et al*., 2013).

As a matter of fact, sustainability aspects are credence attributes because consumers cannot determine by themselves if a wine is produced following sustainable practices (FERNQVIST and EKELUND, 2014; GINON *et al*., 2014; SCHAUFELE and HAMM, 2017) and the expectations that credence attributes have an effect on consumers' perceived quality (FERNQVIST and EKELUND, 2014; SCHAUFELE and HAMM, 2017). Wine is one of the most differentiated products on the market (SCHAUFELE and HAMM, 2017) and the market relies strongly on how consumers perceive wine quality (LOUREIRO, 2003). Sustainability in the wine sector could play an important role in the wine business and it could be a way to both differentiate wines, to deal with food safety constraints and to guarantee the future development of the sector (SELLERS-RUBIO and NICOLAU-GONZALBEZ, 2016; NAIT MOHAND *et al*., 2017). Moreover, eco-labels may offer new attributes for consumers' choices (DELMAS and LESSEM, 2017; KLOHR *et al*., 2013; SCHAUFELE and HAMM, 2017).

As empirical researches pointed out, consumers see vintages, wine producers and the region of origin as signs of quality (DE FRANCESCO *et al*., 2012; GALLENTI *et al*., 2019; JAEGER *et al*., 2013; YANG and PALADINO, 2015; SCHAUFELE and HAMM, 2017). In

particular, origin plays an important role in the European wine sector (LOUREIRO, 2003; VEALE and QUESTER, 2008). According to PERROUTY *et al*. (2006), geographical indication label gives added value to the product and affects both perception of wine quality and its process of purchase (PUCCI *et al*., 2016).
Italy is the first wine producer (European leader of wines certified with geographical indication or organic label[1]) and the second largest consumer country worldwide after France (NOMISMA, 2017). Nevertheless, the Italian wine market has been affected by a deep transformation due to new competitors on the world market (Argentina, Australia, Chile, New Zealand, the United States and South Africa), a constant decrease in the quantities consumed, a lower willingness to pay due to the general economic crisis and a demand for new quality attributes (mainly sustainable and local products) by wine consumers (CRESCIMANNO and GALATI, 2014).
In this framework, despite researches on wine appears to be copious in current literature (see e.g., DAL VECCHIO *et al*., 2018; D'AMICO *et al*., 2016; DI VITA *et al*., 2014; FABBRIZZI *et al*., 2017; MARINELLI *et al*., 2014; MARONE *et al*., 2017; SELLERS-RUBIO and NICOLAU-GONZALBEZ, 2016), to our knowledge, much is still to be studied about on drivers that can push consumers towards both local and sustainable attributes.
Specifically, this paper tries to fill a gap in the literature, with the purpose of understanding the consumers' willingness to consume sustainable and local wine. Specifically, we sought to answer three research questions: What interest do Italian consumers have in sustainable wines? What interest do Italian consumers have in local wines? Which variables are important predictors of consumers' willingness to buy these wines?
To this purpose, the paper is articulated in three sections: sampling procedure and methodological approach, results, discussion and final remarks.

2. MATERIALS AND METHODS

2.1. The questionnaire

In order to explore the drivers of the consumers' inclination to consume sustainable and local wine, a structured survey was developed. Consumers were recruited through invitations to participate in the online survey (performed by Google drive) via social networks (Twitter, Facebook and WhatsApp), University students' networks and email (see e.g. SOGARI *et al*., 2015). Snowball sampling recruitment was also adopted, using interpersonal relations and connections among University students to reach a large number of participants. Data were collected using an online survey between January and July 2015.
Following general studies on food science (e.g. VERBEKE, 2015), in the questionnaire the meaning of sustainable and organic wine used in our survey was explained to respondents.
The online questionnaire[2] consisted of two separate sections.
The first section collected basic socio-demographic information: Gender, Age, Area and Region of residence and education level of respondents.
The second section gathered information on consumption frequencies of wine, type of wine preferred and consumption frequencies of wine out of home. To understand the feelings regarding sustainable wine, according to the literature (D'AMICO *et al*., 2016; SOGARI *et al*., 2016; LOCKSHIN and CORSI, 2012; FORBES *et al*., 2009) the respondents

were asked if an organic label is a guarantee of sustainability of higher quality product, naturalness and food safety with respects the environment. Moreover, it has been asked the willingness to consume sustainable and local wine and the attention paid to wine cellar brand. In addition, to understand the respondent's perceptions concerning local origin of wine, we asked if they thought local origin of wine is a guarantee of territoriality and origin of raw materials (PERITO *et al*., 2019). The last questions collected information on consumer interest towards producer name and nutrition label. It is important to underline that following POMARICI and VECCHIO (2014), most questions of the survey were based on the formula yes/no.

2.2. The sample and data analysis

We surveyed a sample of 1,099 wine consumers in Italy. The questionnaire was validated in a pilot study on a sample of 100 consumers. This pre-test was developed exclusively to discover any possible misinterpretation, error or duplication in the questionnaire.
To explore the drivers of people's inclination to consume sustainable and local wine, and following the current literature (e.g. POMARICI and VECCHIO, 2014; SELLERS, 2016; VERBEKE, 2015) a binary logistic regression model was used. The binary dependent variable yi takes the values "Yes" and "No" and the probability of success $P(Y= Yes|x)$ represents the probability that an individual is willing to consume sustainable and local wine conditioned by variables of the questionnaire. In a first analysis, all potential explanatory variables were included in the logistic model. Many estimated coefficients are associated to non-significant p-values and the relative variables are excluded from the final model. The final model parameterization is selected on the basis of the model AIC, using a mixed "backward" and "forward" stepwise selection strategy.
In formula, the final model is:

$$P(Y=Yes|x) = \beta_0 + \beta_1 Wine + \beta_2 Wine\_out + \beta_3 Lab\_Org + \beta_4 Wine\_company\_name + \beta_5 Local + \beta_6 Market\_name + \beta_9 Nutr \quad (1)$$

Among the parameters of the model, we looked at the β values and at the significance of each factor. The factors were then interpreted according to the odds' ratio, which shows the probability increase/decrease of the wiliness to consume sustainable and local wine when the considered variable increases or decreases.
All computations are carried out using R version 3.5.1 (R DEVELOPMENT CORE TEAM, 2018).

3. RESULTS AND DISCUSSION

3.1. Descriptive results

The sample consists of 564 females and 535 males and 74.4% of people belong to age range 18-45 while, 46.2% of respondents are young (18-35 years) (Table 1). It is important to highlight that the sample analysed in this study is not representative of the whole national population.
About 85% of the respondents come from urban area and 50.41% has a high level of education (i.e. university college or postgraduate degrees).

Table 1. Socio-demographic information of sample (N = 1,099).

Variable		Label	%
Gender			
	Male	gen_m	48.69
	Female	gen_f	51.31
Total			100.00
Age ranges			
		18-25 years	12.83
		26-35 years	33.39
		36-45 years	29.21
		46-55 years	16.20
		56-65 years	6.92
		65 +	1.46
Total			100.00
Area of residence			
	Urban area	urb	84.99
	Rural area	rural	15.01
Total			100.00
Education level			
	Primary or secondary school	low	49.59
	University or postgraduate degree	high	50.41
Total			100.00

*Source: our elaboration on data survey.

The findings show that 88.44% of the sample is inclined to consume organic and local wine and 43.13% of respondents consume wine 2 o 3 times a week. 56.96% prefer red wine and 55.14% of our sample think that the organic label (Lab_Org) is a guarantee of sustainability and a higher quality product. .
Moreover, 51.41% of respondents do not think that the local origin of wine (Local) is a guarantee of territoriality and origin of raw materials. Most respondents do not pay attention to neither wine cellar brand or to producer name. About 51.0% pay attention to nutrition labels when buying wine. Descriptive statistics of the sample are presented in Table 2.

Table 2. Descriptive statistics of sample (N = 1,099).

Variable		Label	%
Readiness to consume sustainable and local wine			
	No	Y	11.56
	Yes		88.44
Total			100.00
Frequency of consumption of wine in terms of times a week or a month		Wine	
	never		0.00

	sometimes a year	1.82
	once a month	4.19
	2 o 3 times a month	11.46
	once a week	35.67
	2 o 3 times a week	43.13
	every day	3.73
Total		100.00
Kind of wine		
	Red	56.96
	White	38.31
	Rosè	4.73
Total		100.00
Frequency of consumption of wine in restaurants, wine bar, etc, in terms of times a week or a month	Wine_out	
	never	4.73
	sometimes a year	18.38
	once a month	17.93
	2 o 3 times a month	19.65
	once a week	18.56
	2 o 3 times a week;	18.38
	every day	2.37
Total		100.00
Do you think that sustainable/organic label is a guarantee of high quality product, naturalness of food, food safety respecting at the same time the environment?	Lab_Org	
	No	44.86
	Yes	55.14
Total		100.00
When you buy wine, do you pay attention to wine cellar brand?	Wine_company_ name	
	No	53.96
	Yes	46.04
Total		100.00
Do you think that local origin of wine is a guarantee of the origin of raw materials supporting at the same time local economies and the culture of landscape?	Local	
	No	51.41
	Yes	48.59
Total		100.00
When you buy wine, do you pay attention to producer name?	Maker name	
	No	67.88
	Yes	32.12
Total		100.00
When you buy wine, do you pay attention to nutrition label?	Nutr	
	No	49.04
	Yes	50.96
Total		100.00

Source: Own elaboration on survey data.

3.2. Model results

Table 3 shows the results of the binary logistic regression model with the estimated coefficients (β), their standard errors (S.E.), Wald X^2-statistics, significance levels, odds ratios (Exp(β)) and goodness-of-fit statistics. The goodness of fit as measured by McFadden's pseudo-R^2 is equal to 0.32. Relatively low value of R^2 is expected for models based on samples with large number of observations (GUJARATI, 2004), as it is happened in our case (1,099 observations). Multi-collinearity was not a major issue in the model as it was tested through Variance Inflation Factors (VIF) (the highest value is equal to 1.19).

Table 3. Parameters of the logistic regression (N = 1,099).

Variable	*β*	S.E.	Wald	Sig.	Exp (*β*)
Intercept	-3.097	0.585	27.977	$1.23\ e^{-07}$	0.045
Wine	0.542	0.109	24.596	$7.07\ e^{-07}$	1.719
Wine_out	0.306	0.085	12.825	0.000342	1.358
Lab_Org	2.709	0.295	84.221	$2\ e^{-16}$	15.018
Wine_company_name	1.883	0.264	50.554	$1.16\ e^{-12}$	6.576
Local	0.971	0.248	15.295	$9.19\ e^{-05}$	2.641
Maker name	0.430	0.271	2.509	0.113	2.509
Nutr	-0.381	0.231	2.705	0.099	2.705
AIC: 553.24					
Pseudo-R^2 = 0.32					
-2Log likelihood statistic = 533.24					

Source: Our elaboration on survey data.

Our findings show that consumers who drink wine frequently (*Wine*) and consume it even outside home (*Wine_out*) are more likely to consume sustainable and local wine, 1.71 times and 1.35 times (respectively), than other people. Therefore, the "wine consume frequency" exerted a positive effect on willingness to consume sustainable and local wine.
Moreover, the *Lab_Org* variable is the most influential factor in the model (Wald $X^2 = 84.2$) followed by *Wine_company_name* variable with Wald X^2=50.5. This last variable (*Wine_company_name*) is referred to the attention that respondent could have to winery's brand when he/she buys wine. Participants who indicated organic label (*Lab_Org*) as guarantee of sustainable product and other aspects (high quality product, naturalness of food, food safety and environmental feeling) are 15.0 times more likely to consume sustainable and local wine than other consumers. In our sample people that pay attention to wine company name (*Wine_company_name*) are 6.57 times more likely to be ready to consume sustainable and local wine than other consumers. It is generally well known by the literature that producer and brand name should interest more consumers when they buy a bottle of wine (POMARICI *et al*., 2017). In addition, participants who indicated local product (Local) as guarantee of quality are 2.6 times more likely to be ready to consume sustainable wine than other consumers. The results are similar to VEALE and QUESTER's (2008) study where origin of product influences consumers' wine quality assessment.

3.3. Discussion

Our results showed a strong interest in respondents towards sustainable and local wine and highlight some important marketing implications for the Italian wine companies. In fact, the findings suggest that producing and marketing wine with sustainability and local characteristics are promising strategies for quality differentiation (SCHAUFELE and HAMM, 2017) and that could allow catching new niches of Italian wine market.
Another interesting result that emerges from current study is that organic label and local origin variables go towards in the same direction in our model, confirming that, in according to LOUREIRO (2003), if the perceived quality of region (local) is negative, wine organic label is not a useful marketing strategy and the people are unwillingness to consume sustainable and local wine. In accordance with SOGARI *et al*. (2015) our results suggest that consumers who perceive sustainable certification as a guarantee of high quality standards have a more positive attitude towards such wine. In fact, consumers may interpret an organic label as a mark of quality (BONCINELLI *et al*., 2019) because an organic certification is indicative of positive health effects (MANN *et al*., 2012), environmental benefits (MUELLER LOOSE and REMAUD, 2013), and great taste (BONCINELLI *et al*., 2019; KIM and BONN, 2015).
Sustainable labelling certification could be an effective strategy to provide consumers with accurate, understandable and trustworthy information to encourage them to buy sustainable wines (GINON *et al*., 2014). Moreover, it is interesting underline that the consumers of our sample inferred that a product is environmentally sustainable in the presence of logos (GOLAN *et al*., 2001). In fact, organic labels are the most influential factor in our model, followed by winery's brand and local label. Also local labels play an important role in differentiating wines because, when the consumers do not know the winery's brand, they base their choice on labelling of origin as a quality standard (LOUREIRO, 2003). In this situation, the consumer relies on the image of the region (negative or positive) that guarantees and promotes that particular label. (LOUREIRO, 2003) Our results emphasized the need to create eco-labels that communicate clearly both the environmental attributes of wine (DELMAS and LESSEM, 2017) and the benefits associated with them.
Even if our sample is not representative of the whole Italian population, the findings could be useful points on which to reflect if we consider that recently the wine market in Italy has been affected by a deep transformation due to both growing international competition and changing of the internal demand. This last aspect is due to new orientation of consumers towards attributes of quality wines (CRESCIMANNO and GALATI, 2014). For these reasons, our findings could be useful for Italian wine producers that want to enter in the sustainable wine market niche. In particular, Italian wine producers could address their marketing strategies towards an adult who drinks wine frequently and preferably in restaurants, pubs and bars, he/she is environmentally friendly and more interested in the local origin of his/her food choices than other consumers.
In this context, wine producers should focus on the communication of their environmental commitment through appropriate marketing tools (POMARICI *et al*., 2016) that help consumers to identify sustainable products (POMARICI and VECCHIO, 2014). In particular, marketers should focus on wine label information (i.e. organic, local and brand) available when consumers inspect the bottle in order to drive the consumers' inclination to consume sustainable and local wine.

4. CONCLUSIONS

This paper has to be considered as an attempt to contribute to the current literature on the willingness to consume sustainable and local wine by Italian consumers and on the main factors that might affect the readiness of consumers.
Profiling consumers who are willing to consume sustainable and local wine could be a first step towards a better understanding of consumers' decisions on sustainable and local food. Our findings indicated that the large majority of the interviewed Italian consumers are willing to consume sustainable and local wine.
The sustainable and local wine consumer of our survey could be described as an adult, who regularly consumes wine, even outside home, more label-conscious and environmentally friendly.
Our findings are even more important if we consider that the Italian wine market (as world market one) has been affected by a deep transformation due to also new orientation of consumers towards attributes of quality wines. In particular, our results emphasize the need to have sustainable labelling that communicates clearly both the environmental attributes of wine and the benefits associated with them. Moreover, our results might be useful to drive marketing strategies because wineries could benefit by promoting these wines as organic and local. Our findings suggest that wine label information (organic, local and brand) might be a useful tool to promote sustainable and local wine and at the same time help to give a positive perception of the whole Italian wine sector.
However, the present study shows some limitations that could be considered and addressed in future research. First of all, the sample is not representative of the Italian population and it suffers from a smaller degree of self-selection. In particular, the questionnaire, to reach the broadest population, was not directed towards specific targets (e.g., university students) and the survey itself is not based on explicit selection mechanism. An additional limitation is that we opted for verbal descriptors to identify the wine attributes, which might mimic a real market in a less realistic way. To avoid this limitation, further studies should simulate real shopping environments where the choice sets are designed with visual labelling elements, such as images or symbols to increase the accuracy of the results.

NOTES

[1]Italy is the largest producer of wine with Protected Designation of Origin (PDO) certification in Europe (405) and organic wine in the world, with 11.9% of organic wine compared to the total of national production of wine (NOMISMA, 2017).

[2]An extract of the questionnaire is available as electronic supplementary material.

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PAPER

OCCURRENCE OF *LISTERIA* SPP. AND ANTIBIOTIC RESISTANCE PROFILES OF *LISTERIA MONOCYTOGENES* FROM RAW MEAT AT RETAIL IN TURKEY

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ABSTRACT

A total of 190 raw meat samples were collected in Ankara to examine the presence of *Listeria* spp. and *L. monocytogenes* and its antibiotic resistance. Of the examined samples, 57 were positive for *Listeria* spp. and among them, 23 were identified as *L. monocytogenes*. Among *L. monocytogenes* strains, 86.96% of isolates were positive for the presence of the *hly*A gene. All *L. monocytogenes* strains were resistant to ampicillin, fosfomycin, nalidixic acid, linezolid, and clindamycin. Multi-drug resistance was observed in 73.91% *L. monocytogenes* strains. In conclusion, the presence of *L. monocytogenes* in raw meat may be indicative of poor hygiene or cross-contamination.

Keywords: *Listeria monocytogenes*, prevalence, antibiotic resistance, raw meat

1. INTRODUCTION

Listeria spp. are Gram-positive, facultatively anaerobic, non-spore forming, rod-shaped bacteria with low G + C content and motile at 10–25°C (NAYAK *et al*., 2015; CORONEO *et al*., 2016). So far, twenty-one species of the genus have been identified (NCBI, 2019). Of them, *Listeria monocytogenes* has been known as the main causative agent of listeriosis in human and other mammals since the 1920s, while *L. ivanovii* is an animal pathogen and rarely infects humans (DOYLE *et al*., 2001; FALLAH *et al*., 2012). The overall rate of listeriosis in humans is low but it can be lethal for high-risk groups like pregnant women, unborn or newly delivered infants, elderly people, severe underlying disease conditions like immune-suppression, organ transplants, patients undergoing treatment for cancer, and AIDS (KHEN *et al*., 2015; ABAY *et al*., 2017; DU *et al*., 2017).

It is difficult to control of contamination by *L. monocytogenes* as it can grow at refrigeration temperature, low oxygen levels, high salt concentration (20% w/v), wide pH values ranging from 4.3 to 9.6, low water content, and hypoxic conditions (ALMEIDA *et al*., 2013; DU *et al*., 2017; KURPAS *et al*., 2018; NOLL *et al*., 2018). This pathogen is also able to survive in vacuum-packed food and modified atmospheres (LAMBERTZ *et al*., 2012). *L. monocytogenes* is a widely distributed in the environment such as soil, contaminated silage, feces of some animals, and in non-treated water. Therefore, it can easily contaminate food products of both animal and plant origin (PESAVENTO *et al*., 2010; ŞANLIBABA *et al*., 2018a). *L. monocytogenes* is also commonly found in food processing environments and various food items including ready-to-eat (RTE) foods, milk and dairy products, meat and its products, unwashed raw vegetables, seafood products, and poultry products (FALLAH *et al*., 2012). *L. monocytogenes* is of particular concern in raw, undercooked or RTE foodstuffs because processed foods are easily contaminated with raw foods in the food-processing environment or at homes (AL-NABUSI *et al*., 2015; OYELAMI *et al*., 2018). Raw meat, such as red meat and chicken, may become contaminated with *L. monocytogenes* either environmentally or during shipping and prolonged storage, particularly if they are stored at above 4°C. The additional handling of raw meats at the retail also results in the transmission of *L. monocytogenes* to raw meats mainly via slicing, weighing, and packaging (KOVACEVIC *et al*., 2012; KURPAS *et al*., 2018). The main concern in raw meat is that it contaminated with *L. monocytogenes* can infect processed foods (PESAVENTO *et al*., 2010).

Antimicrobial resistance (AMR) in foodborne pathogens, especially multidrug resistance, is a great concern for human and animal health at both national and international levels (ALONSO-HERNANDO *et al*., 2012; DU *et al*., 2017). It has been reported that about 33000 deaths occur each year in the European Union due to AMR food-borne pathogens (ANON., 2019a). *L. monocytogenes* is naturally susceptible to a wide range of antibiotics that act on Gram-positive bacteria (GOMEZ *et al*., 2014; WANG *et al*., 2015; BYRNE *et al*., 2016; MOHAMED *et al*., 2016). However, since the first documented report on multi-drug resistant *L. monocytogenes* strain isolated from a patient with meningoencephalitis in France in 1988 (WANG *et al*., 2013; NOLL *et al*., 2018), many strains isolated from food and environmental and clinical samples have shown resistance to one or more antibiotics used for treating listeriosis (ŞANLIBABA *et al*., 2018a). The use of antimicrobials in veterinary medicine is the main cause of the development of AMR foodborne bacterial pathogens including *L. monocytogenes*, as AMR pathogens can easily be transported from animal to human via food consumptions (CONTER *et al*., 2009). The genes responsible for antibiotic resistance could be transferred through movable genetic elements such as conjugative transposons, mobilizable plasmids, and self-transferable plasmids to other foodborne bacteria in the gastrointestinal tract. In *Listeria* spp., efflux pumps have also been reported

as the resistant mechanism (LUNGU *et al*., 2011). *Enterococcus* spp. and *Staphylococcus* spp. serve as a reservoir of resistance genes for *L. monocytogenes* (NATRATILOVA *et al*., 2004). Many studies have been focused on the prevalence of *L. monocytogenes* from raw meat and their antibiotic resistance from the different parts of the world (DIMIC *et al*., 2010; PESAVENTO *et al*., 2010; INDRAWATTANA *et al*., 2011; OSAILI *et al*., 2011; GOMEZ *et al*., 2014; AL-NABUSI *et al*., 2015). However, most of the earlier studies carried out in Turkey (AKPOLAT, 2004; YÜCEL *et al*., 2005; CEYLAN *et al*., 2008; EROL and AYAZ, 2011; DOGRUER *et al*., 2015; ABAY *et al*., 2017; KOCAMAN and SARIMEHMETOĞLU, 2017; ŞANLIBABA *et al*., 2018a; ŞANLIBABA *et al*., 2018b) have been focused on RTE foods, dairy products, vegetables, cooked meat, and poultry. The information on the occurrence of *Listeria* spp., particularly *L. monocytogenes* strains isolated from raw meat and their antimicrobial resistance, is limited. Therefore, the primary aim of this study was: i) to determine the incidence of *Listeria* spp., particularly *L. monocytogenes*, in raw meat samples (chicken meat and red meat) sold in Ankara, Turkey, ii) to identify the isolated strains by phenotypic and genotypic methods, and iii) to assess resistance in the isolated strains against 34 different antibiotics used for treating listeriosis.

2. MATERIALS AND METHODS

2.1. Collection of samples

A total of 190 raw meat samples were randomly purchased from various supermarkets and butchers in the capital city of Turkey over the period of January to April 2018. Sampling locations were randomly selected. The samples were collected only once from each place. These samples consisted of: 1) 80 samples of red meat (minced beef, sliced lamb, and meat cubes) and, 2) 110 samples of chicken meat (legs and wings). All of the samples were non-frozen and kept at refrigeration condition in the retail outlets. While chicken samples analyzed were prepackaged including vacuum and normal atmosphere of packaging, red meat samples were non-packaged. All of the samples were checked for expiry dates and transported to the laboratory under aseptic and refrigerated conditions (+4°C) on the sampling day.

2.2. Isolation and identification of *Listeria* spp.

The two-stage enrichment method, described by the International Organization for Standardization (EN ISO, 11290-1), was used for isolation and identification of *Listeria* spp. Briefly, 25 g of each sample was aseptically weighed and mixed with 225 mL half strength Fraser broth (Merck™, Germany) containing selective supplements as the first enrichment culture in a stomacher bag and homogenized in a stomacher (Seward 400, USA) for 2 min. The homogenized sample was incubated at 30±1°C for 24±2 h. Thereafter, 0.1 mL of pre-enriched Fraser broth was inoculated into 10 mL of full-strength Fraser broth containing selective supplements for second enrichment culture and incubated at 37°C for 48±2 h. After the enrichment procedure, a loopful each of the half- and full-strength Fraser broths was plated on the chromogenic *Listeria* agar (ALOA Agar) (Merck™, Germany) and polymixin acriflavine lithium chloride ceftazidime aesculin mannitol (PALCAM) agar (Merck™, Germany). The plates were incubated at 37°C for 24–48 h. The light blue colonies surrounded by an opaque halo on ALOA agar and gray-green colonies surrounded by

diffuse black zone on PALCAM agar were considered to be of *Listeria* spp. Five presumptive colonies were picked up and further purified on Tryptic Soy agar supplemented with 0.6% of yeast extract (TSA-YE) (Sigma™, Germany) as a non-selective medium. Subsequently, the pinpoint colonies of TSA-YE were subjected to identification procedures, which included Gram's staining, catalase reactions, oxidase tests, carbohydrate utilization, CAMP tests with *S. aureus* and *R. equi*, and motility at 20-25°C.
The isolated *Listeria* species and the reference strains used in this study were inoculated on Tryptic Soy Broth supplemented with 0.6% of yeast extract (TSB-YE) (Sigma™, Germany) and Brain Heart Infusion (BHI) broth (Merck™, Germany) and incubated at 35°C for 24 h. All of the strains used in this study were stored at –20°C with 30% (v/v) glycerol (Merck™, Germany) throughout the study period. The reference strains were obtained from the culture collection of Food Microbiology Laboratory, Department of Food Engineering, Ankara University, Ankara, Turkey.

2.3. Molecular identification of *Listeria* spp. and *L. monocytogenes*

The isolates were subjected to polymerase chain reaction (PCR) analysis to confirm their identity as *Listeria* spp. The genomic DNA of the strains grown at 35°C overnight in TSB-YE was extracted using a genomic DNA extraction kit (Thermo Fisher Scientific™), according to the manufacturer's instructions. The following reference strains were used: *L. monocytogenes* ATCC7644, *Listeria innocua* ATCC12612, *Listeria seeligeri* SLCC3945, *Listeria welshimeri* ATCC35897. The primer pairs designated as U1 (5'–CAGCMGCCGCGGTAATWC–3') and LI1 (5'–CTCCATAAAGGTGACCCT–3'), amplifying a 938-bp region in the 16S rRNA gene sequence of the *Listeri*a genus, were used (USMAN *et al*., 2016). In order to detect the presence of the *hly*A gene, an additional PCR was performed, with DG69 (5'–GTGCCGCCAAGAAAAGGTTA–3') and DG74 (5'-CGCCACACTTGAGATAT–3') as primers specifically amplifying a 636-bp fragment of the *hly*A gene (FALLAH *et al*., 2013). A standardized PCR protocol was followed for the bacterial lysates, in a final volume of 50 μL reaction mixture containing 5 μL PCR buffer, 1 μL 2 mM dNTP mix, 1 μL of each forward and reverse primers, 34.75 μL of sterile distilled water, 0.25 μL of Tag DNA polymerase, 4 μL of 25 mM $MgCl_2$ and 3 μL of the DNA template (BLAIOTTA *et al*., 2002). The PCR amplification was performed in a programmed thermocycler (Techne TC–512, Staffordshire, UK). The PCR conditions were as follows: an initial hold of 2 min at 95°C, followed by 35 cycles each of 45 s denaturation at 95°C, 45 s annealing at 55°C and 2 min extension at 72°C, followed by a final extension for 7 min at 72°C and hold at 4°C. The PCR products were electrophoresed on 1% agarose gels and then stained with ethidium bromide solution (0.5 μg/mL). An O'Gene Ruler™ 10000 bp DNA molecular weight ladder (Fermentas™, Finland) was used as a standard. The gels were visualized under UV light using a Kodak Gel Logic 200 Imaging System (Kodak, USA). Amplified PCR fragments were purified by the PCR purification kit (Thermo Fisher Scientific™) and were sequenced by REFGEN Biotechnology (Ankara, Turkey). Basic local alignment search tool BLAST) was used to compare the sequences against the nucleotide database in National Centre for Biotechnology Information (NCBI) (www. ncbi.nlm.nih.gov.tr/BLAST).

2.4. Antibiotic resistance of *L. monocytogenes* strains

Resistance to different antibiotics was determined for all *Listeria* isolates by the disc diffusion method using Mueller-Hinton agar (Merck™, Germany) as the medium, containing 0.5% defibrinated sheep blood, as described by the Clinical and Laboratory Standards Institute (CLSI) (2011). The selected antibiotics are the ones, commonly used in veterinary and human medicine against listeriosis. The following antibiotic discs were used: penicillin G (10 μg/disc), oxacillin (1 μg/disc), cefotaxime (30 μg/disc), fosfomycin (50 μg/disc), cephalothin (30 μg/disc), furazolidone (50 μg/disc), piperacillin (30 μg/disc), cefuroxime (30 μg/disc), cefoxitin (30 μg/disc), ampicillin (10 μg/disc), amoxicillin/clavulanic acid (20/10 μg/disc), erythromycin (15 μg/disc), clarithromycin (15 μg/disc), tetracycline (30 μg/disc), tigecycline (15 μg/disc), moxifloxacin (5 μg/disc), neomycin (10 μg/disc), ciprofloxacin (5 μg/disc), enrofloxacin (5 μg/disc), levofloxacin (5 μg/disc), nalidixic acid (30 μg/disc), linezolid (30 μg/disc), kanamycin (30 μg/disc), streptomycin (300 μg/disc), gentamicin (120 μg/disc), vancomycin (30 μg/disc), teicoplanin (30 μg/disc), meropenem (10 μg/disc), imipenem (10 μg/disc), clindamycin (2 μg/disc), trimethoprim (5 μg/disc), trimethoprim/sulfamethoxazole (1.25/23.75 μg/disc), chloramphenicol (30 μg/disc), and rifampicin (5 μg/disc). After the incubation at 35°C for 24 h, the diameters of the inhibition zones around each disc were measured. On the basis of the comparative results, the strains were categorized as susceptible, intermediate, or resistant as per the criteria established by CLSI (2011). The breakpoints given for *Staphylococcus* species were used to determine the antibiotic resistance profile of *L. monocytogenes*, as currently there are no resistance criteria given in the CLSI guidelines for *Listeria* spp. (WANG *et al*., 2013; KHEN *et al*., 2015; DU *et al*., 2017; KUAN *et al*., 2017). *E. coli* ATCC25922, *S. aureus* ATCC6538, and *L. monocytogenes* ATCC7644 were used as quality control strains.

2.5. Dendogram construction method

The sequences were aligned with the Multiple Sequence Alignment by CLUSTALW and neighbor-joining method was used for phylogenetic tree.

2.6. Statistical analysis

All statistical analyses were carried out using SPSS 16 package. The analysis of one-way variance (ANOVA) followed by Tukey's test was applied to determine the differences in the prevalence of *Listeria* spp. between the red meat and chicken samples, and also between the antibiotic resistance of *L. monocytogenes* strains. The statistical significance was set at $p<0.05$.

3. RESULTS

3.1. Occurrence and incidence of *Listeria* spp. and *L. monocytogenes*

A total of 190 samples were examined for the presence of *Listeria* spp. using a two-step selective enrichment method as recommended by EN ISO 11290-1. The occurrence of *Listeria* spp. and *L. monocytogenes* in raw meat samples marketed in Turkey is presented in Table 1. *Listeria* spp. was detected in 57 (30.00%) of the samples. The 16S rRNA sequence

analysis indicated *L. monocytogenes* (12.10%; 23/190) to be the most prevalent in the raw meat samples, followed by *L. innocua* (11.05%; 21/190), *L. welshimeri* (6.31%; 12/190), and *L. seeligeri* (0.52%; 1/190). Using neighbour joining method, phylogenetic relationships of 57 *Listeria* spp. were allowed to group into two main clusters. Cluster 1 was composed of 1 isolate. Fifty-six isolates were belonged to cluster 2 (Fig. 1). In addition, *L. monocytogenes* strains were also screened for the virulence-associated *hly*A gene. Among the 23 *L. monocytogenes* strains, only 20 (86.96%) were positive for the presence of the *hly*A gene. LP6, LP16, and LP54 strains of *L. monocytogenes*, isolated from raw chicken samples, were identified as atypical strains since they were devoid of the *hly*A gene.

The prevalence of *Listeria* spp. in the raw chicken meat samples was as follows: 14.54% (16/110) for *L. monocytogenes*, 11.81% (13/110) for *L. innocua*, 6.36% (7/110) for *L. welshimeri*, and 0.90% (1/110) for *L. seeligeri*. On the other hand, the prevalence in the raw red meat samples was as follows: 10.00% (8/80) for *L. innocua*, 8.75% (7/80) for *L. monocytogenes*, and 6.25% (5/80) for *L. welshimeri*.

The samples of raw chicken had the highest occurrence of *Listeria* spp. (33.63%, 37/190), followed by red meat (25.00%, 20/190). In the prevalence of *L. monocytogenes* was significantly higher ($p<0.05$) in the raw chicken meat than that in the raw red meat.

Table 1. Prevalence of *Listeria* species in raw meat samples.

Food Samples	Number of Samples (n)	Number of Positive Samples n (%)				
		***Listeria* spp.**	***L. monocytogenes***	***L. innocua***	***L. welshimeri***	***L. seeligeri***
Raw red meat	80	20 (25.00)	7 (8.75)	8 (10.00)	5 (6.25)	0 (–)[a]
Raw chicken meat	110	37 (33.63)	16 (14.54)	13 (11.81)	7 (6.36)	1 (0.90)
Totals	190	57 (30.00)	23 (12.10)	21 (11.05)	12 (6.31)	1 (0.52)

[a] Not detected.

3.2. Antibiotic resistance of *L. monocytogenes* strains

The antimicrobial resistance of the 23 *L. monocytogenes* strains against 34 different antibiotics was examined using the disk diffusion method according to CLSI (2011) (Table 2). Of the *L. monocytogenes* isolates, 23 were resistant to ampicillin, fosfomycin, nalidixic acid, linezolid, and clindamycin. Frequent resistance was seen against piperacillin (86.96%, 20/23), oxacillin (82.61%, 19/23), kanamycin (82.61%, 19/23), neomycin (78.26%, 18/23), penicillin G (73.92%, 17/23), amoxicillin/clavulanic acid (73.92%, 17/23), levofloxacin (73.92%, 17/23), teicoplanin (73.92%, 17/23), moxifloxacin (69.57%, 16/23), ciprofloxacin (69.57%, 16/23), and furazolidone (52.17%, 12/23). Furthermore, resistance to enrofloxacin (47.83%, 11/23), rifampicin (17.39%, 4/23), streptomycin (17.39%, 4/23), tigecycline (13.04%, 3/23), cefuroxime (13.04%, 3/23), cephalothin (13.04%, 3/23), trimethoprim/sulfamethoxazole (13.04%, 3/23), and cefotaxime (8.69%, 2/23) was also observed. At least one strain was resistant against either of tetracycline, gentamicin, and meropenem (4.35%). In contrast, all strains were susceptible to cefoxitin, erythromycin, clarithromycin, vancomycin, imipenem, trimethoprim, and chloramphenicol. The differences between the antibiotic resistance of *L. monocytogenes* strains were not found to be statistical significance ($p > 0.05$).

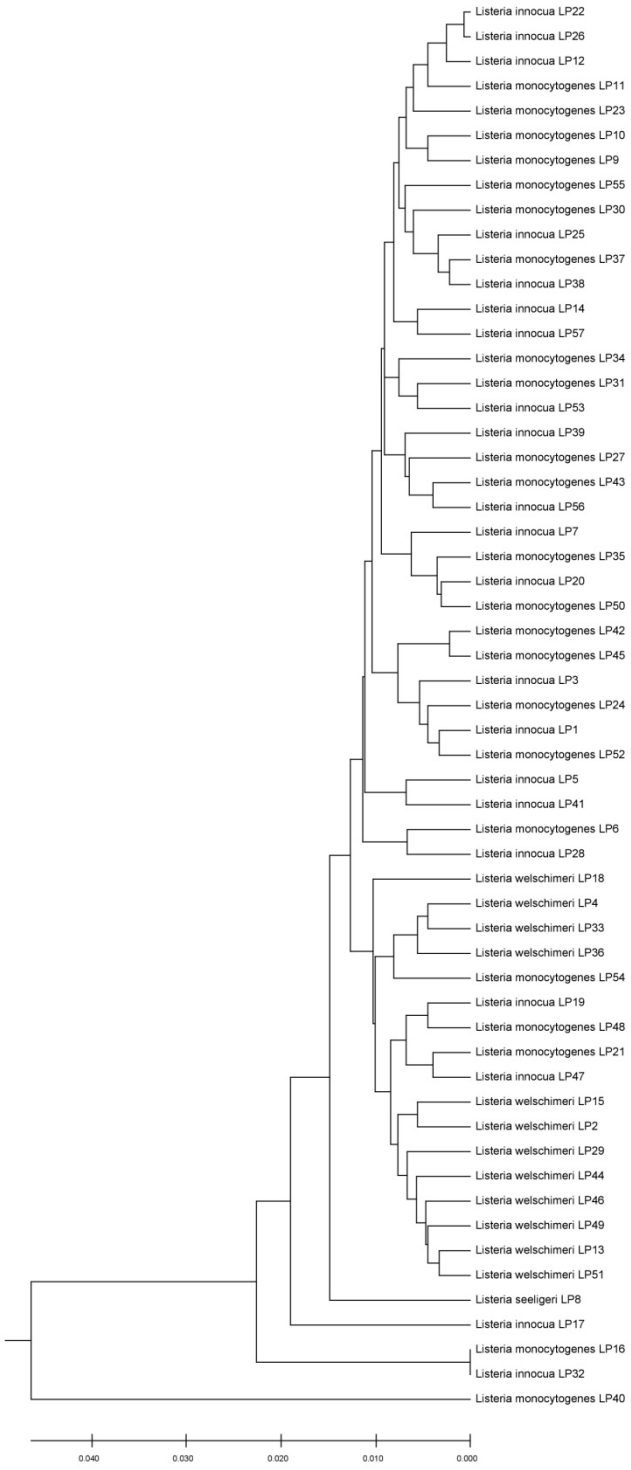

Figure 1. Dendrogram showing the evolutionary relationships among *Listeria* isolates based on the 16S rRNA sequence analysis.

Table 2. Antibiotic susceptibility and resistance (%) of *L. monocytogenes* strains isolated from raw red meat and chicken meat samples.

Antimicrobial Agent [a]	*L. monocytogenes* Strains																	
	RAW RED MEAT (n: 7)						RAW CHICKEN MEAT (n:16)						TOTALS (n:23)					
	S[b]		I[b]		R[b]		S[b]		I[b]		R[b]		S[b]		I[b]		R[b]	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Penicillins																		
Penicillin G	-[c]	-	2	28.57	5	71.43	2	12.50	2	12.50	12	75.00	2	8.69	4	17.39	17	73.92
Oxacillin	-	-	1	14.29	6	85.71	-	-	3	18.75	13	81.25	-	-	4	17.39	19	82.61
Ampicillin	-	-	-	-	7	100.00	-	-	-	-	16	100.00	-	-	-	-	23	100.00
Amoxicillin/clavulanic acid	1	14.29	2	28.57	4	57.14	2	12.50	1	6.25	13	81.25	3	13.04	3	13.04	17	73.92
Piperacillin	2	28.57	-	-	5	71.43	1	6.25	-	-	15	93.75	3	13.04	-	-	20	86.96
Cephalosporins																		
Cephalothin	4	57.14	1	14.29	2	28.57	15	93.75	-	-	1	6.25	19	82.61	1	4.35	3	13.04
Cefotaxime	5	71.43	2	28.57	-	-	13	81.25	1	6.25	2	12.50	18	78.27	2	13.04	2	8.69
Cefuroxime	6	85.71	-	-	1	14.29	14	87.50	-	-	2	12.50	20	86.96	-	-	3	13.04
Cefoxitin	7	100.00	-	-	-	-	16	100.00	-	-	-	-	23	100.00	-	-	-	-
Macrolides																		
Erythromycin	6	85.71	1	14.29	-	-	10	62.50	6	37.50	-	-	16	69.57	7	30.43	-	-
Clarithromycin	5	71.43	2	28.57	-	-	14	87.50	2	12.50	-	-	19	82.61	4	17.39	-	-
Tetracyclines																		
Tetracycline	5	71.43	1	14.29	1	14.29	14	87.50	2	12.50	-	-	19	82.61	3	13.04	1	4.35
Tigecycline	6	85.71	-	-	1	14.29	14	87.50	-	-	2	12.50	20	86.96	-	-	3	13.04
Quinolones																		
Ciprofloxacin	-	-	2	28.57	5	71.43	2	12.50	3	18.75	11	68.75	2	8.69	5	21.74	16	69.57
Levofloxacin	1	14.29	2	28.57	4	57.14	1	6.25	2	12.50	13	81.25	2	8.69	4	17.39	17	73.92
Nalidixic acid	-	-	-	-	7	100.00	-	-	-	-	16	100.00	-	-	-	-	23	100.00
Moxifloxacin	-	-	3	42.86	4	57.14	4	25.00	-	-	12	75.00	4	17.39	3	13.04	16	69.57
Enrofloxacin	4	57.14	2	28.57	1	14.29	3	18.75	3	18.75	10	62.50	7	30.43	5	21.74	11	47.83
Monurol																		
Fosfomycin	-	-	-	-	7	100.00	-	-	-	-	16	100.00	-	-	-	-	23	100.00

[a]The diameters of the zones were compared with the diameters of the Clinic Laboratory Standards Institute (CLSI 2011).

[b]S : Susceptible, I : Intermediately resistant, R : Resistant.

[c]Not detected.

Table 2. Antibiotic susceptibility and resistance (%) of *L. monocytogenes* strains isolated from raw red meat and chicken meat samples (Continued).

Antimicrobial Agent[a]	*L. monocytogenes* Strains																	
	RAW RED MEAT (n: 7)						RAW CHICKEN MEAT (n:16)						TOTALS (n:23)					
	S[b]		I[b]		R[b]		S[b]		I[b]		R[b]		S[b]		I[b]		R[b]	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Oxazolidinones																		
Linezolid	-	-	-	-	7	100.00	-	-	-	-	16	100.00	-	-	-	-	23	100.00
Aminoglycosides																		
Kanamycin	-	-	1	14.29	6	85.71	-	-	3	18.75	13	81.25	-	-	4	17.39	19	82.61
Streptomycin	4	57.14	2	28.57	1	14.29	12	75.00	1	6.25	3	18.75	16	69.57	3	13.04	4	17.39
Gentamicin	5	71.43	2	28.57	-	-	10	62.50	5	31.25	1	6.25	15	65.22	7	30.43	1	4.35
Neomycin	1	14.29	-	-	6	85.71	4	25.00	-	-	12	75.00	5	21.74	-	-	18	78.26
Glycopeptides																		
Vancomycin	7	100.00	-	-	-	-	16	100.00	-	-	-	-	23	100.00	-	-	-	-
Teicoplanin	-	-	2	28.57	5	71.43	-	-	4	25.00	12	75.00	-	-	6	26.08	17	73.92
Carbapenems																		
Meropenem	5	71.43	1	14.29	1	14.29	11	68.75	5	31.25	-	-	16	69.57	6	26.08	1	4.35
Imipenem	7	100.00	-	-	-	-	15	93.75	1	6.25	-	-	22	95.65	1	4.35	-	-
Lincosamides																		
Clindamycin	-	-	-	-	7	100.00	-	-	-	-	16	100.00	-	-	-	-	23	100.00
Sulfonamides/trimethoprim																		
Trimethoprim	4	57.14	3	42.86	-	-	14	87.50	2	12.50	-	-	18	78.26	5	21.74	-	-
Trimethoprim/Sulfamethoxazole	5	71.43	1	14.29	1	14.29	10	62.50	4	25.00	2	12.50	15	65.22	5	21.74	3	13.04
Amphenicol																		
Chloramphenicol	7	100.00	-	-	-	-	16	100.00	-	-	-	-	23	100.00	-	-	-	-
Rifamycins																		
Rifampicin	2	28.57	3	42.86	2	28.57	9	56.25	5	31.25	2	12.50	11	47.83	8	34.78	4	17.39
Nitrofurans																		
Furazolidone	2	28.57	-	-	5	71.43	8	50.00	1	6.25	7	43.75	10	43.48	1	4.35	12	52.17

The diameters of the zones were compared with the diameters of the Clinic Laboratory Standards Institute (CLSI 2011).

[b]S : Susceptible, I : Intermediately resistant, R : Resistant.

[c]Not detected.

Multi-drug resistance, i.e., resistance to three or more antimicrobial agents, was observed in 73.91% (17/23) *L. monocytogenes* strains. While 69.56% (16/23) of the *L. monocytogenes* strains were resistant to four antibiotics, and 60.86% (14/23) of the isolates were resistant to five antibiotics. On the whole, 13 of 23 (56.52%) *L. monocytogenes* strains showed resistance to more than six antibiotics.

4. DISCUSSION

4.1. Prevalence of *Listeria* spp. and *L. monocytogenes* from raw meat samples

As a result of its psychotropic nature, *L. monocytogenes* is of great concern to the meat industry. *L. monocytogenes* contamination can occur in raw meat during processing and storage (ABAY *et al*., 2017). This initial contamination can spread, propagate, and increase during further processing of meat (YANG *et al*., 2017). Normally, only cooked meat is eaten, thus the existence of *L. monocytogenes* in raw meat could be problematic only if the meat is eaten raw or insufficiently cooked. We should not underestimate the risk of listeriosis in this type of food. There is no criterion for routine microbiological testing of raw meat for the presence of *L. monocytogenes* in Turkey (ANON., 2019b). Among the 190 samples tested, 57 were positive for *Listeri*a spp. and the isolation rate of *L. monocytogenes* from raw meat samples (12.10%) was higher than our expectations. The presence of *L. monocytogenes* in raw meat may be attributed to: i) fecal contamination during evisceration, ii) food handlers, and iii) cross-contamination during processing, transportation or marketing (AL-NABUSI *et al*., 2015; GOMEZ *et al*., 2015). This study was also aimed to detect the presence of the *hly*A gene in *L. monocytogenes* strains. This gene is one of the most virulent factors associated with *L. monocytogenes* and essential for intracellular infection (MORENO *et al*., 2014; USMAN *et al*., 2016). It was interesting to note that this virulence gene was detected in 86.96% of the *L. monocytogenes* strains isolated from the raw chicken samples and the rest 13.04% were devoid of it. These strains were named as atypical strains. The presence of this gene in the pathogens thriving on meat suggests a serious risk to human health (ZEINALI *et al*., 2015). To the best of our knowledge, this is the first report on the isolation of atypical *L. monocytogenes* strains, devoid of the *hly*A gene from foods in Turkey. Our results are consistent with those reported by KAUR *et al*. (2007), OSAILI *et al*. (2011), MORENO *et al*. (2014), USMAN *et al*. (2016) and ZEINALI *et al*. (2015), who identified some atypical *L. monocytogenes* strains from food. The *hly*A gene has an important role in the invasion process of *L. monocytogenes* (ZEINALI *et al*. 2015). The occurrence of some mutations alternating the genes responsible for pathogenesis may be the reason of the absence of *hly*A. This may explain why some species do not cause infections. Further studies are needed to determine the presence of other virulence genes in these strains.

The overall incidence of *Listeria* spp. in all raw meat samples was 30%, which is higher than that reported in Italy (21.4%) (PESAVENTO *et al*., 2010), but lower than that documented in Japan (58.7%) (INDRAWATTANA *et al*., 2011), Iran (34.7%) (FALLAH *et al*., 2012), Canada (70%) (AL-NABUSI *et al*., 2015), and Nigeria (80%) (OYELAMI *et al*., 2018). Our study showed that the overall incidence of *L. monocytogenes* in raw meat was 12.10%. The prevalence of *L. monocytogenes* was higher in raw chicken meat (14.54%) than raw red meat (8.75%). The rate of *L. monocytogenes* contamination in raw meat, from different parts of the world, was found to be 23.3% in Morocco (ENNAJI *et al*., 2008), 15.4% in Japan (INDRAWATTANA *et al*., 2011), 18.2% in Jordan (OSAILI *et al*., 2011), 14.1% in

Iran (FALLAH *et al*., 2012), 12% in China (WANG *et al*., 2013), 43.8% in Canada (AL-NABUSI *et al*., 2015), and 28% in Nigeria (OYELAMI *et al*., 2018). These differences suggest that *L. monocytogenes* contamination rates may be affected by geographical location, weather conditions, environmental conditions, the actions of food handlers, monitoring studies and isolation methods. In previous studies carried out in Turkey (AKPOLAT, 2004; YÜCEL *et al*., 2005; CEYLAN *et al*., 2008; EROL and AYAZ, 2011; DOGRUER *et al*., 2015; KOCAMAN and SARIMEHMETOĞLU, 2017), the contamination rate of *L. monocytogenes* in raw meat samples was found between 4.7% and 32.76%, which is partially consistent with our study. Related to these past results, we observed no increase in the incidence of *L. monocytogenes* in raw meats from Turkey. The reason for this consistency in the incidence of *L. monocytogenes* in raw meats may be the compliance with the Good Manufacturing Practices (GMPs), and Hazard Analysis and Critical Control Point (HACCP) systems.

4.2. Antibiotic resistance in *L. monocytogenes* strains

Earlier, *L. monocytogenes* was considered to be susceptible to a wide range of antibiotics (LUNGU *et al*., 2011; ALONSO-HERNANDO *et al*., 2012). However, there has been an increasing number of reports about *L. monocytogenes* strains resistant to one or more antibiotics from food, clinical, and environmental products since 1988 (GOMEZ *et al*., 2014; WANG *et al*., 2015).

Many strains of *L. monocytogenes* have been reported to be completely or partly resistant to fluoroquinolones, cephalosporins, aztreonam, pipemidic acid, fosfomycin, and macrolides, and other antibiotics, especially those of the third and fourth generations (RUIZ-BOLIVAR *et al*., 2011; WANG *et al*., 2013; GOMEZ *et al*., 2014; BRYNE *et al*., 2016). In the current study, the antimicrobial resistance tests of *L. monocytogenes* strains revealed that all of the *L. monocytogenes* strains were resistant to nalidixic acid, fosfomycin, ampicillin, linezolid, and clindamycin. We observed that 69.57%, 73.92%, 69.57% and 47.83 of *L. monocytogenes* strains were resistant to ciprofloxacin, levofloxacin, moxifloxacin, and enrofloxacin, respectively, which belong to the fluoroquinolones class of antibiotics. These high rates of resistance against fluoroquinolones could be ascribed to the frequent use of these antibiotics in animal feeds to treat infections (FALLAH *et al*., 2012; WANG *et al*., 2015). However, unexpectedly our isolates of *L. monocytogenes* showed low resistance to cephalothin (13.04%), cefuroxime (13.04%), and cefotaxime (8.69%), and all of them were susceptible to cefoxitin (100%).

The members of penicillin group antibiotics include ampicillin, oxacillin, penicillin G, amoxicillin, and piperacillin. They are the most active β-lactam compounds that inhibit the synthesis of bacterial cell wall peptidoglycan (ETABU and ARIKEKPAR, 2016). *L. monocytogenes* is naturally susceptible to β-lactams (LUNGU *et al*., 2011; BYRNE *et al*., 2016). Clinicians usually treat human listeriosis with the standard antibiotic therapy that includes high doses of penicillin, ampicillin, and amoxicillin alone or combined with gentamicin (KORSAK *et al*., 2012; GOMEZ *et al*., 2014; SHI *et al*., 2015; OLAIMAT *et al*., 2018). In the present study, *L. monocytogenes* strains showed high resistance to ampicillin (100%), piperacillin (86.96%), oxacillin (82.61%), penicillin G (73.92%), and amoxicillin/clavulanic acid (73.92%). This finding is highly significant as far as the treatment of human listeriosis is concerned. However, our results were not similar to the previous studies conducted in Turkey (TERZI *et al*., 2015; KOCAMAN and SARIMEHMETOĞLU, 2017), which reported low resistance to these antibiotics. Thus our observations are of great medical concern. Trimethoprim alone or combined with

sulfamethoxazole is generally used in the case of allergy to beta-lactams and rifampin, erythromycin, vancomycin, linezolid, and meropenem can also be used as possible alternatives (AL-NABUSI *et al*., 2015; ŞANLIBABA *et al*. 2018a). It is worth noting that our results showed that resistance to trimethoprim/sulfamethoxazole (13.04%) and meropenem (4.35%) was found to be low in this study. In addition, resistance to trimethoprim was not observed in this study, in line with WANG *et al*. (2015), OBAIDAT *et al*. (2015), and KUAN *et al*. (2017).

Clindamycin interferes with bacterial protein synthesis in a similar way to erythromycin and chloramphenicol (SHI *et al*., 2015). Therefore, owing to the similar mode of action, a cross-resistance among clindamycin, erythromycin, and chloramphenicol can sometimes be detected (RUIZ-BOLIVAR *et al*., 2011; MORENO *et al*., 2014). In this study, no resistance to chloramphenicol and erythromycin was observed; this concords with the findings of YÜCEL *et al*. (2005), ENNAJI *et al*. (2008), and CHEN *et al*. (2010). In contrast, resistance to clindamycin was 100%. This, to the best of our knowledge, seems to be the first report from Turkey showing the cross-resistance of these antibiotics. These results also concord with the theory of a specific enzyme that inactivates clindamycin, as previously reported for *Staphylococcus* spp. (RUIZ-BOLIVAR *et al*., 2011; MORENO *et al*., 2014).

Rifampin is the main antibiotic used in the treatment against *Mycobacterium tuberculosis* and Gram-positive bacteria (RUIZ-BOLIVAR *et al*., 2011). It has also been recommended to treat listeriosis (OLAIMAT *et al*., 2018). Fortunately, the resistance rate (17.39%), found in our work, does not seem to be alarming and is in agreement with FALLAH *et al*. (2013). Vancomycin is one of the last therapeutic options for the treatment of human listeriosis especially in case of bacteremia and endocarditis (OBAIDAT *et al*., 2015). Fortunately, all *L. monocytogenes* strains were found susceptible to the vancomycin in accordance with the results of RAHIMI *et al*. (2010), OKADA *et al*. (2011), WANG *et al*. (2015), AL-NABUSI *et al*. (2015), and BRYNE *et al*. (2016). The resistance of the isolates against vancomycin is a contrary finding to that obtained by IEREN *et al*. (2013) and FALLAH *et al*. (2013). Tetracycline and tigecycline are the members of tetracyclines, whose target of antimicrobial activity in bacteria is the ribosome (ETEBU and ARIKEKPAR, 2016). Tetracycline resistance has most frequently been reported in *Listeria* spp. of different origins (PESAVENTO *et al*., 2010; FALLAH *et al*., 2012; KORSAK *et al*., 2012). This might have arisen due to the extensive and prolonged use of these antimicrobials in human medicine and as growth promoters in animals (BRYNE *et al*., 2016). In contrast, these results could not be confirmed by us, because only one and two of the examined *L. monocytogenes* strains were resistant to tetracycline and tigecycline, respectively. The rare occurrence of resistance to tetracycline (4.35%) and tigecycline (13.04%) is in accordance with RAHIMI *et al*. (2010), AL-NABUSI *et al*. (2015), and NOLL *et al*. (2018). Tetracyclines are not used as the first drug of choice for listeriosis treatment and their use is also not recommended in children and pregnant women (RUIZ-BOLIVAR *et al*., 2011). While *L. monocytogenes* strains showed resistance to kanamycin (82.61%) and neomycin (78.26%), low resistance to streptomycin (17.39%) and gentamicin (4.35%) was also observed. The high frequency of resistance to kanamycin and neomycin was unexpected and might be in part due to the excessive use of these antibiotics in veterinary medicine in Turkey. These results are in agreement with ALONSO-HERNANDO *et al*. (2012) and SHI *et al*. (2015). However, some authors have reported high sensitivity of *L. monocytogenes* to kanamycin (OKADA *et al*., 2011; WANG *et al*., 2013; JAMALI *et al*., 2015; WU *et al*., 2015). In the present study, there was no significant association between the different *L. monocytogenes* strains in terms of antibiotic resistance ($p< 0.05$).

The prevalence of multidrug resistance *L. monocytogenes* strains isolated from raw meat was 73.91% (data not shown). The multi-resistance patterns reported in other countries are as follows: 16.4% in Iran (RAHIMI *et al*., 2010), 48% in Colombia (RUIZ-BOLIVAR *et al*., 2011), 64.3% in Nigeria (IEREN *et al*., 2013), 2.9% in Spain (GOMEZ *et al*., 2014), 21.25% in China (SHI *et al*., 2015), 21% in Germany (NOLL *et al*., 2018), and 81% in Malaysia (KUAN *et al*., 2017). These differences among multidrug resistance in *L. monocytogenes* strains could result from the differences in the use of antimicrobials at the regional level.
The result of our study suggested that the overall incidence of antibiotic resistance in *L. monocytogenes* is alarming. Further, the high resistance rate observed against antibiotics commonly used to treat listeriosis, such as penicillin, oxacillin, ampicillin, piperacillin, and amoxicillin/clavulanic acid, is also a great concern.

5. CONCLUSIONS

Our results regarding the occurrence of *L. monocytogenes* in raw meat and the presence of virulence-associated genes in the strains indicate an alarming situation to the public health. The counts of *L. monocytogenes* in raw meat at the retail level are crucial for contaminating with cooked foods. Retail centers must be controlled legally monitored. This study is the first report of *hly*A negative *L. monocytogenes* strains isolated from food in Turkey. In this study, we have demonstrated that all *L. monocytogenes* strains were resistant to ampicillin, fosfomycin, nalidixic acid, linezolid, and clindamycin against Gram-positive bacteria. The controlled use of antibiotics for therapeutic purposes may be important to limit the emergence of resistant *L. monocytogenes* strains in the world.

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PAPER

DRYING CHARACTERISTICS OF 'ANKARA' PEAR SLICES

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ABSTRACT

This study evaluated the effects of drying temperature and pre-treatment on the rehydration capacity and color parameters of sliced pears (*cv. Ankara*). Drying trials were conducted at 55, 65, and 75°C. Pre-treatment consisted of immersion of pear slices in a citric-acid solution or blanching in hot water. Pre-treatment was found to have a significant effect on both rehydration capacity and color, with higher temperatures and pre-treatment resulting in decreases in drying time and increases in rehydration capacity. Effective diffusivity values ranged between 1.12×10^{-10} and 2.94×10^{-10} m^2/s. Blanched pear slices had the lowest E_a values (15.51 kJ/mol), followed by the samples immersed in citric acid (28.03 kJ/mol) and the untreated samples (33.48 kJ/mol). The Midilli *et al.* model displayed the best fit to the drying data of five models tested based on the statistical criteria evaluated. Natural color of fresh pear was best preserved with lower drying temperatures and pre-treatment with citric acid.

Keywords: color properties, convective drying, moisture content, rehydration capacity

1. INTRODUCTION

Pear is one of the most important fruits in Turkey and around the world. In 2017, Turkey accounted for approximately 503,004 tons of the 24.17 million tons of pears produced world-wide, making it the 5th largest pear producer behind China (16.53 million tons), Argentina (930,340 tons), the United States (677,891 tons) and Italy 772,577 tons) (FAOSTAT, 2019). Turkey is also a center of genetic diversity, with over 600 of the more than 5,000 varieties found throughout the world (KARADENİZ, 1999). One of the most important pear varieties in Turkey is the 'Ankara' pear, which originated in Ankara and is grown mainly in Turkey's Central Anatolia region, especially in the province of Ankara (ERDOĞAN *et al.*, 2007). 'Ankara' pear trees produce medium-sized, green fruit with smooth surfaces, thin skins, short, thick stalks and juicy, fragrant flesh that melts in the mouth. The fruit are also easy to store (DUMANOĞLU *et al.*, 2006; ERDOĞAN *et al.*, 2007).
Vegetables and fruits contain basic nutrients that are important for human health. Because fruits and vegetables are cultivated on a seasonal basis and have a high-water content that makes them easily perishable, various preservation techniques have been developed so that fruits and vegetables can be consumed throughout the year (QUILES *et al.*, 2005). Dehydration, although a highly complicated product-processing technique (MASKAN, 2000), is the basic method used for reducing moisture levels in order to minimize on-going microbial reactions, prevent deterioration (KROKIDA and MARINOS-KOURIS, 2003), and increase the shelf life (DAS *et al.*, 2001) of agricultural products. Of the many drying methods available, convective drying, which represents one of the most common of all postharvest technologies, allows for high-quality products that preserve close to their original color (DOYMAZ, 2004).
Pears are consumed in various forms, both fresh and dried. Dried pears are consumed directly as snacks and are also widely used as inputs in the food industry. The design, operation, and maintenance of fruit-drying systems require a good understanding of drying characteristics. Studies have evaluated drying characteristics of different varieties of pears, such as 'd'Anjou' (PARK *et al.*, 2002) and 'Deveci' (DOYMAZ, 2013), as well as different techniques, including convective drying (GONZÁLEZ-MARTÍNEZ, 2006) air-drying (DOYMAZ, 2013; DOYMAZ and İSMAIL, 2012), osmo-vacuum drying (AMIRIPOUR *et al.*, 2015), mid-infrared-freeze drying (ANTAL *et al.*, 2017), and microwave-vacuum drying (TASKIN *et al.*, 2019). However, the literature includes no data on the drying behavior of the 'Ankara' pear variety, whose texture varies greatly from that of other varieties, especially the 'Deveci' pear. Thus, this study was carried out to examine how drying temperature and pre-treatment by either immersion in a citric acid solution or blanching in hot water affect the drying characteristics and quality parameters (i.e. moisture content, rehydration capacity, color) of 'Ankara' pear.

2. MATERIALS AND METHODS

2.1. Material

The pears used in this study (*cv. Ankara*) were obtained from a local market in Ankara, Turkey. Pears were kept refrigerated at 5ºC and removed 12 hours prior to the trials to obtain equilibrium. Pears were then sliced into sound, homogenous samples of 5±0.5 mm thickness and randomly distributed among 3 groups according to pre-treatment, as follows: Citric Acid: pear slices were immersed in a citric-acid solution (5 g/L) for 3 min at

room temperature; Blanching: pear slices were blanched in 85°C water for 3 min and then rinsed with running water; Untreated: pear slices received no pre-treatment.

2.2. Drying

Pears were dried according to SACILIK *et al.* (2010) using a convective hot-air dryer (57 x 68 x 57 cm) comprised of a perforated basket (576 cm^2 x 12 cm), an adjustable fan, an electric heater, and a load-cell system attached to a PC (Fig. 1). Drying runs were carried out at 55, 65 and 75 °C, with a constant air velocity of 1 m/s (IZLI *et al.*, 2019). A minimum of 250 g of pear slices was used for each run. Pear slices were dried with tissue paper and then placed uniformly into the basket, which was positioned in the drying system after it had been allowed to idle for 20 min to reach thermal stabilization. Initial moisture content of pears was measured at 120 ºC using an HB43-S Halogen Moisture Analyzer (Mettler Toledo, Switzerland) and recorded as 572.04% d.b. (85.12% w.b.). During the drying process, moisture loss from samples in the drying basket was measured using a load cell and continuously recorded using specially developed software connected to a PC. Once moisture-loss measurements were completed, dried samples were evaluated for rehydration capacity and color.

2.3. Effective diffusivity and activation energy

A falling-rate drying period can be observed in drying pear slices, with moisture and/or vapor migration controlled by diffusion. In this case, Fick's second law can be derived as follows (CRANK, 1975; SACILIK and UNAL, 2005):

$$\frac{\partial M_l}{\partial t} = \nabla(D_{eff} \nabla M_l) \qquad (1)$$

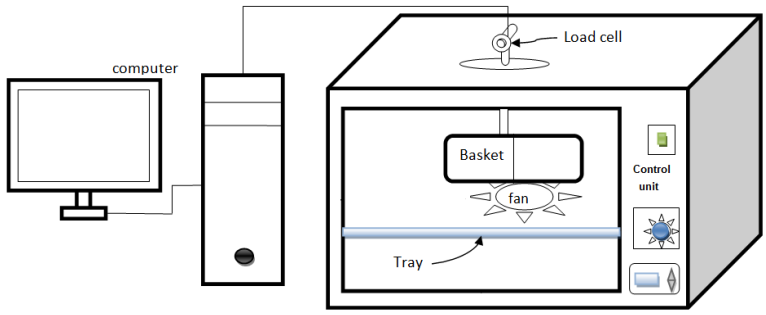


Figure. 1. The diagram of drying system.

where M_l is the local moisture content in % d.b., t is the drying time in min, and D_{eff} is the effective diffusivity in m^2/s. Assuming moisture migration to be realized through diffusion, shrinkage to be negligible, and diffusion coefficients and temperatures to be constant (CRANK, 1975) yields the following equation:

$$M_R = \frac{M - M_e}{M_0 - M_e} = \frac{8}{\pi^2} \sum_{n=0}^{\infty} \frac{1}{(2n+1)^2} \exp\left(\frac{-(2n+1)^2 \pi^2 D_{eff} t}{4h^2} \right) \quad (2)$$

For long drying periods, by considering only the first term in the series and, given the relatively small size of M_e as compared to M and M_0 reducing moisture ratio (M_R) to M/M_0 Equation 2 can be simplified to yield Equation 3:

$$\ln \frac{M}{M_0} = \ln \frac{8}{\pi^2} - \left(\frac{\pi^2 D_{eff} t}{4h^2} \right) \quad (3)$$

where M_R is the dimensionless moisture ratio, M is the moisture content at any time in % d.b., M_e is the equilibrium moisture content in % d.b., M_0 is the initial moisture content in % d.b., h is the half-thickness of the slab in sample in m, and n is a positive integer.
Effective diffusivity and drying air temperature are correlated using the Arrhenius equation (Equation 4):

$$D_{eff} = D_0 \exp\left(-\frac{E_a}{RT_a} \right) \quad (4)$$

where D_0 is the pre-exponential factor of the Arrhenius equation in m^2/s, E_a is the activation energy in kJ/mol, R is the universal gas constant in kJ/mol.K, and T_a is the absolute air temperature in K.

2.4. Modelling of drying data

Drying data were fitted to five selected models (Table 1). Moisture ratios were determined using the following equation:

$$M_R = \frac{M - M_e}{M_0 - M_e} \quad (5)$$

where M_R is the moisture ratio, M, M_e and M_0 are, respectively, the moisture content at any time, the equilibrium moisture content, and the initial moisture content in % d.b.
M_R was further reduced to M/M_0, given the continuous fluctuation of relative humidity during the drying processes, (DIAMENTE and MUNRO, 1993). Data were analyzed by using Statistica 6.1 (StatSoft Inc., USA) software package. Drying rate constants and model coefficients were calculated according to Levenberg-Marguardt, and the statistical validity of the selected drying models was assessed according to the criteria put forth in Equations 6, 7 and 8 (SACILIK *et al.*, 2010; YURTLU, 2011):

$$P = \frac{100}{N} \sum_{i=1}^{N} \frac{\left| M_{R,ex,i} - M_{R,pre,i} \right|}{M_{R,\exp,i}} \quad (6)$$

$$RMSE = \left[\frac{1}{N} \sum_{i=1}^{N} \left(M_{R,ex,i} - M_{R,pre,i} \right)^2 \right]^{1/2} \quad (7)$$

$$\chi^2 = \frac{\sum_{i=1}^{N} (M_{R,ex,i} - M_{R,pre,i})^2}{N - z} \quad (8)$$

where $M_{R,ex,i}$ is the ith experimental moisture ratio, $M_{R,pre,i}$ is the ith predicted moisture ratio, N is the number of observations, and z is the number of constants.
R^2 was used as the primary comparison criteria. Goodness of fit was also examined based on P, $RMSE$ and χ^2 (YURTLU, 2011).

Table 1. Selected drying models.

Model no	Model name	Model	References
1	Page	$M_R = \exp(-kt^m)$	Agrawal and Singh (1977)
2	Logarithmic	$M_R = a \exp(-kt) + c$	Yagcioglu *et al.* (1999)
3	Two-term	$M_R = a \exp(-kt) + b \exp(-k_0 t)$	Henderson (1974)
4	Approximation of diffusion	$M_R = a \exp(-kt) + (1 - a) \exp(-kbt)$	Yaldiz and Ertekin (2001)
5	Midilli *et al.*	$M_R = a \exp(-kt^m) + bt$	Midilli *et al.* (2002)

2.5. Rehydration capacity and color parameters of pear slices

Rehydration capacity is of paramount importance for dried products. In this study, rehydration capacity was determined by immersing 10 g of dried pear slices into 85 °C water for 3 min, drying the pear surfaces with paper towels, and measuring the mass of the rehydrated sample using an electronic digital scale (±0.001 g), with rehydration capacity expressed as the ratio of the mass of the rehydrated sample to the mass of the dried sample (PRAKASH *et al.*, 2004).
Color properties are also among the important quality parameters of dried fruits (ELICIN and SACILIK, 2005). In this study, color measurements were obtained from 5 points on the surface of each pear sample using a Minolta CR-300 Chromameter, and the average measurement was calculated. Hue angles and color differences between raw and dried samples were calculated with the help of Equation 9 and Equation 10 (SACILIK and UNAL, 2005):

$$H = \tan^{-1}\left(\frac{b^*}{a^*}\right) \quad (9)$$

$$\Delta E = \sqrt{(L_0 - L_f)^2 + (a_0 - a_f)^2 + (b_0 - b_f)^2} \quad (10)$$

where H is the hue angle°, ΔE is the color difference, L_0, a_0 and b_0 are the color lightness, green-red and blue-yellow values of raw pear slices, and L_f, a_f and b_f are the color lightness, green-red and blue-yellow values of dried pear slices.

3. RESULTS AND DISCUSSION

3.1 Hot-air drying curves of pears

Pear (*cv. Ankara*) drying characteristics are presented in Figs. 2, 3 and 4 according to drying temperature and pre-treatment procedures. As the Figs show, pear moisture content was observed to decrease continuously over time from 572.04% d.b. to between 4.43% d.b. and 19.22% d.b. Moisture content was significantly affected by drying temperature, citric-acid treatment, and blanching. Untreated pears required drying times of 1,560, 1,080 and 900 min at 55, 65 and 75 ºC, respectively, to reach their final moisture content, as compared to 1,140, 900 and 660 min for pear samples pre-treated with citric acid and 840, 720 and 600 min for samples blanched in hot water. These Figs. – representing decreases in drying time of 46% at 55ºC and 33% at 65ºC and 75ºC for blanched pears as compared to untreated pears – demonstrate that water diffusion increases with pre-treatment. Similar results have been reported by DOYMAZ (2010) for Amasya red apples, by DOYMAZ (2013) for pear, by VARDIN and YILMAZ (2018) for pomegranate arils, and by PANDEY *et al.* (2019) for green peas.

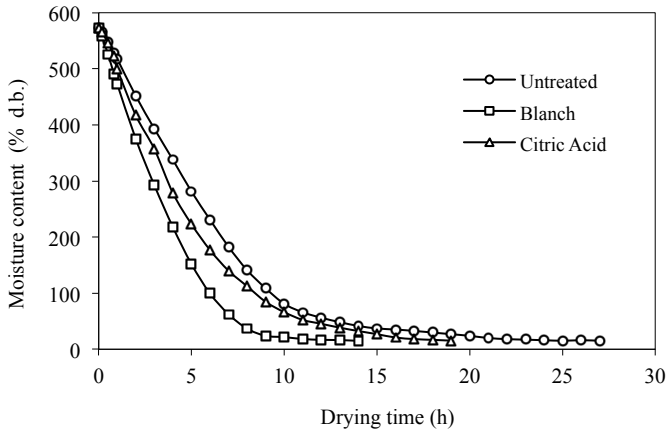


Figure 2. Drying curves for 'Ankara' pear at 55°C.

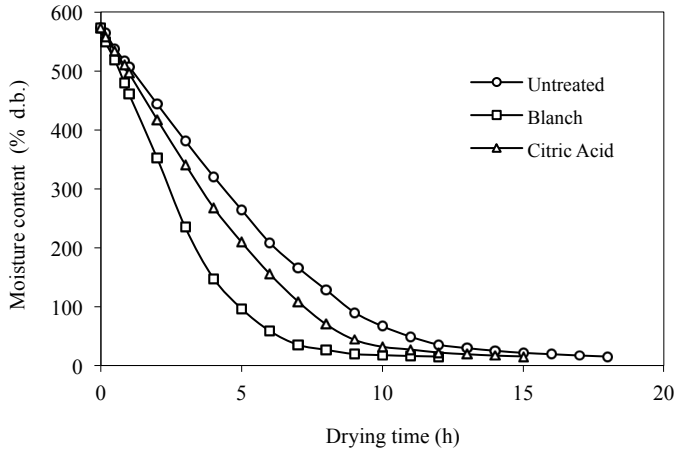


Figure 3. Drying curves for 'Ankara' pear at 65°C.

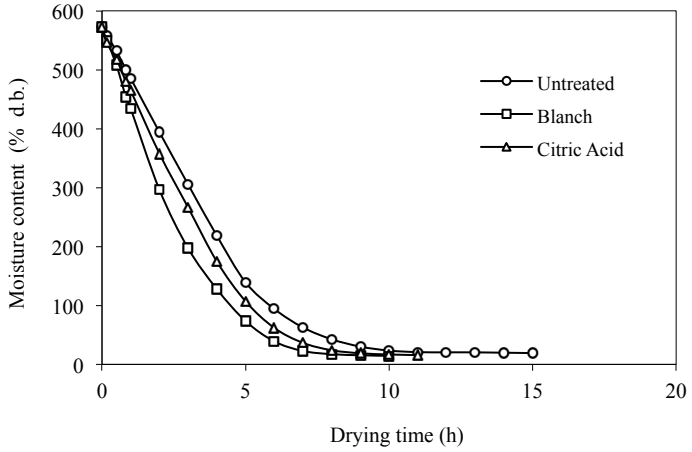


Figure 4. Drying curves for 'Ankara' pear at 75°C.

3.2. Effective diffusivity and activation energy

From Equation 3, a plot of ln(M_R) vs. the time provides a straight line with a slope *s* of:

$$s = \frac{\pi^2 D_{eff}}{4h^2} \tag{11}$$

The highest D_{eff} values were obtained for the blanched pear samples, followed by the citric-acid treated and the untreated samples (Table 2). D_{eff} values were observed to increase with increases in air temperature due to accelerated moisture diffusion, which could be due to an increase in water permeability caused by cracks in the sample surfaces. The D_{eff} values obtained for 'Ankara' pear slices in the present study are comparable to values ranging from 1.59×10^{-10} to 7.64×10^{-10} m^2/s obtained for 'd'Anjou' pear at 40 ºC - 80 ºC (PARK *et al.*, 2002), from 2.27×10^{-10} to 4.97×10^{-10} m^2/s for "organic apple" at 40 ºC - 60 ºC (SACILIK and

ELICIN, 2006), from 2.66×10^{-10} to 4.56×10^{-10} m^2/s for Üryani plum at 50ºC - 70ºC (SACILIK *et al.*, 2006), from 0.85×10^{-10} to 2.18×10^{-10} m^2/s for pear slices at 55ºC - 75ºC (DOYMAZ, 2012), and from 8.56×10^{-11} to 2.25×0^{-10} m^2/s for 'Deveci' pear slices at 50ºC - 71ºC (DOYMAZ, 2013). Activation energy values were obtained by plotting $\ln(D_{eff})$ vs. 1/T (Fig. 5), which yielded a straight line indicating an Arrhenius dependence on temperature. Using Equation 4, activation energy values for untreated pear samples, pear samples treated with citric acid, and blanched pear samples were obtained using Equations 12, 13 and 14, respectively, as follows:

Table 2. Effective diffusivity for 'Ankara' pear at various air temperatures.

	Air temperature, °C	D_{eff} x10^{10}, m^2/s	R^2
Untreated	55	1.12	0.9747
	65	1.56	0.9878
	75	2.26	0.9713
Citric acid	55	1.45	0.9951
	65	1.90	0.9839
	75	2.61	0.9821
Blanched	55	2.12	0.9709
	65	2.44	0.9756
	75	2.94	0.9825

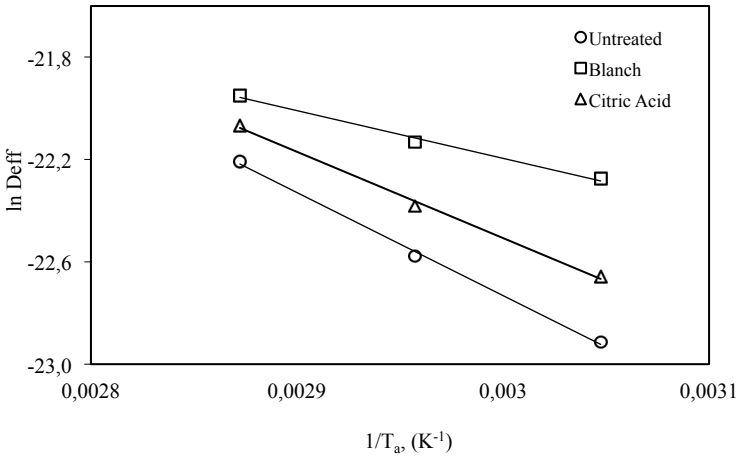


Figure 5. Arrhenius-type relationship between D_{eff} and T_a.

Untreated samples:

$$D_{eff} = 2.36\times10^{-5}\exp\left(-\frac{4026.23}{T}\right) \quad (12)$$

(R^2=0.9979),
Citric acid-treated samples:

$$D_{eff} = 4.15\times10^{-6}\exp\left(-\frac{3371.19}{T}\right) \quad (13)$$

(R^2=0.9965),
Blanched samples:

$$D_{eff} = 6.19\times10^{-8}\exp\left(-\frac{1865.72}{T}\right) \quad (14)$$

(R^2=0.9916).

The highest value of activation energy was obtained for the untreated samples (E_a=33.48 kJ/mol), followed by the citric-acid treated samples (E_a=28.03 kJ/mol) and blanched samples (E_a=15.51 kJ/mol). These values are in line with the range (15-40 kJ/mol) specified by Rizvi (1986) for various foods.

3.3. Parameter estimation

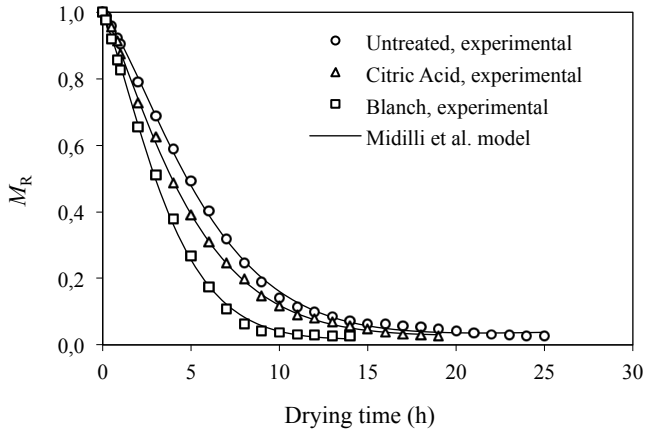
Estimated values of drying models and comparison criteria (R^2, *P*, *RMSE* and χ^2) are given in Table 3. Selected models offered a good fit to data. Of the 5 models examined, the MIDILLI *et al.* had highest R^2 and lowest *P*, *RMSE* and χ^2 values, indicating it to be the best model in terms of fitness to data. Comparisons of the experimental data and the predicted moisture ratios obtained using the MIDILLI *et al.* model for 'Ankara' pear slices at 55, 65 and 75°C are presented in Fig 6. As the Fig. show, there is very good conformity between the actual and the predicated data, confirming the goodness of fit of the MIDILLI *et al.* model.

3.4. Quality parameters (rehydration and color retention)

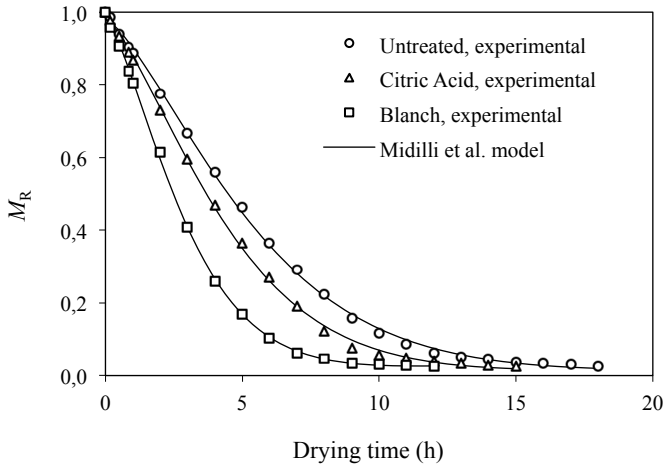
Air temperature as well as pre-treatment, either with a citric-acid solution or by blanching, significantly affected the rehydration capacity of 'Ankara' pears (Table 4). The highest rehydration values were observed for the blanched pear slices dried at 75ºC. At every temperature examined, the blanched pear slices showed the greatest rehydration capacity, followed by the samples treated with citric acid and the untreated samples. Increases in air temperatures during drying resulted in increases in rehydration capacity, with increases of 5.43%, 4.64% and 10.54%, respectively, for untreated samples, samples treated with citric acid, and blanched samples when temperatures were increased from 55 to 75 ºC. This finding can be explained by an increase in the rate of moisture removal with increases in air temperature, which leads to less shrinkage and thus an accelerated rate of rehydration. Similar results have been reported by AMIRIPOUR *et al.* (2015), HEBDA *et al.* (2019) and SINGH *et al.* (2006).
Table 5 shows the Hunter color values for pears by air temperature and pre-treatment procedures. The lowest *a** values and the highest *L** and *H* values were observed at 55°C regardless of pretreatment. *H* and *L** values decreased with increases in temperature, whereas *a** values increased with increases in temperature, demonstrating that browning occurred as a result of temperature increases. Similar results were reported by WANG and CHAO (2003), ELICIN and SACILIK (2005) and SACILIK and ELICIN (2006).

Table 3. Statistical criteria of the models for 'Ankara' pear.

	°C	Model no	Model coefficients	R^2	P (%)	RMSE	χ^2
Untreated	55	1	k=0.0985; m=1.2518	0.9974	25.54	$1.84x10^{-2}$	$3.65x10^{-4}$
		2	a=1.0627; k=0.164; c=-0.0126	0.9932	23.20	$3.06x10^{-2}$	$10.15x10^{-4}$
		3	a=-0.0405; k_0=0.5464; b=1.4754; k_1=0.2142	0.9980	21.22	$1.69x10^{-2}$	$3.11x10^{-4}$
		4	a=6.901; k=0.113; b=0.9935	0.9915	28.90	$3.42x10^{-2}$	$12.67x10^{-4}$
		5	a=0.99; k=0.082; m=1.3664; b=0.0015	0.9993	9.65	$1.02x10^{-2}$	$1.14x10^{-4}$
	65	1	k=0.0982; m=1.3124	0.9988	10.84	$1.33x10^{-2}$	$1.97x10^{-4}$
		2	a=1.12; k=0.1527; c=-0.0858	0.9942	26.81	$2.98x10^{-2}$	$9.92x10^{-4}$
		3	a=05257.; k_0=0.1863; b=0.5265; k_1=0.1863	0.9894	29.24	$4.14x10^{-2}$	$19.24x10^{-4}$
		4	a=-7.4478; k=0.3419; b=0.908	0.9920	10.02	$1.34x10^{-2}$	$1.99x10^{-4}$
		5	a=0.9795; k=0.0836; m=1.395; b=0.00061	0.9992	7.24	$1.15x10^{-2}$	$1.49x10^{-4}$
	75	1	k=0.156; m=1.3316	0.9962	44.90	$2.24x10^{-2}$	$5.47x10^{-4}$
		2	a=1.0545; k=0.273; c=0.0065	0.9904	35.97	$3.65x10^{-2}$	$14.54x10^{-4}$
		3	a=2.2259; k_0=0.3951; b=-1.2366; k_1=0.6568	0.9965	42.11	$2.24x10^{-2}$	$5.51x10^{-4}$
		4	a=9.9874; k=0.1899; b=0.9696	0.9878	50.74	$4.11x10^{-2}$	$18.46x10^{-4}$
		5	a=0.9826; k=0.1336; m=1.4489; b=0.00165	0.9994	9.72	$0.96x10^{-2}$	$1.03x10^{-4}$
Citric acid	55	1	k=0.1352; m=1.1923	0.9991	12.76	$1.09x10^{-2}$	$1.32x10^{-4}$
		2	a=1.0648; k=0.1897; c=-0.0213	0.9970	13.48	$2.08x10^{-2}$	$4.83x10^{-4}$
		3	a=0.5246; k_0=0.2011; b=0.5246; k_1=0.2009	0.9965	8.83	$2.31x10^{-2}$	$5.99x10^{-4}$
		4	a=-5.7317; k=0.121; b=1.0676	0.9955	17.59	$2.57x10^{-2}$	$7.36x10^{-4}$
		5	a=1.0034; k=0.131; m=1.2332; b=0.0012	0.9997	3.73	$0.64x10^{-2}$	$0.45x10^{-4}$
	65	1	k=0.1254; m=1.3216	0.9987	12.84	$1.40x10^{-2}$	$2.23x10^{-4}$
		2	a=1.1207; k=0.1869; c=-0.0836	0.9937	29.66	$3.20x10^{-2}$	$11.74x10^{-4}$
		3	a=0.4054; k_0=0.2271; b=0.6489; k_1=0.2271	0.9891	30.74	$4.35x10^{-2}$	$21.85x10^{-4}$
		4	a=-6.8642; k=0.4215; b=0.899	0.9987	11.83	$1.44x10^{-2}$	$2.39x10^{-4}$
		5	a=0.9798; k=0.1081; m=1.4065; b=0.0007	0.9991	8.66	$1.24x10^{-2}$	$1.78x10^{-4}$
	75	1	k=0.198; m=1.3054	0.9982	13.01	$1.69x10^{-2}$	$3.41x10^{-4}$
		2	a=1.1148; k=0.2576; c=-0.0823	0.9931	30.98	$3.49x10^{-2}$	$14.66x10^{-4}$
		3	a=0.5247; k_0=0.3119; b=0.5247; k_1=0.3119	0.9886	31.51	$4.67x10^{-2}$	$26.72x10^{-4}$
		4	a=-5.384; k=0.147; b=1.1161	0.9929	32.32	$3.53x10^{-2}$	$14.97x10^{-4}$
		5	a=0.9738; k=0.1699; m=1.4089; b=0.0011	0.9988	8.65	$1.48x10^{-2}$	$2.69x10^{-4}$
Blanched	55	1	k=0.1757; m=1.2674	0.9987	17.28	$1.41x10^{-2}$	$2.29x10^{-4}$
		2	a=1.0863; k=0.2434; c=-0.0475	0.9944	32.44	$3.03x10^{-2}$	$10.62x10^{-4}$
		3	a=-0.0407; k_0=0.2745; b=1.01; k_1=0.2746	0.9922	26.57	$3.71x10^{-2}$	$16.11x10^{-4}$
		4	a=-5.789; k=0.4965; b=0.8918	0.9988	16.57	$1.41x10^{-2}$	$2.29x10^{-4}$
		5	a=0.9843; k=0.1584; m=1.3390; b=0.00098	0.9990	9.79	$1.32x10^{-2}$	$2.03x10^{-4}$
	65	1	k=0.2161; m=1.2946	0.9987	17.90	$1.44x10^{-2}$	$2.45x10^{-4}$
		2	a=1.0827; k=0.2986; c=-0.0391	0.9927	28.95	$3.56x10^{-2}$	$15.06x10^{-4}$
		3	a=-0.4799; k_0=0.3298; b=0.5734; k_1=0.3298	0.9911	21.92	$4.11x10^{-2}$	$20.21x10^{-4}$
		4	a=-4.5469; k=0.6298; b=0.859	0.9989	16.06	$1.37x10^{-2}$	$2.21x10^{-4}$
		5	a=0.9813; k=0.1921; m=1.3988; b=0.0021	0.9995	2.65	$9.61x10^{-2}$	$1.11x10^{-4}$
	75	1	k=0.281; m=1.2235	0.9996	11.58	$0.83x10^{-2}$	$0.82x10^{-4}$
		2	a=1.08; k=0.3377; c=-0.0442	0.9963	24.31	$2.55x10^{-2}$	$7.96x10^{-4}$
		3	a=-2.7851; k_0=0.7071; b=3.7845; k_1=0.5749	0.9996	11.08	$0.86x10^{-2}$	$0.93x10^{-4}$
		4	a=0.1349; k=0.3568; b=0.9989	0.9914	28.21	$3.91x10^{-2}$	$18.68x10^{-4}$
		5	a=0.9936; k=0.2729; m=1.257; b=0.00103	0.9996	7.70	$0.78x10^{-2}$	$0.75x10^{-4}$



(a)



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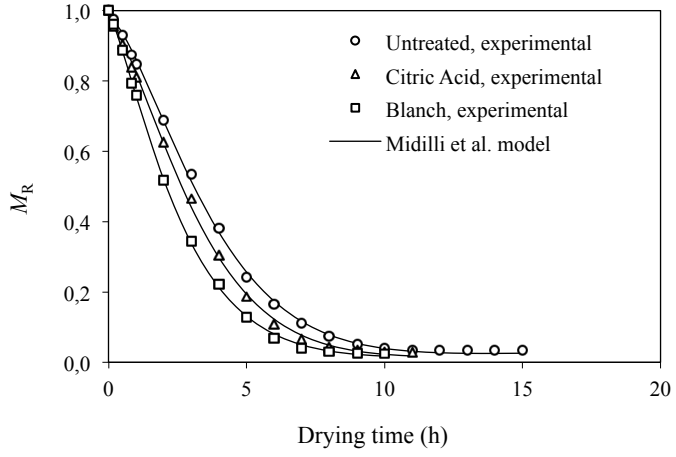

(c)

Figure 6. Conformity of the Midilli *et al.* for 'Ankara' pear at 55°C (a), at 65°C (b) and at 75°C (c).

Table 4. Rehydration capacity for 'Ankara' pear at various temperatures.

	Air temperature, °C	Rehydration capacity
Untreated	55	3.31
	65	3.35
	75	3.49
Citric acid	55	3.45
	65	3.54
	75	3.61
Blanched	55	3.51
	65	3.69
	75	3.88

Table 5. Color values for 'Ankara' pear at various temperatures.

	Air temperature	Hunter color values				
	°C	*L**	*a**	*b**	*ΔE*	*H°*
Untreated	55	72.69	5.04	33.27	10.09	81.40
	65	68.04	4.46	26.33	14.62	80.13
	75	66.38	6.57	29.22	15.43	77.50
Citric acid	55	78.16	2.91	34.03	8.97	85.25
	65	75.69	5.79	34.96	9.41	80.58
	75	67.71	5.20	31.98	12.85	80.56
Blanched	55	69.45	6.26	30.73	12.54	78.43
	65	64.72	6.99	30.77	16.61	77.65
	75	63.98	7.69	34.37	16.29	77.60

In terms of pre-treatment, the present study found samples pre-treated with citric acid had higher H and L^* values as compared to blanched and untreated samples at each air temperature tested. Moreover, the samples treated with citric acid had smaller ΔE values than both the blanched and untreated samples, indicating that pre-treatment with citric acid helped to preserve the original color of pear slices. Overall, the natural color of fresh pear was best preserved when slices were pre-treated with a citric-acid solution and dried at the lowest air temperature (55°C).

4. CONCLUSIONS

In conclusion, drying temperature and pre-treatment with either a citric-acid solution or by blanching in hot water significantly affected the moisture content, rehydration capacity and color parameters of 'Ankara' pear slices. Blanched pear slices required shorter drying times than samples treated with citric acid as well as untreated samples. When compared to untreated pears, blanched pear slices required 46% less time for drying at 55ºC and 33% less time at 65ºC and at 75ºC. D_{eff} values were observed to decrease with decreases in

temperature and were lower for untreated pears than for pre-treated pears. E_a values were highest for untreated samples (33.48 kJ/mol), followed by citric acid-treated (28.03 kJ/mol) and blanched samples (15.51 kJ/mol). Based on the evaluated statistical criteria, the MIDILLI *et al.* model showed the best fit to the drying data of all the models tested. Rehydration capacity of pear slices was seen to decrease with decreases in drying temperature. The natural color of fresh pear slices was best retained when the samples were pre-treated with citric acid and dried at the lowest air temperature.

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