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Bacterial conjugated linoleic acid bio-fortification of synbiotic yogurts using *Propionibacterium freudenreichii* as adjunct culture

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PAPER

Abstract

In this study, *Propionibacterium freudenreichii* was used for *in situ* production of conjugated linoleic acid (CLA) in yogurt. Firstly, effects of process variables, including strain type, percentage of milk fat, percentage of inoculum, quantity of sunflower oil, concentration of inulin, temperature of fermentation and time of storage at 4°C, on production of CLA by *Propionibacterium freudenreichii* were investigated using screening method of the Plackett–Burman design. Then optimisation of CLA production process was conducted using three major factors of milk fat percentage, inulin concentration and storage time at 4°C using central composite design. Analysis of variance established that the models were highly significant ($P \leq 0.05$). The model demonstrated that the production of CLA was affected by these three factors. Optimised CLA production by *Propionibacterium freudenreichii* ssp. *shermanii* in yogurts was achieved after 17 days of storage at 4°C in skim-milk containing 1.75% (w/w) fat and 2.25% (w/v) inulin as prebiotic. Reconfirmation test established that at the highlighted optimum conditions, the highest concentration of produced CLA was 6.4 mg g^{-1} lipid in yogurt, which is a 256% increase in total CLA production, compared with control samples. Results demonstrated that *Propionibacterium freudenreichii* ssp. *shermanii* not only leads to production of synbiotic yogurts containing inulin but also increases CLA production in yogurts.

Keywords: conjugated linoleic acid, probiotic, *Propionibacterium freudenreichii,* yogurt

Introduction

In addition to nutritional and sensory characteristics of food products, health beneficial aspects are other important criteria for consumers to choose food products. One of the best manners to receive essential nutrients with minimum side effects is enrichment of food products (Grunert, 2005). Functional foods play important roles in this area as tendency to consume functional foods has increased recently. Such characteristics are found in a new group of products called synbiotics, which contain probiotics and prebiotics simultaneously (Holzapfel and Schillinger, 2002). Various food products are established as probiotic carriers, of which fermented dairy products, such as yogurt and cheese, include the largest proportion in research and marketing (Pandey and Mishra, 2015). Propionic acid bacteria (PAB) are widely applied as beneficial probiotic bacteria in several food technologies

(Zárate *et al.*, 2011) because of their ability to produce important metabolites, for instance, propionic acid (Van Wyk *et al.*, 2018), folate (Rad *et al.*, 2016), vitamins B_2, B_7, B_{12} and K (Abou Ayana *et al.*, 2016; Zárate, 2012) and bacteriocins (Ahmadi *et al.*, 2015) are used in industrial and commercial scales (Farhadi *et al.*, 2012; Kouya *et al.*, 2008). Use of PAB in production of dairy products such as yogurt increases product viscosity through the production of exopolysaccharides and inhibits growth of undesirable microorganisms in the product through the production of propionic acid and bacteriocins. This increases shelf life of the product. In addition, growth of PAB does not interfere with the growth of lactic acid bacteria (LAB) in dairy products (Ekinci and Gurel, 2008; Gorret *et al.*, 2001).

Conjugated linoleic acid (CLA) is another valuable metabolite produced by PAB in culture media (Van Wyk *et al.*, 2018; Yang *et al.*, 2017). In fact, CLA is a fatty acid naturally found in milk fats and dairy products such as yogurt, butter and cheese (Van Wyk *et al.*, 2018). The compound belongs to a group of omega-6 fatty acids, and is a geometric isomer of linoleic acid (LA; Yang *et al.*, 2017). Beneficial properties of CLA include preventing increase of body fats (Corbo *et al.*, 2014), anti-carcinogenesis properties (colon, prostate, skin and breast cancers) (Masso-Welch *et al.*, 2004), antioxidant properties (Zárate, 2012), lowering of blood serum cholesterol (Hernandez, 2013), anti-inflammation properties (Olson *et al.*, 2017), anti-diabetic properties (Balci Yuce *et al.*, 2017) and regulation of the system. Daily intake of 3 g of CLA is recommended to prevent cancers; however, the CLA content of dairy products is only 0.5–9.9 mg g^{-1} of fats (Zárate, 2012). Commercially, most of CLA is produced through the chemical isomerisation of LA, in which harmful by-products are produced as well. In the chemical production method, various isomers of CLA are produced (Ogawa *et al.*, 2001). Studies have verified that c9t11-CLA, t9t11-CLA and t10c12-CLA isomers prevent diseases in the human body and include medical uses (Yang *et al.*, 2017). Dairy PAB has the potential to convert unsaturated fatty acids cis-9 cis-12 LA (c9c12-18:2) to cis9-trans-11 (c9t11-18:2), trans-10-cis-12 (t10c12-18:2) and trans-9-cis-11(t9c11-18:2) conjugate isomers (Hennessy *et al.*, 2012). Thus, it is possible to produce dairy products with high CLA levels by developing products fermented by PAB, which produces increased CLA levels by converting LA present in milk to CLA.

Environmental and growth factors greatly affect CLA production in dairy products (Yang *et al.*, 2017). Several studies have been conducted on the effects of process variables on microbial production of CLA, including probiotic strains (*Lactobacillus* sp., *Bifidobacterium* sp., *Propionibacterium* (*P.*) sp., *Leuconostoc* sp., *Lactococcus* sp., *Enterococcus* sp. and *Pediococcus* sp.) (Fukuda *et al.*, 2006; Kim, 2003; Ross *et al.*, 2010), inoculum size (Yang *et al.*, 2017), pH-value (Cousin *et al.*, 2016), incubation and fermentation temperatures (Khan *et al.*, 2011), added prebiotics (Ogawa *et al.*, 2001), LA-rich sources (Xu *et al.*, 2004), dissolved oxygen (Kim *et al.*, 2000) and storage time at 4°C (Akalin *et al.*, 2007). Therefore, optimisation of conditions is critical for the growth and production of CLA by PAB (Khodaiyan *et al.*, 2008).

The aim of this study was to investigate factors affecting CLA production in yogurts by *P. freudenreichii* ssp. *freudenreichii* and ssp. *shermanii* using the Plackett–Burman design (PBD). In addition, effect of variables (bacterial strains, milk fat concentration, inoculum percentage, prebiotic (inulin) concentration, sunflower oil quantity, fermentation temperature and storage time at 4°C) on production of CLA was investigated. To optimise the most important affecting factors, response surface methodology (RSM) design was used in yogurts containing *P. freudenreichii* ssp. *shermanii* for production of CLA.

Materials and Methods

Materials

Skim-milk powder and 40% (w/w) fat cream were kindly gifted by Pak Dairy, Tehran, Iran. Inulin powder with an average degree of polymerisation of ≥25 was provided by Ava Salamat Javid, Tehran, Iran. Sunflower oil (Margarine Foods, Tehran, Iran) was purchased from supermarkets. CLA standard was purchased from Sigma, St. Louis, MO, USA. All analytical reagents and chemicals were purchased from Merck, Darmstadt, Germany. All solvents used were of analytical or High Performance Liquid Chromatography (HPLC) grade.

Preparation of cultures

A commercial yogurt starter culture (YoFlex Express 1.0) containing *Streptococcus thermophilus* (ST) and *L. delbrueckii* ssp. *bulgaricus* (LB) was selected because of the mild acid-production activity of PAB used in this study. The YoFlex Express 1.0 was purchased from Chr. Hansen, Horsholm, Denmark, and used based on manufacturer's recommendations. Commercial starter culture (PS-4) containing *P. freudenreichii* ssp. *shermanii* was purchased from Chr. Hansen, Horsholm, Denmark. Cultures were obtained in freeze-dried (DVS) form and stored at –18°C. The PAB (PS-4) was weighed to prepare an initial count of 8 log colony-forming unit (CFU) mL^{-1}. Pre-cultures were prepared by dissolving each culture in 60 mL of sterilised skim-milk and activating them at 42°C for 20 min before use. The *P. freudenreichii* ssp. *freudenreichii* PTCC No. 1674 was provided by the Research and

Technology Department of Ministry of Sciences (Persian Type Culture Collection), Tehran, Iran. The strain was sub-cultured in sodium lactate medium (SLM) containing 1% (v/v) sodium lactate syrup, casein peptone 10 g L^{-1} and yeast extract 10 g L^{-1} at 30°C under micro-aerobic conditions (Grinstead and Barefoot, 1992)

Milk preparation

After preparing of reconstituted milk with 13% (w/v) of commercial skim-milk powder in distilled water (DW), the milk was pasteurised at 90°C for 30 min and cooled in an ice bath to temperature below 35°C to prevent possible heat shocks to probiotic bacteria. For preparing various percentages of milk fats, Pearson square method was used.

Fermentation

In this study, values of independent variables in yogurt samples were calculated based on the design of experiments (PBD and RSM) at each stage. After inoculation, yogurt samples were transferred into 100-mL polypropylene cups, and milks were incubated at 30–43°C (based on the design of experiments) using laboratory oven until a pH of 4.6 was reached. pH values of yogurt samples were determined with a pH meter 605 (Methrohm AG, Herisau, Switzerland). Then samples were quickly cooled using ice bath and stored at 4°C. Three yogurt samples were prepared to verify the model and compare productions of CLA by *P. freudenreichii* ssp. *shermanii*. The control yogurt, which contained traditional yogurt starter cultures (ST and LB) only, was not supplemented with *P. freudenreichii* ssp. *shermanii* (PS4) and prebiotics (inulin). Other samples (YC and PS4) included yogurt starter culture and *P. freudenreichii* ssp. *shermanii*, and in the third yogurt sample (YC, PS4 and inulin), *P. freudenreichii* ssp. *shermanii* was added in addition to traditional starter cultures and 2.27% (w/v) inulin. Fat content of milk in all three yogurt samples was 1.75% (w/w). Analyses were conducted after an overnight storage of yogurt samples and after 7, 16 and 21 days of storage at 4°C.

Count of viable bacteria

Cell count of the starter cultures (ST and LB) and probiotics (PAB) was conducted in duplicate after incubation time. Yogurt samples (1 mL) were added to 9 mL of 0.15% (w/v) sterile peptone water (Merck, Germany) and viable bacteria were counted as formed colonies using the pour plate method. LB and ST were plated in MRS agar and M17 agar (Merck, Germany) (Dave and Shah, 1996). The MRS agar was acidified to pH 5.4 using acetic acid. Sodium lactate agar was used for selective enumeration of PAB (Tharmaraj and Shah, 2003). The incubation temperature for *L. delbrueckii* ssp. *bulgaricus*, *S. thermophilus* and *P. freudenreichii* ssp. *shermanii*, respectively, were 45°C for 72 h, 37°C for 24 h and 30°C for 5–7 days under anaerobic conditions using gas generating pack A (Merck, Darmstadt, Germany), except for *S. thermophilus*.

Lipid extraction and CLA analysis

Extraction of CLA was conducted based on the method by Lin *et al*. (1999), in which yogurt was mixed with chloroform–methanol in a ratio of 2:1 (v/v), and was refrigerated and centrifuged for 6 min at 4,500 × g. The organic phase layer was collected and dehydrated with 0.3 g of sodium sulphate and stored in refrigerator for 24 h. The middle phase was separated from sodium sulphate using decantation and used in experiments. To remove the organic solvents (chloroform-methanol), rotary evaporator was used to dry off. Thereafter, in order to saponify fatty acids 1 mL solution of 1N sodium hydroxide in methanol was added into the solution and then it was incubated at 100°C for 15 min. Then hydrochloric acid solution in methanol was added to methylate present fatty acid, and the mixture was incubated at 60°C for 20 min using water bath. At this stage, 2 mL of distilled water was added and homogenised for 15 min using vortex mixer to release methyl esters from methanol, followed by formation of polar bonds between methanol and water. Then n-hexane was added and homogenised to transfer methyl esters from aqueous phase to organic phase. After removing aqueous phase, anhydrous sodium sulphate was mixed with organic phase and 1 µL of this mixture was injected into gas chromatographic columns (Capillary BP10; Philips Scientific Model 4410, UK) fitted with a flame ionisation detector. The column was 25 m in length and 0.22 mm in diameter with a thickness of 0.25 µm. The initial temperature of the column was 150°C with 1-min holding time, injection temperature was 250°C, final temperature was 230°C with 10-min duration and a temperature ramp of 5°C in 1 min. In this study, the total quantity of CLA (mg g^{-1} lipid) was reported as the sum of the production of two isomers (c9t11-18:2 and t10c12-18:2).

Experimental design

This study was conducted progressively at three levels step by step. As mentioned previously, different factors might affect bio-production of CLA in yogurt samples by PAB. The first optimisation step included identification of variables with significant effects on CLA production by PAB using PBD. After identification of effective and

significant variables in CLA production, effective factors identified at three levels were optimised using central composite design (CCD) under RSM designations. Moreover, the best conditions for independent variables in CLA production by PAB were provided and the quantities of CLA production in PAB yogurt samples were compared with those in control yogurt samples, which only contained starter cultures (YoFlex Express 1.0).

Plackett–Burman design

Effective factors and their levels were selected based on the literature review. The selected variables, including media compositions (e.g. strain type, milk fat percentage (MFP), inoculum percentage, sunflower oil quantity and inulin concentration) and environmental factors (e.g. incubation and fermentation temperatures and storage time at 4°C), are shown in Table 1. High levels (+) and low levels (–) represent two different levels of independent variables.

RSM design

The RSM is a set of statistical techniques for designing experiments, constructing models, assessing effects of factors and searching for the optimal conditions of the factors for optimal responses. In general, RSM is a great tool for optimising conditions when several factors are involved in production of a product (Cousin *et al*., 2016; Grinstead and Barefoot,1992; Khodaiyan *et al*., 2008). A combination of factors that produces a specific optimal response can be identified using design factor and RSM (Khodaiyan *et al.*, 2008). For additional accurate predictions on the optimum conditions of CLA bioproduction and to minimise the number of test sets, CCD under RSM was designed. In this study, all factors were used at three levels (Table 2). Experimental ranges of the three significant variables for CCD trials are shown in Table 2.

Statistical analysis

Statistical analysis of the results was conducted using MINITAB statistical software v.16 (Minitab, USA), and response surface plots were drawn. Data were statistically treated using analysis of variance (ANOVA). All data were presented as the mean value ± standard deviation (SD) of independent experiments on various days. In general, $P \leq 0.05$ was established statistically significant.

RESULTS AND DISCUSSION

Selection of the most important affecting factors using the Plackett–Burman design

The primary purpose of screening experiments is to select important major effects from less important ones.

Table 1. Process variables, selected levels and eight trials of the Plackett–Burmann design to study the impact of seven factors (and finding main variables) on microbial production of CLA in synbiotic yogurt.

Run	Independent variables							Response		
	A Strains	B Milk fat % (w/w)	C Inulin % (w/v)	D Sunflower oil (g L^{-1})	E Inoculum size (%)	F Temperature (°C)	G storage time (days)	cis-9,trans-11 CLA mg g^{-1} lipid	trans-10, cis-12 CLA mg g^{-1} lipid	Experimental total CLA mg g^{-1} lipid	Predicted total CLA mg g^{-1} lipid
1	PFF**	1	0	0.1	1	43	14	4.1 ± 0.11	0.2 ± 0.13	4.3 ± 0.14	4.3
2	PFF	3	0	0	2	30	14	4.4 ± 0.17	ND	4.4 ± 0.17	4.4
3	PFF	3	2	0	1	43	1	4.6 ± 0.09	0.8 ± 0.05	5.4 ± 0.07	5.3
4	PFS*	3	2	0.1	1	30	14	4.5 ±0.11	0.2 ±0.14	4.7 ± 0.14	4.7
5	PFF	1	2	0.1	2	30	1	4.6 ± 0.14	1.1 ± 0.12	5.7 ± 0.05	5.6
6	PFS	3	0	0.1	2	43	1	4.8 ± 0.10	ND§	4.8 ± 0.10	4.8
7	PFS	1	2	0	2	43	14	5 ± 0.09	ND	5.0 ± 0.09	4.9
8	PFS	1	0	0	1	30	1	5.5 ± 0.07	ND	5.5 ± 0.07	5.4

*PFS: *Propionibacterium freudenreichii* ssp. *shermanii* (PS4) (code-1).
**PFF: *Propionibacterium freudenreichii* ssp. *freudenreichii.*
§ND: The amount was less than detection limit.
CLA: conjugated linoleic acid.

Table 2. Main process variables, range and 17 trials of central composite design to study the impact of main and interaction effects on optimisation of microbial production of CLA in synbiotic yogurt.

Run	X1 – Milk fat % (w/w)	X2 – Inulin % (w/v)	X3 – Storage time (days)	cis-9,trans-11 CLA (mg g^{-1} lipid)	trans-10,cis-12 CLA (mg g^{-1} lipid)	Total CLA (mg g^{-1} lipid)
1	1.00	1	1	4.2	ND	4.2
2	3.50	1	1	4	0.1	4.1
3	1.00	3	1	4.1	ND*	4.1
4	3.50	3	1	3.9	0.1	4.0
5	1.00	1	21	5	0.3	5.3
6	3.50	1	21	4.9	0.2	5.1
7	1.00	3	21	4.9	0.9	5.8
8	3.50	3	21	4.7	0.5	5.2
9	1.00	2	11	5.2	0.4	5.6
10	3.50	2	11	4.9	0.4	5.3
11	2.25	1	11	4.8	0.6	5.4
12	2.25	3	11	5.1	0.4	5.5
13	2.25	2	1	4.4	ND	4.4
14	2.25	2	21	5.2	0.6	5.8
15	2.25	2	11	5.2	0.8	6.0
16	2.25	2	11	5.4	0.5	5.9
17	2.25	2	11	5.1	0.7	5.8

*ND: The amount was less than detection limit.
CLA: conjugated linoleic acid.

In this study, Student's *t*-test was conducted to demonstrate significance of each factor (*t*-value = coefficient/standard error (S_b)) (Khosravi-Darani and Zoghi, 2008). The tabulated *t*-value (degree of freedom = 6) at $P \leq 0.05$ was 1.94. Each variable linked to *t*-value higher than the tabulated *t*-value (1.94 for $P \leq 0.05$) was significant. Table 3 refers to statistical calculations of CLA production in yogurt samples by PAB. Results established that MFP, prebiotic (inulin) concentration and storage time at 4°C were significant due to their *t*-values being higher than 1.94. Based on Table 3, addition of 2% (w/v) inulin to yogurts increased the production of CLA. This increase might be due to the prebiotic role of inulin, which was an important factor in growth and maintenance of probiotics and caused longer survival of *P. freudenreichii* during the storage period at 4°C as well as greater production of CLA in yogurt. Mohanty *et al.* (2018) reported that prebiotics, especially inulin, were good candidates of functional foods. Salem *et al.* (2007) demonstrated that addition of 1% inulin to dairy cheese promoted growth and longer survival of existing strains. In another study done by Effat et al. (2019), it was reported that addition of 1–3% prebiotics, such as inulin, to milk increased survival and viability of the probiotic *Propionibacterium* strains. Table 3 shows that storage of yogurt containing *P. freudenreichii* at 4°C for 14 days increased the production of CLA. The PAB may adapt and survive at acidic pH of 2 (Van Wyk *et al.*, 2018). Owing to the fact that yogurt samples containing *P. freudenreichii* had pH higher than 2, *P. freudenreichii* was able to grow and produce CLA during the storage time. Akalin *et al.* (2007) reported increase in CLA production in yogurts during storage for 28 days. In addition, results in Table 3 indicate that yogurt samples containing 1% fat (w/w) with *P. freudenreichii* increased CLA production. Biohydrogenation pathway is also a mechanism for the formation of CLA in yogurts (Ha *et al.*, 1989). In order to convert LA to CLA in this pathway, LA isomerase plays an important role. Starter cultures, such as PAB, did not affect CLA formation without presence of LA. Increase in the proportion of milk fat and LA in yoghurt with *P. freudenreichii* increased production of CLA. Kishino *et al.* (2002) found that *Lactobacillus plantarum* AKU 1009a could produce high content of CLA (3.88 mg mL^{-1}) in nutrient media with 0.06% (w/v) LA. Khosravi-Darani *et al.* (2014) reported that CLA content in probiotic yogurts containing PAB increased by 40% from average 8.01 mg g^{-1} fat in non-treated yogurts to 11.03 mg g^{-1} fat in probiotic yogurts containing grape seed oil as a source of LA.

Optimisation of CLA production using response surface methodology

After selecting the most important affecting factors, central composite design and RSM method were used to

Table 3. Statistical data for analysis of variance of CLA production in yogurt by PAB.[a]

Factors	Coefficient	*t*-value
A (Strains)	–0.025	–0.35
B (Milk fat (%) w/w)	–0.150	–2.14
C (Inulin (%) w/v)	0.225	3.14
D (Sunflower oil, g/L)	–0.100	–1.42
E (Inoculum size, %)	0	0
F (Temperature, °C)	–0.1000	–1.42
G (Storage time, days)	0.375	5.28

[a]A_0 = 4.9 (mean of experimental CLA), standard error, S_b = 0.07, estimated error, S^2_e = 0.04, tabulated *t*-value (degree of freedom 6) at $P \leq 0.05$ is 1.94.
CLA: conjugated linoleic acid; PAB: propionic acid bacteria.

optimise the three factors (MFP, prebiotic concentration and storage time at 4°C). Design matrix for these factors in optimisation sets is described in Table 2. Results of RSM in the form of ANOVA are provided in Table 4. $P < 0.05$ demonstrates that the model terms are significant. The ANOVA results established that quadratic regression for the production of CLA by *P. freudenreichii* ssp. *shermanii* in yogurt models was significant. The lack-of-fit test was insignificant ($P = 0.314$) and only 1.8% of the total variations were not explained by the model (R^2 = 98.2%). The quadratic model was based on Eq. (1):

$$Y = 2.626 + 0.741X_1 + 1.001X_2 + 0.187X_3 - 0.155 (X_1)^2 - 0.242 (X_2)^2 - 0.005 (X_3)^2 - 0.04X_1X_2 - 0.006X_1X_3 + 0.01X_2X_3, \quad (1)$$

where Y, X_1, X_2 and X_3 were equivalent experimental response, MFP, inulin concentration and storage time at 4°C, respectively. Effects of various levels of variables on CLA production in yogurts by *P. freudenreichii* ssp. *shermanii* can be achieved using Eq. (1). Based on *t*-test and *P*-value, Table 4 shows that MFP, inulin concentration and storage time at 4°C significantly affected production of CLA, while the three affecting factors were not significant ($P \leq 0.05$).

Effects of inulin and milk fat percentage on CLA production

Figure 1 shows the effects of MFP, concentration of inulin and storage time at 4 °C in yogurt on production of CLA by *P. freudenriechii* in surface plots. In surface plot, response is plotted for two independent variables at a time, while other variables are fixed. Quantities of fat and free LA in milk and presence of inulin play important roles in survival of probiotic bacteria such as PAB as well as production of CLA in yogurts (Akalin *et al.*, 2007; Xu *et al.*, 2005). Figure 1a shows that increase in MFP up to 2.1% (w/w) increased production of CLA in yogurt by *P. freudenreichii* ssp. *shermanii*; however, production of CLA decreased at higher fat proportions. Results were similar to those established by Wang *et al.* (2007), who reported that the maximum production of CLA (78.8 μg mL^{-1}) was produced by *P. freudenreichii* ssp. *shermanii* in MRS media containing 12 mg mL^{-1} of sunflower oil as a source of LA. However, production of CLA decreased at higher concentrations of sunflower oil. Wang *et al.* (2007) demonstrated that at 9.6 mg mL^{-1} sunflower oil in SLM media, 73.9 μg mL^{-1} CLA was produced by *P. freudenriechii*. Again, the concentration of CLA decreased significantly when concentration of oil was higher than 9.6 mg mL^{-1}.

Table 4. Analysis of variance results for CLA production in yogurt by *P. freudenreichii* ssp. *shermanii*.

Source of variation	Degree of freedom	Sum of squares	Mean square	*P*
Regression	9	7.839	0.871	0.000
Linear	3	4.290	0.769	0.000
Square	3	3.404	1.256	0.000
Interaction	3	0.145	0.048	0.157
Lack of fit	5	0.123	0.024	0.314
Pure error	2	0.020	0.010	
Total	16	7.982		

Factors	Degree of freedom	Coefficient estimate	Standard error	*P*
Intercept	1	2.626	0.391	0.000
X_1	1	0.741	0.270	0.029
X_2	1	1.001	0.368	0.030
X_3	1	0.187	0.023	0.000
X_1^2	1	–0.155	0.055	0.027
X_2^2	1	–0.242	0.087	0.027
X_3^2	1	–0.005	0.000	0.000
X_1X_2	1	–0.040	0.040	0.356
X_1X_3	1	–0.006	0.004	0.182
X_2X_3	1	0.010	0.005	0.088

CLA: conjugated linoleic acid; PAB: propionic acid bacteria.

Nieman (1954) reported that free fatty acids disrupted permeability of cytoplasmic membranes in gram-positive bacteria and negatively affected the production of CLA. Wang *et al.* (2007) reported antibacterial activity of LA. Other studies have demonstrated that free fatty acids have negative and inhibitory effects on production of CLA by bacteria such as *Lactobacillus plantarum*, *P. freudenreichii* and *Lactobacillus* spp. (Alonso *et al.*, 2003; Lin, 2000; Lin *et al.*, 1999). As shown in Fig. 1a, production of CLA by *P. freudenreichii* ssp. *shermanii* in yogurts increased with increase in the concentration of inulin to nearly 2% (w/v). Increase in concentration

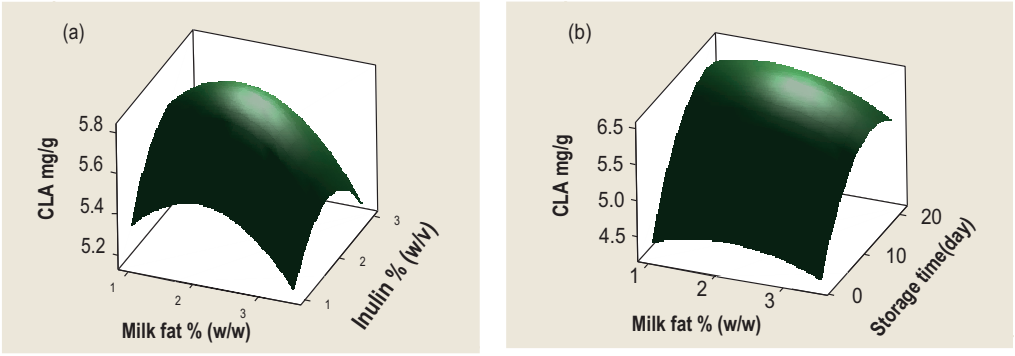

Figure 1. Surface plot of interactive effect on CLA production in yogurt by *P. freudenreichii* ssp. *shermanii*. (a) Effect of inulin and milk fat percentage; (b) effect of storage time of yogurt at 4°C and milk fat percentage.

of inulin by 2% (w/v) or more decreased production of CLA. The figure also shows that high concentrations of inulin had negative effects and decreased the production of CLA. Addition of high concentrations of inulin to yogurts favoured further survival of yogurt starter culture bacteria, resulting in greater decrease in yogurt pH. At lower pH, probiotic bacteria, such as PAB, have less ability to grow and function and hence CLA production decreases by these bacteria. Results of this study are similar to the results of a study done by Effat *et al.*(2019), who reported that increasing inulin concentration in yogurts from 3% to 5% decreased survival rate of probiotic bacteria.

In another study performed by Akalin *et al.* (2007), significant increase in CLA levels was reported when fructooligosaccharides (FOS) were added to yogurts and a 2.90-fold increase was observed in total CLA production in yogurts manufactured with 2% FOS using *Bifidobacterium animalis*.

Effects of yogurt storage time at 4°C and MFP on CLA production

Figure 1b shows that at a constant MFP, production of CLA in yogurts increased with increasing storage time at 4°C. Increase in the concentration of CLA continued until day 16 of storage of yogurt at 4°C, and then concentration of CLA decreased mildly. Studies have been conducted on the effects of yogurt storage time at 4°C on CLA production by different probiotics with various results. The results obtained by Boylston and Beitz (2002) indicated no significant change in yogurts' CLA content during storage for 7 days. In another study, Shantha *et al.* (1995) also showed stability in yogurts' c9t11-CLA isomer concentration at refrigerated storage for 42 days. In a study done by Akalin *et al.* (2007), relative decrease was reported in the concentration of c9t11-CLA isomer after 28 days. The major reason for decrease in yogurts' CLA concentration at storage time included oxidative

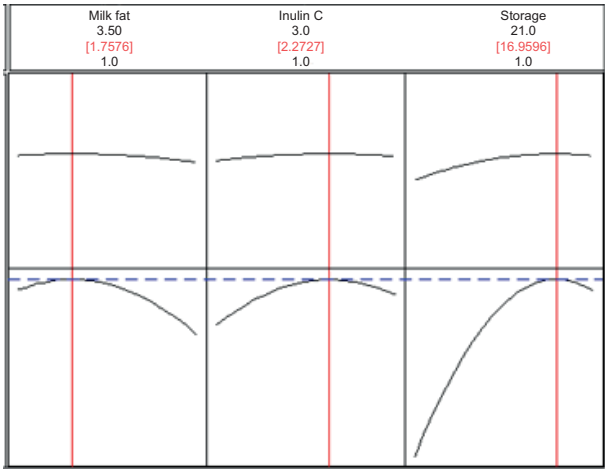

Figure 2. Optimisation plot of CLA production in yogurt by *P. freudenreichii* ssp. *shermanii*.

reactions that caused destruction of conjugated double bond system. Figure 2 points the best conditions for the production of CLA in yogurts by *P. freudenreichii* ssp. *shermanii*. The best values for the three variables of MFP (X_1), inulin concentration (X_2) and storage time at 4°C (X_3) included 1.75% (w/w), 2.27% (w/v) and 17 days, respectively; the highest CLA production by *P. freudenreichii* ssp. *shermanii* was seen in yogurts containing inulin (X_2).

Verification of the model

To verify the model, yogurt samples were prepared under optimal conditions of MFP (1.75% w/w), inulin concentration (2.27% w/v) and storage time at 4°C (~17 days) in three replicates, and the quantity of CLA in yogurts containing *P. freudenreichii* ssp. *shermanii* under optimal conditions was compared with two other yogurt samples from Section 2.4. The highest quantity of CLA included 6.4 ± 0.2 mg g^{-1} lipid. Model and regression didn't establish significant lack of fit between experiments and

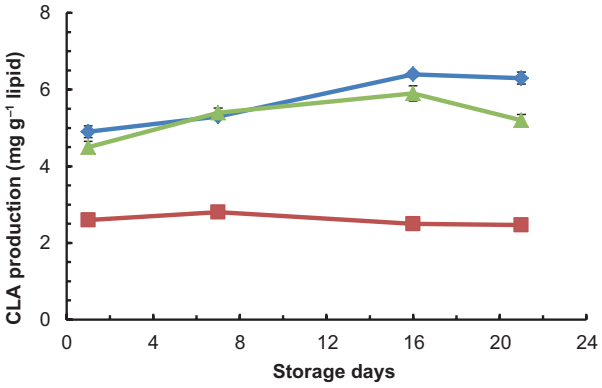

Figure 3. CLA production during storage of yogurt samples at 4°C. Yogurts: YC (■), YC + PS4 (▲), YC + PS4 + inulin (◆).

predicted values of CLA production by *P. freudenreichii* ssp. *shermanii* in yogurts (6.70 mg g^{-1} lipid; Fig. 3).

As seen in Fig. 3, the highest production rate of CLA occurred in yogurt samples containing *P. freudenreichii* ssp. *shermanii*, compared with control yogurt within the first 24 h of storage. This production rate of CLA was equal to 4.9 and 4.5 mg g^{-1} lipid, respectively, for the yogurt samples of *P. freudenreichii* ssp. *shermanii* and inulin and those without inulin, while this value of CLA was 2.6 mg g^{-1} lipid for control yogurts. Similar results were reported in a study done by Wang *et al.* (2007), which resulted in the highest production of CLA in three culture media of SLM, MRS and skim-milk at 24 h. Figure 3 shows that on early days of storage of yogurt samples (up to day 6), no significant differences were seen in the production of CLA by *P. freudenreichii* ssp. *shermanii* for yogurt samples (with or without inulin), with 189% and 191% increase in production of CLA, respectively, compared with control yogurt samples. On day 16 of storage, quantity of CLA in yogurts containing inulin reached to 6.4 mg g^{-1} lipid, increasing by 256%, compared with control samples. For yogurts without inulin, this increase was 239%. In yogurt samples containing *P. freudenreichii* and inulin, decreased concentration of CLA was observed after day 16, similar to the results of optimisation shown in Fig. 2. As previously stated, oxidative reactions that destroyed conjugated double bond systems were the major reasons for decrease in CLA concentration.

Microbiological viable count analysis

Bacterial count results of the three yogurt samples produced using the co-culture method during 3 weeks are compared with each other in Table 5. Viable counts of *S. thermophilus* in control yogurt samples without inulin during 21 days of storage decreased from 9.41 log CFU mL^{-1} on day 1 to 8.70 log CFU mL^{-1} on day 21. In this yogurt sample, a decrease in *L. delbrueckii* ssp. *bulgaricus* was seen from 8.19 log CFU mL^{-1} to 5.87 log CFU mL^{-1}, which was much higher for *L. delbrueckii* ssp. *bulgaricus* than for *S. thermophilus* in all samples. Low storage temperatures and over acidification have been reported for this decrease (Ekinci and Gurel, 2008).

Table 5. Viable cell count of starter cultures in fermented skim-milk during 21 days of storage at 4°C.[a]

Viable count	Storage time (days)	YC (log CFU mL^{-1})	YC + PS4 (log CFU mL^{-1})	YC + PS4 + Inolin (log CFU mL^{-1})
Streptococcus thermophilus	1	9.41 ± 0.02	9.43 ± 0.02	9.35 ± 0.04
	7	9.08 ± 0.03	9.42 ± 0.04	9.19 ± 0.07
	16	9.04 ± 0.06	9.31 ± 0.01	9.27 ± 0.02
	21	8.70 ± 0.05	8.76 ± 0.01	8.64 ± 0.1
Lactobacillus delbrueckii ssp. *bulgaricus*	1	8.19 ± 0.08	8.04 ± 0.04	8.32 ± 0.05
	7	7.12 ± 0.09	7.12 ± 0.09	8.02 ± 0.09
	16	6.43 ± 0.03	7.08 ± 0.06	7.07 ± 0.01
	21	5.87 ± 0.04	6.19 ± 0.02	6.08 ± 0.05
P. freudenreichii ssp. *shermanii*	1	—	9.18 ± 0.04	9.32 ± 0.5
	7	—	7.97 ± 0.01	9.05 ± 0.03
	16	—	6.16 ± 0.05	8.00 ± 0.01
	21	—	5.98 ± 0.08	6.33 ± 0.09

[a]Mean ± standard deviation (SD).
YC: yogurt starter culture containing *Streptococcus thermophiles* and *Lactobacillus delbrueckii* ssp. *bulgaricus*.
YC + PS4: yogurt starter culture containing *Streptococcus thermophiles, Lactobacillus delbrueckii* ssp. *bulgaricus* and *P. freudenreichii* ssp. *shermanii*.
YC + PS4 + inulin: yogurt starter culture containing *Streptococcus thermophiles, L. delbrueckii* ssp. *bulgaricus, P. freudenreichii* ssp. *shermanii* and 2.25% inulin added to yogurt sample.
CFU: colony-forming unit.

Similar results are reported from other studies (Ekinci and Gurel, 2008; Güler-Akin and Akin, 2007; Ranadheera *et al.*, 2012). Results presented in Table 5 indicate that a similar decrease was observed in the viable count of *S. thermophiles* and *L. delbrueckii* ssp. *bulgaricus* during 21 days of storage in yogurt containing *P. freudenreichii* ssp. *shermanii* (YC and PS4). This decrease was less pronounced for *L. delbrueckii* ssp. *bulgaricus.* Results demonstrated that addition of *P. freudenreichii* ssp. *shermanii* to yogurt samples did not affect negatively yogurt starter cultures (ST and LB). Comparison of yogurt samples with and without inulin demonstrated that presence of prebiotics, such as inulin, could significantly affect the count number of *P. freudenreichii* ssp. *shermanii*. As shown in Table 5 for yogurts containing inulin (YC, PS4 and inulin), number of *P. freudenreichii* ssp. *shermanii* after 16 days of storage was 8.00 log CFU mL^{-1}. In yogurts without inulin, this value was 6.16 log CFU mL^{-1}. Other studies (Capela *et al.*, 2006; Oliveira *et al.*, 2009) have reported positive effects of prebiotics, such as polydextrose, oligofructose and maltodextrin, on the survival of probiotics.

Conclusions

In this study, *P. freudenreichii* ssp. *shermanii* was used with traditional yogurt starter cultures (ST and LB) to enrich and produce CLA in yogurts. Results from PBD design demonstrated that three factors of MFP, inulin concentration and storage time at 4°C significantly affected CLA production in yogurts by *P. freudenreichii* ssp. *shermanii* using RSM design and optimising conditions of the three highlighted factors. The highest production of CLA (6.4 mg g^{-1} lipid) in yogurts was achieved with 1.75% (w/w) of fat, 2.25% (w/v) of inulin and 17 days of storage at 4°C, establishing a 256% increase in total CLA production compared with control yogurt samples. In conclusion, results of this study have revealed that *P. freudenreichii* ssp. *shermanii* exerts no negative effects on growth of yogurt starter cultures, and inulin could be beneficial for further survivals of *P. freudenreichii*.

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Conflict of interest

There was no conflict of interest to declare.

Compliance with ethical standards

The authors do not have any kind of interests. Research does not involve Human Participants and/or Animals. Informed consent is not applicable.

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The occurrence of aflatoxin M_1 in industrial and traditional fermented milk: A systematic review study

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Review

Abstract

Aflatoxin M_1 (AFM_1) is a toxic secreted into the milk of animals fed with diets contaminated by aflatoxin B_1, which can cause some adverse health effects in humans. The occurrence of AFM_1 in dairy products varies based on several factors, including the fermentation process. In this article, the published citations from January 2000 to October 2020 regarding the AFM_1 occurrence in industrial and traditional fermented milk were systemically reviewed. According to the findings, a reducing trend in the AFM_1 contamination of fermented milk was observed over the years, mainly in traditional products. Despite this trend, further control measures besides the preventative approaches are needed to deal with the high levels of AFM_1 in fermented milk.

Keywords: AFM_1; yogurt; fermented milk; occurrence; contamination; food safety; traditional dairy products

Introduction

Aflatoxins are toxic, secondary metabolites synthesized by some fungi species in the genus *Aspergillus*, mainly those belonging to the species *A. flavus*, *A. nomius*, and *A. parasiticus* (Ismaiel *et al.*, 2020). Aflatoxins are considered the most important mycotoxins, given their carcinogenic and hepatotoxic effects on animals and humans (Bhat *et al.*, 2010). Among several types of aflatoxins, the most frequent ones found as natural contaminants of foodstuffs are aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), and G_2 (AFG_2) (Nejad *et al.*, 2019). While AFB_1 possesses the highest toxicity, this toxin is also classified as a Group 1 carcinogen by the International Agency for Research on Cancer (2002). In addition, AFM_1 and AFM_2 are produced by hepatic biotransformation of AFB_1 and AFB_2, respectively, and maybe shed through the urine and milk of animals (Campagnollo *et al.*, 2016; Imamura *et al.*, 2015).

Milk and milk products have high nutritional and biological value, contributing to a balanced diet for human beings. Among dairy products, fermented milk is important, as it is consumed by a wide range of people, from infants to elders (Barukcic *et al.*, 2018). Some fermented milk has, in their composition, probiotics that lead to improved digestibility, besides some other health-promoting factors, such as bioactive peptides and bacteriocins (Black, 2011). As AFM_1 is highly stable through pasteurization, ultra-high temperature processing, and

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other processing methods used in dairy production, the toxin may be found not only in processed milk but also in dairy products (Jalili and Scotter, 2015). Yogurt and other fermented milk products are typically manufactured by fermentation of lactic acid in milk, both traditional and industrialized products with different levels of AFM_1, given the range of pH values and fermentation conditions (Govaris *et al.*, 2002). However, studies related to AFM_1 contents in these fermented products are scarce and controversial (Campagnollo *et al.*, 2016; Mahmood Fashandi *et al.*, 2018; Mousavi Khaneghah *et al.*, 2017). Figure 1 presents an overview processing steps of fermented milk and some relevant points regarding the AFM_1 contamination of these products.

When fermented milk is produced using milk contaminated with AFM_1, the mycotoxins are not eliminated at once, as they are resistant to most processing steps (Behfar *et al.*, 2012). Therefore, to safeguard human health, maximum limits of AFM_1 residues recommended in most countries range from 0 to 1.0 µg/L of milk (Iqbal *et al.*, 2015). In the European Union (EU), the tolerable limit for AFM_1 in milk is no more than 0.05 µg/L (European Commission, 2006), while in the United States and Brazil, a maximum level of 0.5 µg/L is accepted (Agência Nacional de vigilância sanitária, 2011; Food and Drug Administration, 2000).

Besides yogurts, other fermented products are also susceptible to AFM_1 contamination, including traditional ones, such as *Lala*, kefir, and Doogh. *Lala* is traditional African fermented milk produced by natural fermentation or mesophilic cultures (Kuboka *et al.*, 2019). Kefir is a dairy product rich in vitamins, essential amino acids, and minerals, made by fermenting the kefir grains (Gamba *et al.*, 2016). Kefir is the most common probiotic product consumed in Europe and is associated with beneficial health effects related to homeostasis balance (Otles and Cagindi, 2003). Doogh is an Iranian fermented product made from yogurt added with potable water, sodium chloride, and probiotic cultures (Kiani *et al.*, 2018). While several studies have been dedicated for evaluating the AFM_1 levels in milk and other dairy products (Fallah, 2010; Kim *et al.*, 2011; Rahmani *et al.*, 2018), no systematical review was conducted to summarize the findings. Therefore, the current investigation was undertaken to systematically review the literature published in the last 20 years regarding the prevalence of AFM_1 in industrial and traditional fermented milk globally.

Literature Search

A systematic literature search was conducted among some international databases such as PubMed, Science Direct, and Google Scholar (as gray literature) using the following key terms: "aflatoxins" OR "aflatoxin M_1" OR "mycotoxins" AND "Occurrence" OR "Contamination" OR "prevalence" OR "incidence" OR "fermented milk" OR "dairy products" OR "cultured dairy" OR "yogurt" OR "Kefir." All relevant articles published from January 2000 to October 2020 that investigated the prevalence of AFM_1 in fermented milk were retrieved and screened for eligibility. In addition, the reference lists of

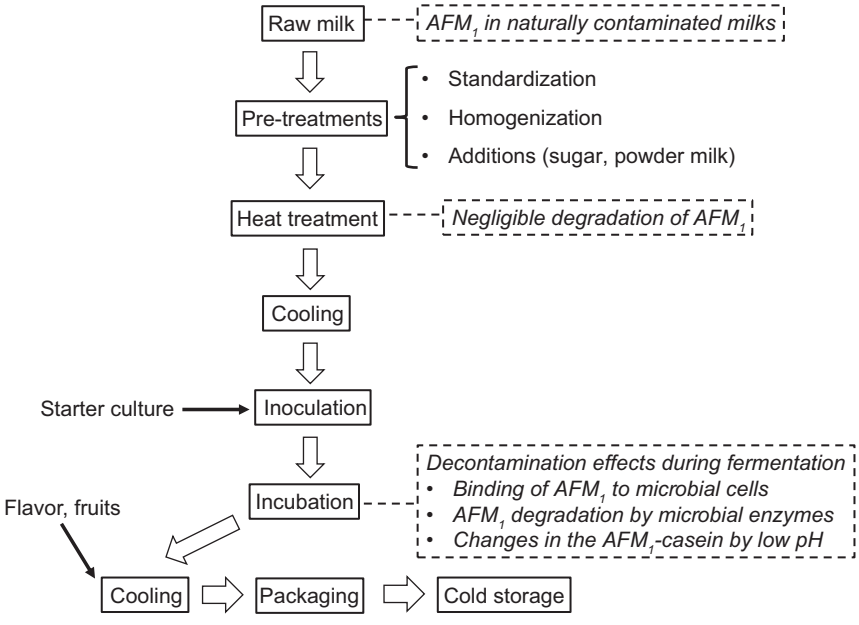

Figure 1. General processing flow chart of fermented milk and relevant steps regarding the aflatoxin M_1 (AFM_1) contamination during manufacture (in italic).

included articles were also manually searched to identify other suitable studies.

After excluding unsuitable articles due to irrelevant content, 150 full texts of potentially eligible articles were downloaded. Then, the downloaded citations were examined for inclusion and criteria of final eligibility. Inclusion criteria were: (1) availability of full-text article, (2) original cross-sectional research studies (not reviews), (3) reporting of AFM_1 prevalence among fermented, milk-based products, (4) indicating an accurate analytical method, and (5) published in the English in order to avoid any mistake during translation from other languages. The citations that did not meet these criteria were excluded. A total of 100 articles were excluded based on the above-mentioned exclusion criteria according to PRISMA, as detailed in Figure 2. Finally, 50 articles that fulfilled the inclusion criteria were included in this review.

The Occurrence of Aflatoxin M_1 in Fermented Milk

Table 1 presents the worldwide prevalence of AFM_1 in yogurt and other fermented milk products during the last 20 years. Several studies reported a high prevalence of AFM_1 in yogurt and other traditional products in African countries. This is consistent with the high prevalence of AFB_1 reported in feedstuff used for dairy cows and AFM_1 in milk in the African continent (Muaz *et al.*, 2021). In addition to the climatic conditions that favor fungal growth in several geographic areas in Africa, the lack of effective regulation of aflatoxins in the food chain and the low public awareness of this risk are among important factors that contribute to high prevalence of aflatoxins in African countries (Wild *et al.*, 2015). In Egypt, 63% of the yogurt samples exceed the EU's AFM_1 levels (Aiad and Aboelmakarem, 2013). The mean prevalence of AFM_1 in Egyptian yogurt samples was higher in the winter than in the summer. Coherently, higher AFM_1 levels in milk samples have also been reported in the winter season in different countries (Bilandzic *et al.*, 2014; De Roma *et al.*, 2017; Fallah, 2010; Ruangwises and Ruangwises, 2010). The reasons for such a higher prevalence of AFM_1 in milk and fermented products during the winter are not well established but may involve higher consumption of AFB_1-contaminated feed by dairy cows during this period, as well as differences in the feed storage and diet composition, and rainfall effects (Fallah, 2010; Hajmohammadi *et al.*, 2020). After incubation of *Lactobacillus acidophilus* and *Bifidobacterium lactis* into the fermented products, a decrease in mycotoxin prevalence was observed at the end of the storage period (Ibrahim *et al.*, 2016). In this regard, the percentages of Egyptian Zabadi yogurt samples exceeding the European limits in 2016 and 2017 were 12.5 and 18.7, respectively. However, these prevalence data were lower in this product than in milk and cheese, mainly in the winter (Ismaiel *et al.*, 2020). In Nigeria, 20

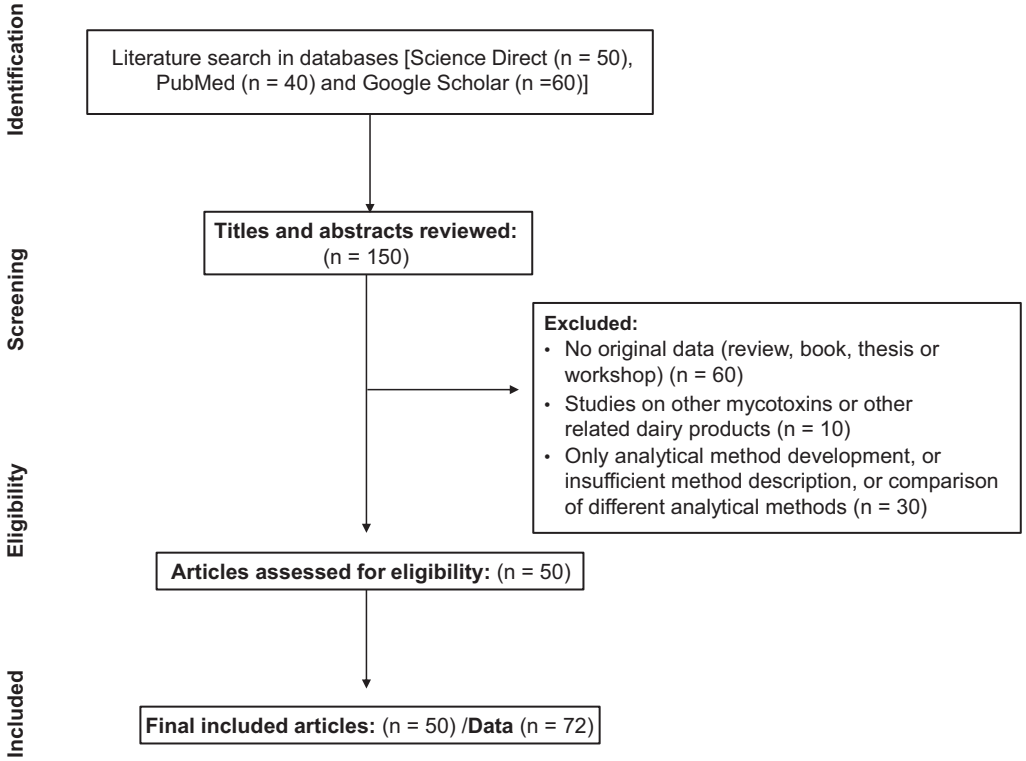

Figure 2. Flow chart describing the search and selection of articles evaluated in the study.

Table 1. Occurrence of aflatoxin M_1 in yogurt and other fermented milks reported in the last 20 years.

Country	Type of product	Samples analyzed (n)	Positive samples		LOD (ng/kg or L)	Concentration (ng/kg or L)		Analytical method	Reference
			n	%		Range	Mean		
Africa:									
Egypt	Yogurt[a]	30	8	26	5	11.40–98.80	28.41	ELISA	Aiad and Aboelmakarem (2013)
Egypt (Winter)	Yogurt	24	12	50	NR	56.60–84.14	64.68	HPLC	Ibrahim *et al.* (2016)
Egypt (Summer)	Yogurt	24	12	50	NR	31.46–66.05	39.13	HPLC	Ibrahim *et al.* (2016)
Egypt (2016 production)	Yogurt *Zabady*	32	4	12	50	130–240	185	HPLC	Ismaiel *et al.* (2020)
Egypt (2017 production)	Yogurt *Zabady*	32	6	19	50	100–170	130	HPLC	Ismaiel *et al.* (2020)
Nigeria	Yogurt	2	10	10	1	583.5–647.0	0.62 µg/L	HPLC	Anthony *et al.* (2016)
Burundi	Yogurt	6	6	100	NR	8,200–63,200	33,500	ELISA	Udomkun *et al.* (2018)
Kenya	Yogurt	8	3	37	NR	< LOD—690	NR	ELISA	Langat *et al.* (2016)
Kenya	Yogurt	21	12	57	2	17–1,100	134	ELISA	Lindahl *et al.* (2018)
Kenya	*Lala*	27	8	30	2	12–160	48	ELISA	Lindahl *et al.* (2018)
Kenya	Yogurt	17	13	77	2	26–270	96	ELISA	Lindahl *et al.* (2018)
Kenya	*Lala*	8	5	63	2	10–340	111	ELISA	Lindahl *et al.* (2018)
Kenya	Yogurt	NR	NR	NR	5	NR	379.3	ELISA	Kuboka *et al.* (2019)
Kenya	*Lala*	NR	NR	NR	5	NR	379.3	ELISA	Kuboka *et al.* (2019)
Congo Republic	Yogurt	2	3	67	NR	4,800–26,000	16,100	ELISA	Udomkun *et al.* (2018)
Americas:									
Brazil	Yogurt	53	47	72	3	10–529	NR	HPLC	Iha *et al.* (2011)
Brasil	Yogurt	3	3	100	3	75.1–112.9	94	HPLC	Iha *et al.* (2013)
Asia:									
Qatar	Yogurt	21	16	76	NR	4.16–38.21	31.32	ELISA	Hassan *et al.* (2018)
China	Yogurt	27	15	55	5	4.0–47.0	17.2	HPLC	Guo *et al.* (2019)
China	Yogurt	NR	NR	NR	0.6	NR	NR	ELISA	Zhou *et al.* (2019)
South Korea	Yogurt	55	15	27	20	20–150	51	HPLC	Yoon *et al.* (2016)
South Korea	Yogurt	60	50	83	2	3–172	29	ELISA	Kim *et al.* (2000)

(*continues*)

Table 1. Continued

Country	Type of product	Samples analyzed (n)	Positive samples		LOD (ng/kg or L)	Concentration (ng/kg or L)		Analytical method	Reference
			n	%		Range	Mean		
Iran	Pasteurized yogurt	40	40	100	NR	2.1–61.7	15.1	ELISA	Barjesteh *et al.* (2010)
Iran	Yogurt	10	10	100	NR	7–53	25	ELISA	Barjesteh *et al.* (2010)
Iran	Yogurt	68	45	66	12	15–119	32	HPLC	Fallah (2010)
Iran	Traditional yogurt	60	14	23	12.5	15–36	17	HPLC	Fallah *et al.* (2011)
Iran	Industrial yogurt	61	30	49	12.5	15–102	26	HPLC	Fallah *et al.* (2011)
Iran	Traditional Doogh	65	9	14	12.5	13–29	NR	HPLC	Fallah *et al.* (2011)
Iran	Industrial Doogh	71	16	22.5	12.5	13–53	NR	HPLC	Fallah *et al.* (2011)
Iran	Yogurt	60	48	80	10	19.7–319.4	130.5	ELISA	Rahimi (2012)
Iran	Yogurt	60	59	98	NR	6.2–87	51.7	ELISA	Issazadeh *et al.* (2012)
Iran	Yogurt	13	13	100	NR	5–36	13.5	ELISA	Arast *et al.* (2012)
Iran	Yogurt	40	14	35	NR	11.4–115.8	130.5	ELISA	Nilchian and Rahimi (2012)
Iran	Yogurt	80	77	96	5	< LOD—100	29.1	ELISA	Mason *et al.* (2015)
Iran	Yogurt	42	10	24	1	6.3–21.3	15.1	ELISA	Bahrami *et al.* (2015)
Iran	Doogh	44	6	14	NR	7.0–12.1	9.0	ELISA	Bahrami *et al.* (2015)
Iran	Yogurt	90	90	100	NR	5.0–83.0	32.1	ELISA	Nikbakht *et al.* (2016)
Iran	Yogurt	18	15	83	NR	7.8–12.1	10.3	ELISA	Sohrabi and Gharahkoli (2016)
Iraq	Yogurt	32	32	100	NR	0.16–42.74	16.92	ELISA	Najim and Jasim (2014)
Iraq	Traditional yogurt	20	15	75	NR	22.2–172.9	103.9	HPLC	Mossawei *et al.* (2016)
Iraq	Yogurt	20	10	50	NR	30.5-107.4	58.37	HPLC	Mossawei *et al.* (2016)
Kuwait	Yogurt	2	1	50	10	NR	NR	HPLC	Ivastava *et al.* (2001)
Lebanon	Yogurt	64	21	33	5	5–50	NR	ELISA	El Khoury *et al.* (2010)
Lebanon	Yogurt	NR	NR	72	NR	NR	24.55	ELISA	Hassan and Kassaify (2014)
Lebanon	Yogurt	28	18	64	3.2	15–545	91	HPLC	Daou *et al.* (2020)
Lebanon	Strained yogurt	27	24	89	3.2	37–1,843	201	HPLC	Daou *et al.* (2020)
Lebanon	Yogurt *Ayran*	9	8	89	3.2	20–315	242	HPLC	Daou *et al.* (2020)
Malaysia	Yogurt	5	2	40	2	7.5–31	25.4	ELISA	Nadira *et al.* (2016)

Pakistan	Yogurt	96	59	61	4	4.0–615.8	90.4	HPLC	Iqbal and Asi (2013)
Pakistan (Winter)	Yogurt	51	13	25	4	NR	53	HPLC	Iqbal *et al.* (2013)
Pakistan (Summer)	Yogurt	45	8	18	4	NR	19	HPLC	Iqbal *et al.* (2013)
Pakistan (Winter)	Plain yogurt	36	15	42	0.4	NR	63.6	HPLC	Iqbal *et al.* (2017)
Pakistan (Winter)	Flavored yogurt	30	17	57	0.4	NR	50.5	HPLC	Iqbal *et al.* (2017)
Paquistão (Summer)	Plain yogurt	30	11	37	0.4	NR	59.6	HPLC	Iqbal *et al.* (2017)
Pakistan (Summer)	Flavored yogurt	25	10	40	0.4	NR	45.3	HPLC	Iqbal *et al.* (2017)
Taiwan	Yogurt	24	3	12	5	7–44	NR	HPLC	Lin *et al.* (2004)
Turkey	Yogurt	40	32	80	50	61.61–365.64	NR	ELISA	Gurbay *et al.* (2006)
Turkey	Plain yogurt	104	68	65	NR	1–100	NR	ELISA	Akkaya *et al.* (2006)
Turkey	Yogurt with fruits	21	7	33	NR	1–100	NR	ELISA	Akkaya *et al.* (2006)
Turkey	Yogurt	52	29	56	NR	1–150	NR	ELISA	Akkaya *et al.* (2006)
Turkey	Yogurt	80	70	87	5	10–475	66.1	ELISA	Atasever *et al.* (2011)
Turkey	Yogurt *Ayran*	80	72	90	5	6–264	36.5	ELISA	Atasever *et al.* (2011)
Turkey	Yogurt	50	10	20	2	40.62–72.04	55.28	ELISA	Temamogullari and Kanici (2014)
Turkey	Yogurt	19	17	89	100	16–107.2	47.92	HPLC	Sarica *et al.* (2014)
Turkey	Yogurt	60	2	3	5	24–28	NR	HPLC	Sahin *et al.* (2016)
Turkey	Yogurt *Ayran*	60	1	2	5	NR	5	HPLC	Sahin *et al.* (2016)
Europe:									
Italy	Yogurt	120	73	61	1	1.0–32.2	9.1	HPLC	Galvano *et al.* (2001)
Portugal	Plain yogurt	48	2	4	10	43.0–45.0	44.0	HPLC	Martins and Martins (2004)
Portugal	Yogurt with fruits	48	16	33	10	19.0–98.0	51.12	HPLC	Martins and Martins (2004)
Serbia	Fermented milks	302	NR	NR	6	25–500	190	ELISA	Keskic *et al.* (2016)
Spain	Yogurt	72	2	3	25	NR	38.34	ELISA	Cano-Sancho *et al.* (2010)
Spain	Yogurt	6	2	33	25	NR	21.6	ELISA	Cano-Sancho *et al.* (2015)

[a]Without any further designation, the term "yogurt" applies to industrial products.
ELISA: enzyme-linked immunosorbent assay; HPLC: high-performance liquid chromatography; LC-MS/MS: liquid chromatography coupled to tandem mass spectrometry. NR: not reported.

samples of yogurt were analyzed, and 10% were contaminated with AFM_1 (Anthony *et al.*, 2016). In a study carried out in Nairobi, the capital of Kenya, AFM_1 was analyzed in samples of fermented milk and yogurt, and contamination was observed in levels above 0.05 μg/L (Langat *et al.*, 2016). In Dagoretti and the Westland area belonging to Nairobi, 77 and 57% of *Lala* and yogurt, respectively, contained detectable levels of AFM_1 (Lindahl *et al.*, 2018). In Nairobi, a study with pasteurized yogurt and *Lala* revealed that all samples had AFM_1 above the detection limit (5ng/kg). After undergoing an additional experimental fermentation, both products showed a significant reduction in AFM_1 prevalence (Kuboka *et al.*, 2019). The prevalence of AFM_1 in yogurt and milk samples was evaluated in Burundi in the Republic of the Congo, and 29% of them showed levels much higher than the limits recommended by the EU (Udomkun *et al.*, 2018).

Few studies considering the prevalence of AFM_1 in fermented milk produced in the Americas and European countries were conducted (Table 1). In Brazil, 95% of the samples of yogurt or dairy-based drinks from the Ribeirão Preto region were contaminated with AFM_1 (Iha *et al.*, 2011). Interestingly, while the naturally contaminated yogurts from were incubated for 12 h, there was a reduction about 6% in the toxin levels (Iha *et al.*, 2013). The fermentation process in yogurts contributes to reducing the concentration of AFM_1 due to factors such as low pH, production of organic acids, and the presence of bacteria that synthesize lactic acid and other byproducts of fermentation (Govaris *et al.*, 2002).

As for the European countries, AFM_1 was detected in the Cataluña region of Spain among 2.8% of the samples analyzed, the only one region that showed contamination above that determined by the EU (Cano-Sancho *et al.*, 2010). In another study, however, 33% of yogurt samples from the same Spanish region were contaminated with AFM_1, with none of them exceeding the European limits (Cano-Sancho *et al.*, 2015). In samples of yogurt from Italian supermarkets analyzed in 1996, 61% showed levels of AFM_1, but similar to Spain, none of them exceeded the limits determined by the EU (Galvano *et al.*, 2001). In a study carried out in Portugal, 4.2% of the samples of plain yogurt and 33.3% of the samples of strawberry yogurt were contaminated with this toxin (Martins and Martins, 2004). In Serbia, the mean concentrations of AFM_1 in dairy products and fermented dairy drinks in 2015 were 0.018 and 0.019 μg/kg, respectively, with 5.86 and 2.64% of the samples exceeding the limits determined by the EU. It was also observed that the toxin levels were more significant in the winter and autumn in both products (Keskic *et al.*, 2016).

The majority of data describing the prevalence of AFM_1 in fermented milk were provided by studies in Asian countries (Table 1). In Qatar, the incidence of AFM_1 in yogurts was analyzed using an immunoenzymatic assay (ELISA); 76.1% of the samples were positive. However, none of them showed contamination levels above the EU maximum limits, posing no public health threats in this country (Hassan *et al.*, 2018). AFM_1 prevalence in yogurts produced with buffalo milk in different dairy factories in Southern China were evaluated, and none of the samples had levels greater than the limit of 500 ng/kg determined in the country(Guo *et al.*, 2019). Another study carried out with cow milk showed AFM_1 levels inside the limit determined by this country and the EU (Zhou *et al.*, 2019). In South Korea, 27.27% of the yogurt samples showed AFM_1, but none of them was above the limit determined by the Korean Ministry of Food and Drug Safety (0.5 μg/kg) (Kim-Soo *et al.*, 2016). However, in a previous study, 83% of yogurt samples were contaminated by this toxin (Kim *et al.*, 2000).

In the Mazandaran province of Iran, 100% of the pasteurized yogurt and local yogurt samples were contaminated with AFM_1 (Barjesteh *et al.*, 2010). However, in another study in Iran, 20.6% of yogurt samples were contaminated with levels above the limits determined by the local regulations (0.05 μg/L) and were greater in the winter than the summer (Fallah, 2010). Moreover, samples of traditional and industrial yogurt and Doogh were evaluated, and the AFM_1 incidences in both these industrial products were greater in the autumn and winter than in traditional ones. As for Doogh samples, the contamination levels were low, and no significant seasonal effect was observed (Fallah *et al.*, 2011). Seasonal factors may influence the presence of the toxin in these products, as some studies observed higher levels of contamination in milk samples in the autumn and winter compared with summer and spring (Kamkar, 2005). These variations may be related to the procedures during processing, degree of milk contamination, type of yogurt, fermentation conditions, geographic regions, season, country, and analytical methods used to detect these toxins (Di Guan *et al.*, 2011). In general, yogurts have shown lower contamination levels with AFM_1 than cheese (Rabie *et al.*, 2019), as the fermentation process contributes to reducing the concentration of AFM_1 because of low pH and the production of fermentation-related byproducts such as organic acids, including lactic acid, among other factors (Campagnollo *et al.*, 2016). In milk, AFM_1 binds to casein, and the modifications on its structure caused by pH reduction during fermentation may lead to changes in this bound (Govaris *et al.*, 2002). However, the exact mechanisms involved in the mycotoxin decontamination during the fermentation process are not entirely understood. Several experimental data indicate that aflatoxin reduction in fermented products occurs through its binding to the cell wall components of starter cultures, as reviewed by Muaz *et al.* (2021), or through degradation

of the toxins by microbial enzymes into less toxic substances (Guo *et al.*, 2020). The most studied bacteria with practical AFM_1-binding abilities are lactic acid bacteria belonging to the genus *Levilactobacillus* spp. (former *Lactobacillus* sp.) such as *L. rhamnosus* and *L. plantarum* (Sadiq *et al.*, 2019). Regarding bio-detoxification, several species in the genera *Pseudomonas*, *Rhodococcus*, *Streptomyces*, *Bacillus*, and *Pleurotus* have been reported to be capable of degrading aflatoxins (Guo *et al.*, 2020). However, these bacterial species are not allowed to be used as starter cultures in fermented foods. The combination of fermentation with some emerging technologies, such as ultrasound, ohmic heating, and cold plasma, has been proposed, aiming at improving aflatoxin's detoxification (Gavahian *et al.*, 2021).

In Shahr-e Kord, Iran, AFM_1 was detected in 35% of the yogurt samples, but not above the EU's acceptable limit (Nilchian and Rahimi, 2012). In Gilan, another province of Iran, 63.33% of the yogurt samples were above the EU limits (Issazadeh *et al.*, 2012). In central Iran, yogurt samples showed mean AFM_1 contamination levels of 13.55 ng/kg (Arast *et al.*, 2012). In Isfahan, 80% of the yogurt samples were contaminated with this toxin, and 5% of them were above the limit determined by the EU (Rahimi, 2014). In traditional Iranian yogurts, these toxin levels were more significant than in industrialized products (Mason *et al.*, 2015). Still, in Iran, aflatoxins levels were evaluated in yogurt and Doogh samples, with 23.8 and 13.6%, respectively, yielding positive results (Bahrami *et al.*, 2016). However, in Iran, 100% of the yogurt samples collected in 2014 were contaminated, with 22.22% above the AFM_1 limits determined by the EU (Nikbakht *et al.*, 2016). On the other hand, 83.3% of the yogurt samples were positive for AFM_1 in another study, although none of them was above the limits determined by the Institute of Standards and Industrial Research of Iran (50 ng/L) (Sohrabi and Gharahkoli, 2016).

In Bagdad, the capital of Iraq, 100% of the yogurt samples from supermarkets were contaminated with AFM_1 (Jasim and Najim, 2014). A study carried out with local and imported yogurts in Iraq found that 75 and 50%, respectively, of the samples contained AFM_1 (Al-Mossawei *et al.*, 2016). In Kuwait, one sample out of two yogurt samples produced in a local farm was contaminated with AFM_1 (Ivastava *et al.*, 2001). In Lebanon, 32.81% of the samples analyzed showed the presence of AFM_1, with 6.25% of them exceeding the limits determined by the EU (El Khoury *et al.*, 2011). Still, in Lebanon, 72% of the yogurt samples analyzed showed AFM_1, with 13% above the recommended limits (Hassan and Kassaify, 2014). In another study carried out in Lebanon with different yogurt types, it was observed that 64.3% of the samples were positive for AFM_1, and 35.7% were above the limits recommended by the EU. Strained yogurt, popularly consumed by the Lebanese population, showed 88.9% contaminated samples, with 81.5% above the EU acceptable limits. The authors suggested that these findings may be due to low-quality powdered milk in the production, leading to high levels of contamination in the final product. As for the yogurt drink Ayran, 88.9% of the samples were positive, with 44.4% above the EU recommended limits (Daou *et al.*, 2020).

In Malaysia, 40% of the yogurt samples collected in January 2014 were contaminated with AFM_1, although none of them was above the limits determined by the EU (Nadira *et al.*, 2017). A study carried out in the winter and summer in Pakistan showed that 37 and 29% of the samples of yogurt, respectively, were contaminated with this toxin, and were above the country limits (0.05 μg/L) (Iqbal *et al.*, 2013). In Punjab, a province of Pakistan, 47% of the yogurt samples were above the legal limits (Iqbal and Asi, 2013). Corroborating these findings, another study carried out in the winter and summer showed that plain yogurt and flavored yogurt samples were contaminated with AFM_1 by 20 and 16%, respectively, and were above the levels determined by the EU during the summer. In the winter, 27.7 and 40%, respectively, were above the EU limits, posing a considerable threat to the population's health (Iqbal *et al.*, 2017). In Taiwan, 12.5% of the samples of yogurt beverages were contaminated with AFM1 but at low levels (Lin *et al.*, 2004).

On the other hand, in Ankara, Turkey's capital, 32% of the yogurt samples showed AFM_1 levels above the country's limit (Gurbay *et al.*, 2006). Also, in Turkey, 11.53% of the yogurt samples, 9.52% of fruit yogurt samples, and 21.15% of strained yogurt samples showed AFM_1 levels greater than those allowed by the existing regulations in the country (50 ng/kg) (Akkaya *et al.*, 2006). Corroborating this finding, 20% of the yogurt samples evaluated in other studies showed contamination levels above the acceptable limits by Turkish Food Codex (2008) (50 ng/kg) (Atasever *et al.*, 2011; Temamogullari and Kanici, 2014). Another study in Ankara showed that 89.5% of the yogurt samples were contaminated with AFM_1. Only 5 were above the limit determined by the local regulations (Sarica *et al.*, 2015). On the other hand, in Turkey, only two yogurt samples and one sample of Ayran showed AFM_1, but the levels were below the EU limits (Sahin *et al.*, 2016).

Concluding Remarks

Several studies regarding the prevalence of AFM_1 in industrial and traditional fermented milk were conducted worldwide in the past 20 years, indicating

high frequencies of positive samples at low levels of contamination among different industrial and traditional fermented milk products. A decreasing trend in the contamination of fermented milk products was observed over the years, mainly in traditional products. However, AFM_1 contamination in fermented milk at levels higher than the recommended tolerable limits was reported in African and Asian countries. Continuous monitoring and controlling actions from both manufacturers and regulatory bodies are essential to reduce the AFM_1 contamination levels in industrial and traditional fermented milk. Further studies to improve fermentation performance to reduce the AFM_1 contents in contaminated milk and other similar products are recommended.

Conflict of interest statement

The authors declare that there are no conflicts of interest relevant to this study.

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An assessment of *Cuminum cyminum* (Boiss) essential oil, NaCl, bile salts and their combinations in probiotic yogurt

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Abstract

This article is prepared to investigate the impacts of *Cuminum cyminum* essential oil (CEO), NaCl, bile salts, and their combinations on the viability of *Lactobacillus casei* in probiotic yogurt.

The water distillation method was used to extract the CEO, and GC/MS was used to determine its constituents. Then, the CEO's antibacterial activity, together with NaCl and bile salts, was investigated via the microdilution technique by determining the minimum inhibitory concentration (MIC) against *L. casei.* Further, the stress effects of 50% MIC on CEO, NaCl, and bile salts were examined by comparing the stress treatments with the control in terms of the *L. casei* population, pH, acidity, and syneresis percentage in probiotic yogurt during storage in the refrigerator for 28 days.

According to the results, the *L. casei* population and pH decreased in all the treatments during the storage time, such that the intensity of the decrease in the control and CEO treatments was lesser than in other stress treatments ($P<0.05$). The acidity and percentage of syneresis during the storage time increased for all the treatments, with the increase being less in control and CEO than in the other stress treatments ($P<0.05$). The control and CEO treatments scored the highest in the sensory evaluation ($P<0.05$).

Applying stresses below the MIC resulted in the survival of *L. casei* in the recommended amount (10^5–10^6 CFU mL^{-1}) in the probiotic yogurt until the end of 28 days.

Keywords: *Cuminum cyminum*, probiotic, yogurt, *Lactobacillus casei,* Stress, Essential oil

Introduction

Today, particular attention is being given to functional foods, which have nutritional value as well as positive effects on human health. People are excited about eating products containing probiotics. However, products containing probiotics are amongst the ones in which there have been health allegations. These allegations are even advertised in the media during the last few years (Zendeboodi *et al*., 2020). Various studies on probiotics for humans' advantages have focused on the innovative formulation, and some even provided valuable information on probiotics linked to health and well-being. (Roobab *et al*., 2020). Probiotics are microorganisms that improve the gut microbial balance. These often include the *Lactobacillus* and *Bifidobacterium* species,

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as they have a historically prolonged and reliable value. Also, identified as generally recognized as safe (GRAS), all are the predominant inhabitants in the human intestines. (Al-Okbi and Mohamed, 2012; Lucatto *et al*, 2020). *Lactobacillus casei* is a gram-positive, mesophilic, microaerophilic, catalase-negative, and spore-free bacterium and a facultative hetero-formative bacterium with high acid production capacity (Fontana *et al*., 2018). *L. casei* is a probiotic strain linked with antihypertensive antioxidant, antihypocholesterolemic and anticarcinogenic, characteristics (Balthazar *et al*., 2018; Garcia *et al.,* 2019).

Fermented milk, such as yogurts, has the potential to act as a medium for producing value-adding materials because of its favorable sensory attributes, nutritional properties, and high-grade harmony, and ample content of essential nutrients; besides, yogurt consumption enhances gut macrobiotic activity, mitigates immune responses, and increases gastrointestinal functionality by adjusting lactose intolerance. Technologists and manufacturers have reviewed distant fortifications by combining probiotics and nutraceutical compounds (Alizadeh Khaledabad *et al*., 2020; Lucatto *et al*., 2020).

Today, probiotic yogurt is the most popular and widely consumed probiotic product in the world. The survival of probiotic bacteria in yogurt and similar products is an important challenge during storage in probiotic products. The minimum acceptable concentration of probiotic strains for beneficial and therapeutic effects should be at least 10^6–10^7 CFU g^{-1} or mL in the final product (Azizkhani and Parsaeimehr, 2018). The main problem in production was maintaining the survival rate of probiotic strains during storage of product with due attention to high acidity, oxygen stress, and nutrient deficiencies. The main reasons for reducing the viability of probiotic strains in the stomach were low pH and bile salts in the intestines (El-Shafei *et al*., 2010). Many studies have been carried out on the viability of probiotics under the stomach's acidic conditions, bile salts of the small intestine (Sahadeva *et al*., 2011), and survival rate of probiotics were studied in the cold storage of foods (Mortazavian *et al*., 2007). Various methods such as microencapsulation, the addition of prebiotics and essential oils (Capela *et al*., 2006), and different procedures were used to increase the survival rate of probiotic strains during storage of functional products applied stresses were less than the minimum inhibitory concentration (MIC). They produced resistance-inducing genes (Maragkoudakis *et al*., 2006). Some essential oils improve probiotics' viability, and others may also decrease the viability of probiotics (Calsamiglia *et al*., 2007).

In many cases, the viability of probiotic bacteria is not sufficient, and it is necessary to evaluate the viability of probiotic bacteria in yogurt by applying different procedures to increase the resistance of bacterial cells against stresses. Hence, the usage of herbal essential oils and probiotic strains in dairy products is a new strategy to overcome pathogenic bacteria and stimulate probiotics. This study aimed to evaluate the effects of stresses less than the MIC of *Cuminum cyminum* Boiss. essential oil (CEO), NaCl (NC), and bile salts (BS) or a combination of them on the viability of probiotic *L. casei* and later we monitored through the storage time, physicochemical and sensory properties of probiotic yogurt.

Materials and Methods

Materials

Cow's milk (3% fat) was supplied from Pegah, Tehran Plant, and Starter culture of yogurt (*Streptococcus thermophilus* and *Lactobacillus bulgaricus* sub spp. *delbrukii*) was purchased from Hanson Company, Denmark.

Extraction and analysis of the essential oil

The *Cuminum cyminum* was collected from the Kerman province of Iran, and the Iranian Institute approved its scientific name of Botanical Garden Research. The plant›s essential oil was extracted by the water vapor distillation method, and then it was analyzed by colorimeter attached to a mass spectrometer (Model HP-6890, USA). HP-5MS capillary column with 30 m length, 0.25 mm inner diameter, and 0.32 µm inner layer thicknesses was used. The regulated programming for identification and quantification was set up as follows: the temperature was elevated from 60 to 265°C, with a flow rate of 2.5°C per min, and then the column was maintained at 265°C for 30 min. The injection room temperature was 250°C, and the flow rate of helium as a carrier gas was 1 mm/min. Finally, the flame ionization detector (FID) identified the essential oil components with an electrical capacity of 70 eV and an ionization source temperature of 250°C.

Preparation of inoculums

Lactobacillus casei (ATCC39392) was supplied from the Microbial Collection of Pasteur Institute of Iran. It remained in laboratory samples in a glycerol stock at –70°C and transferred in Brain Heart Infusion (BHI) broth (Merck, Germany) at 37°C without shaking. Working cultures were prepared from stock cultures in two successive transfers (1% inoculum) in BHI broth at 37°C for 18 h. *L. casei* cells were inoculated from working cultures to BHI broth. After 18 h incubation at 37°C, optical density (OD) (absorbance) of 0.1 at 600 nm,

using a Spectronic 20 spectrophotometer (Varian, USA) was applied for determining the population of *L. casei.* Cell concentration of *L. casei* was 2.2×10^{10} CFU mL^{-1} for inoculation. The enumeration of *L. casei* was performed according to the serial dilution method and cultivating on (BHI) agar (Merck, Germany) after incubation for 24 h at 37°C.

Determination of minimum inhibitory concentration (MIC) of *Cuminum cyminum* Boiss. essential oil, NaCl, and bile salt

A 96-well plate with a volume of 300 µL was used in this experiment. Sequential concentrations of essential oil of *C. cyminum* Boiss (0, 500, 1000, 2000, 3000, 4000, 5000, 7500, and 10. 000 mg L^{-1}), bile salt (0%, 0.05%, 0.02%, 0.03%, 0.06%, 0.07%, 0.08%, 0.1%, 0.2% and 0.3%) and NaCl (0%, 1%, 2%, 3%, 4% and 5%) were used in De Man, Rogosa and Sharpe agar (MRS) broth (Merck, Germany). Media contained 5% DMSO and were transferred to 96-well plates. Then, 250 µL of different concentrations of essential oil, NaCl, and bile salts along with 20 µL of *L. casei* suspension (5×10^6 CFU mL^{-1}) were added to all well. The contents of each well were mixed with a shaker for 2 min. The microplates were closed by Parafilm and then incubated for 24 h at 37°C in an anaerobic jar (Merck, Germany). At the end of incubation time, turbidity or non-turbidity was evaluated in the wells.

Adaptation and challenging conditions

Cultures were at the logarithmic phase. Bacterial cells were separated by centrifugation (Hettich, Germany) and resuspended in fresh BHI broth (non-adapted control culture). The inoculation content of *L. casei* was 1×10^{10} CFU mL^{-1}. Adaptation time was conducted in the same medium at 37°C (I) for 120 min with 1 mL included in 100 mL of 5% DMSO (CEO), (II) 10 g per 100 mL in Mueller Hinton broth medium (Merck, Germany) (NC), (III) 0.05 g per 100 mL in Mueller Hinton broth medium (BS), (IV) 1 mL in 100 mL containing 5% DMSO plus 10 g per 100 mL in Mueller Hinton broth medium (CEONC), (V) 1 mL in 100 mL containing 5% DMSO plus 0.05 g per 100 mL in Mueller Hinton broth medium (CEOBS), (VI) 10 g per 100 mL plus 0.05 g per 100 mL in Mueller Hinton broth medium (NCBS) and (VII) 1 mL in 100 mL containing 5% DMSO, 10 g per 100 mL plus 0.05 g per 100 mL in Mueller Hinton broth medium (CEONCBS). After centrifugation, adapted and non-adapted cells were inoculated to yogurt (10^8 per mL). Enumeration of *L. casei* was conducted by serial dilution method (Most Probable Number) at 0, 7, 14, 21, and 28 days of storage to evaluate survival rate. All the plates were incubated at 37°C for 48 h. Furthermore, all experiments have been done twice.

Physicochemical properties of yogurt samples

The experiments for physicochemical properties were pH, acidity%, and syneresis%. pH and titratable acidity concluded based on the method described by Yangilar and Yildiz (2018). Moreover, the percentage of syneresis was averaged according to the method defined by Wacher-Rodarte *et al*. (1993). Five milliliters of the yogurt sample was centrifuged at 2.208 g for 20 min at 4°C, and the volume of isolated whey was calculated after 1 min. Lastly, we displayed the Syneresis rate (%) as the separated whey volume per 100 g of yogurt (Wacher-Rodarte *et al*., 1993).

Sensory evaluation

Sensorial tests were executed based on a 5-point Hedonic scale. The lowest score was intensely disliked, and the highest score was 5 as remarkably like samples (Shahdadi *et al*., 2015). Sensory properties were measured as follows: flavor, texture, and overall acceptability. Sensory evaluations were carried out during 28 days of storage. Ten trained panelists performed judgments.

Analytical study

All experiments were performed in completely randomized design as triplicates and the result was reported as mean ± SD. The comparisons of data mean were performed by Tukey test. Two-way ANOVA was used for determination of significance or non-significance of data ($P<0.05$).

Results

The chemical combination of *Cuminum cyminum* Boiss. essential oil

Accordingly, 15 compounds were identified that constitute 100% of the essential oils in total. The most abundant essential oil component was Propanal, 2-methyl-3-phenyl. The concentration of them was 24.2%. Next, Gamma-Terpinene, Phenylethanediol, and 2-Beta-Pinene had the highest level of essential oil components, with 18.94%, 18.88%, and 12.59%, respectively (Table 1).

MIC results

The MIC values of the CEO, NC, and BS against *L. casei* were 1%, 4%, and 0.3% (v/v), respectively.

Table 1. GC/MS results of *Cuminum cyminum* (Boiss) essential oil

No.	Name	Area (%)
1	α-Pinene	1.12
2	Sabinene	0.74
3	2-Beta-Pinene	12.59
4	β-Myrcene	0.93
5	l-Phellandrene	1.10
6	Cymene	6.91
7	Limonene	1.73
8	Gamma-terpinene	18.94
9	(E)-4-(Cyclohex-1'-enyl)but-2-en-1-ol	1.88
10	Propanal, 2-methyl-3-phenyl-	24.22
11	2-Caren-10-al	8.80
12	Phenylethanediol	18.89
13	Gamma-cadinene	0.57
14	Trans-beta-farnesene	0.72
15	Carotol	0.86

Survival of *Lactobacillus casei*

The results showed that the interaction effects of treatment and storage time on the *L. casei* population (Figure 1) were significant (P<0.05). The viability of *L. casei* decreased in all treatments except for control and sample under CEO stress at 7 days of storage time (P<0.05). The viability of *L. casei* of all samples significantly decreased during 28 days of storage (P<0.05). Following 28 days of storage time, the highest survival rate of *L. casei* was detected for CEO (6.05±0.03 log CFU mL^{-1}) and control (6.01±0.02 log CFU mL^{-1}) treatments, which was significantly different from others (P<0.05). No significant difference was observed within NC and BS treatments (P>0.05). Nevertheless, the lowest survival rate of *L. casei* was observed in the stress condition treated with CEONCBS at all days of storage time (P<0.05).

pH

The data for pH (Figure 2) showed that the effect of time and type of samples were significant (P<0.05). The pH of all the yogurt samples decreased significantly during the storage period (P<0.05), and higher pH was attributed to samples under stress (except for the CEO) (P<0.05). At the end of 28 days of storage, the highest and lowest pH values for CEONCBS and the control treatments were 4.23±0.06 and 3.91±0.06, respectively (P<0.05). The comparison among all stress treatments showed the highest and lowest pH of samples were attributed to CEO and CEONCBS during storage time, respectively (P<0.05). No significant difference was observed between NC and BS treatments on the same day of storage (except on day 14) (P>0.05).

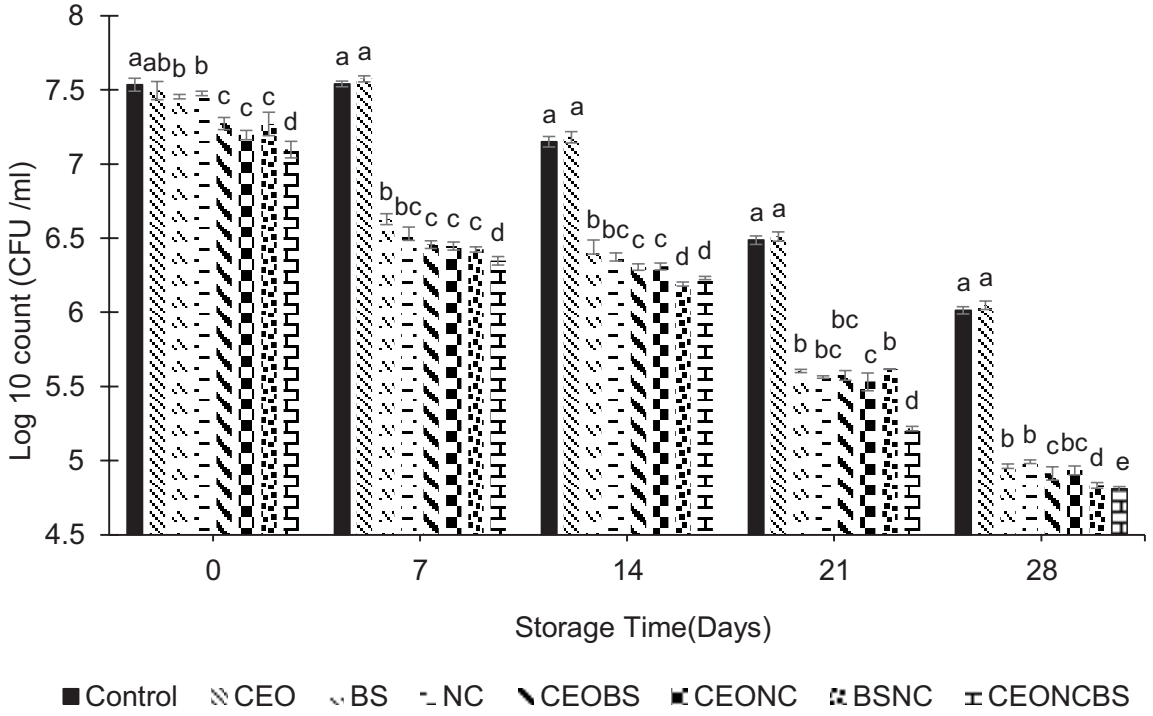

Figure 1. Effect of *Cuminum cyminum* essential oil (CEO), NaCl (NC), bile salts (BS), and their combinations on the survival of *L. casei* ATCC-39392 (log CFU mL^{-1}). Deviation bars designate the standard error of the method (n = 3).

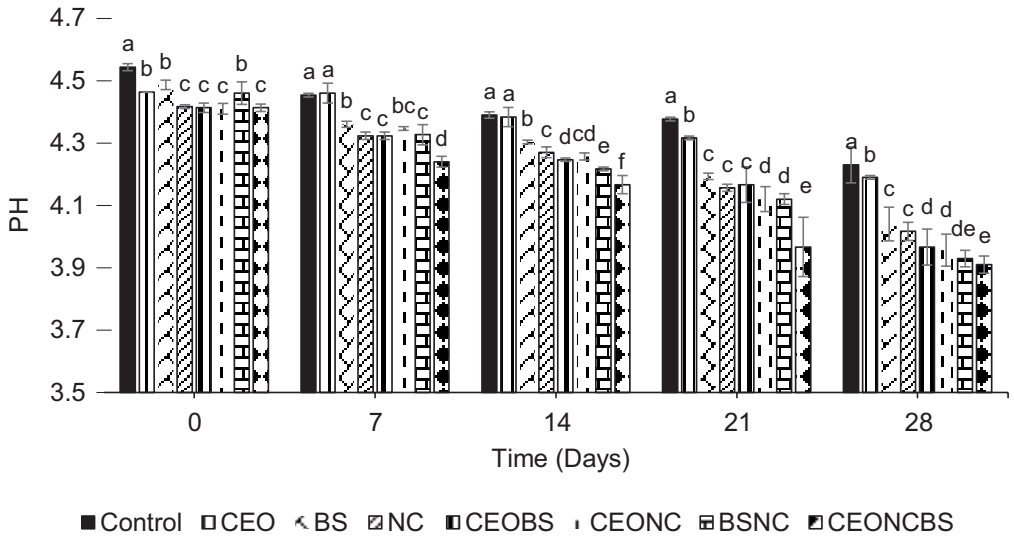

Figure 2. Effect of *Cuminum cyminum* essential oil (CEO), NaCl (NC), bile salts (BS), and their combinations on changes in pH of probiotic yogurt. Deviation bars designate the standard error of the method (n = 3).

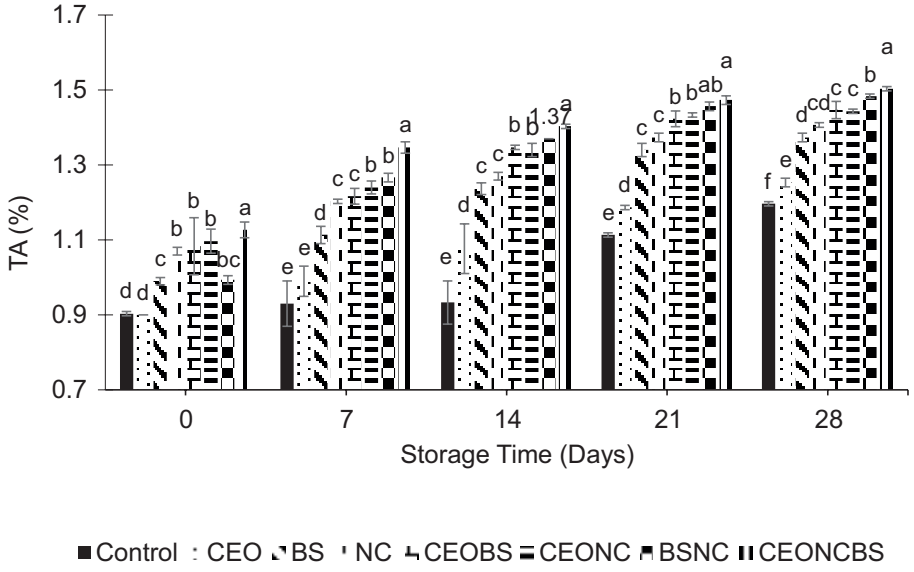

Figure 3. Effect of *Cuminum cyminum* essential oil (CEO), NaCl (NC), bile salts (BS), and their combinations on acidity changes of probiotic yogurt. Deviation bars designate the standard error of the method (n = 3).

Titratable acidity (TA%)

The titratable acidity (Figure 3) results showed that interaction between group and time was significant (P<0.05). The acidity of all samples significantly increased during storage time (P<0.05). The acidity of the control sample was less than those of stress treatments (P<0.05). Treatments CEO and CEONCBS had the highest and lowest acidity, respectively (P<0.05). After 28 days of the storage time, the lowest and highest acidity values were related to treatments under the stress of CEONCBS (1.19±0.008) and control (1.50±0.006), respectively. No significant difference was observed between NC and BC treatment during 28 days of storage (P>0.05).

Syneresis

Results showed that exchange within-group and time was notable (P<0.05). As shown in Figure 4, the amount of syneresis significantly rose in all samples during storage time (P<0.05). The increase in intensity for control and CEO treatments was less than that for stress treatments (P<0.05). Among the stressful treatments, the treatments under stress with CEO and CEONCBS had the lowest and highest percentage of syneresis, respectively, during 28 days of storage (P<0.05). No significant difference was observed between treatments with NC and BC on the same day of storage time (P>0.05). After 28 days of storage time, the lowest and the

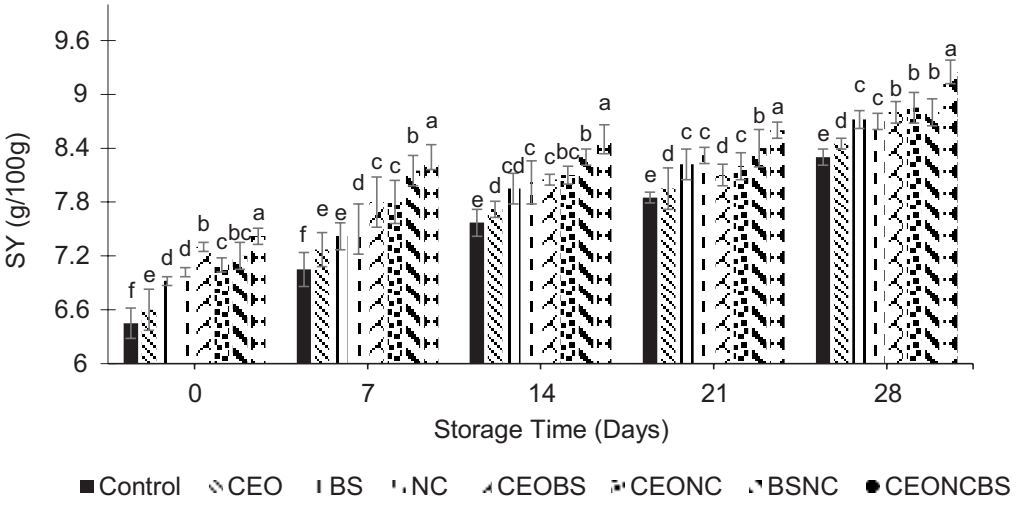

Figure 4. Effect of *Cuminum cyminum* essential oil (CEO), NaCl (NC), bile salts (BS), and their combinations on syneresis changes in probiotic yogurt. Deviation bars designate the standard error of the method (n = 3).

highest percentages of syneresis were observed for control (8.30±0.09) and stress with CEONCBS (9.25±0.13) treatments, respectively.

Sensory properties

Table 2 showed the results of sensory characteristics (flavor, texture, and general acceptance) during 28 days of storage time. The interaction between group and time on sensory characteristics was significant (P<0.05). The sensory scores of examples decreased during storage time. A more severe drop in sensory score was observed for stress treatments except for the CEO (P<0.05). Flavor scores for all treatments showed there were no significant differences among all samples on the first day of storage (P>0.05). The most leading and lowest scores were attributed to control (4±0) and CEONCBS (3.11±0.33) at 28 days of storage time, respectively (P<0.05). The texture feature results showed no significant difference within samples on the first day of storage (P>0.05). The highest and lowest scores were attributed to control (4.33±0.50) and CEONCBS (3.22±0.44) at 28 days of storage time, respectively (P < 0.05). Overall acceptance of samples indicated no significant difference among all samples on the first day of storage (P > 0.05). The highest and lowest scores were attributed to control (4±0) and CEONCBS (3.220 ± 0.44) at 28 days of storage time, respectively (P < 0.05).

Discussion and Conclusion

The effect of MIC of the essential oils and salts in return to the survival of *L. casei* was quite high; the inhibitory effect of concentrations less than MIC was also seen on pathogenic bacteria. Hence, the usage of concentrations of essential oils, NaCl, and bile salts less than MIC may also eliminate pathogens without harm to probiotics (Calsamiglia *et al*., 2007).

The viability of probiotic strains during production, food storage, and passage through the gastrointestinal tract is a major challenge in fermented dairy products. Researchers reported that enumeration of probiotic strains for beneficial and therapeutic effects should be at least 10^6 to 10^7 CFU g^{-1} or mL in products (Azizkhani and Parsaeimehr, 2018). Treatments with stresses of NC, BS, CEONC, CEOBS, and CEONCBS maintained viability until 21 days of storage. However, control with CEO sample-maintained viability up to 28 days of storage time. The survival rate of *L. casei* in control and stress treatments with the CEO increased at 7 days of storage. A rise in the survival rate of *L. casei* was observed to reduce pH at 7 days of storage. The increased survival rate of *Lactobacillus acidophilus* LA5, *Lactobacillus fermentum*, and *Bifidobacterium* Bb-12 in yogurt at 7 days of storage was reported by Azizkhani and Parsaeimehr . The bacterial population decline was attributed to the accumulation of organic acid during growth and fermentation. The main reasons for reducing pH are converting lactose to lactic acid, type of starter culture, duration of storage, and fermentation temperature (Singh *et al*., 2011). Several studies reported a decline in probiotic strains' survival rate during storage time (Azizkhani and Parsaeimehr, 2018; Yangilar and Yildiz, 2018). Indeed, decreased pH of products during storage was due to activation of the beta-galactosidase enzyme at 0–5°C, as well as post-acidification. pH values decreased to 4.2. The viability of probiotic bacteria is affected by increased

Table 2. Sensory properties of probiotic yogurt samples with *Cuminum cyminum* essential oil (CEO), NaCl (NC), bile salts (BS), and their combinations.

Sensory properties	Yogurt samples	Storage time (Days)				
		0	7	14	21	28
Flavor	Control	5 ± 0^{a}	5 ± 0^{a}	5 ± 0^{a}	4.67 ± 0.50^{a}	4 ± 0^{a}
	CEO	5 ± 0^{a}	5 ± 0^{a}	5 ± 0^{a}	4.75 ± 0.44^{a}	4 ± 0^{a}
	BS	5 ± 0^{a}	4 ± 0^{b}	4 ± 0^{b}	4 ± 0^{b}	3.33 ± 0.50^{b}
	NC	5 ± 0^{a}	4 ± 0^{b}	4±0^{b}	3.78 ± 0.44^{b}	3.33 ± 0.50^{b}
	CEOBS	5 ± 0^{a}	4 ± 0^{b}	4 ± 0^{b}	3.78 ± 0.44^{b}	3.33 ± 0.50^{b}
	CEONC	5 ± 0^{a}	4.33 ± 0.50^{b}	3.78 ± 0.44^{b}	3.78 ± 0.44^{b}	3.33 ± 0.50^{b}
	NCBS	4.67 ± 0.50^{a}	4 ± 0^{b}	3.78 ± 0.44^{b}	3.22 ± 0.44^{c}	3.11 ± 0.33^{b}
	CEONSBS	4.75 ± 0.44^{a}	4.33 ± 0.50^{b}	4.33 ± 0.50^{b}	3.22 ± 0.44^{c}	3.11 ± 0.33^{b}
Texture	Control	5 ± 0^{a}	5 ± 0^{a}	5 ± 0^{a}	4.67 ± 0.23^{a}	4.33 ± 0.50^{a}
	CEO	5 ± 0^{a}	5 ± 0^{a}	4.56 ± 0.53^{b}	4.33 ± 0.50^{bc}	4.33 ± 0.50^{a}
	BS	5 ± 0^{a}	4 ± 0^{b}	4.33 ± 0.50^{bc}	4.33 ± 0.50^{bc}	3.44 ± 0.53^{b}
	NC	5 ± 0^{a}	4.33 ± 0.50^{b}	4.33 ± 0.50^{bc}	4.33 ± 0.50^{bc}	3.22 ± 0.44^{b}
	CEOBS	4.56 ± 0.53^{a}	4 ± 0^{b}	4 ± 0^{c}	4 ± 0^{c}	3.44 ± 0.55^{b}
	CEONC	5 ± 0^{a}	4 ± 0^{b}	4 ± 0^{c}	4 ± 0^{c}	3.44 ± 0.44^{b}
	NCBS	4.67 ± 0.50^{a}	4.33 ± 0.50^{b}	4 ± 0^{c}	3.22 ± 0.44^{d}	3.44 ± 0.44^{b}
	CEONSBS	4.67 ± 0.50^{a}	4 ± 0.70^{b}	3.78 ± 0.44^{b}	3.22 ± 0.44^{d}	3.22 ± 0.44^{b}
Overall acceptability	Control	5 ± 0^{a}	5 ± 0^{a}	4.89 ± 0.33^{a}	4.67 ± 0.50^{a}	4 ± 0^{a}
	CEO	5 ± 0^{a}	5 ± 0^{a}	4.56 ± 0.53^{a}	4.33 ± 0.50^{ab}	3.78 ± 0.44^{ab}
	BS	5 ± 0^{a}	4 ± 0^{b}	4 ± 0^{b}	4 ± 0.70^{b}	3.22 ± 0.44^{b}
	NC	5 ± 0^{a}	4 ± 0.70^{b}	4 ± 0^{b}	4 ± 0^{b}	3.22 ± 0.44^{b}
	CEOBS	4.75 ± 0.44^{a}	4 ± 0^{b}	4 ± 0^{b}	4 ± 0^{b}	3.22 ± 0.44^{b}
	CEONC	5 ± 0^{a}	4 ± 0^{b}	4 ± 0^{b}	4 ± 0^{b}	3.22 ± 0.44^{b}
	NCBS	4.67 ± 0.50^{a}	4.33 ± 0.50^{b}	4 ± 0^{b}	3.56 ± 0.53^{c}	3.22 ± 0.44^{b}
	CEONSBS	4.67 ± 0.50^{a}	4 ± 0.70^{b}	4.33 ± 0.50^{b}	3.22 ± 0.44^{c}	3.22 ± 0.44^{b}

Each observation is a mean ± SD of three replications. In each column and row, means with the same letters had no significant difference at $P > 0.05$.

hydrogen ions compared to lactate ions (Kailasapathy, 2006). Compared to treatments with stress, treatment with CEO had higher population viability of *L. casei* than others during storage time ($P < 0.05$). The susceptibility of microorganisms to essential oils depends on the details of essential oil and the type of microorganisms. The antimicrobial activity of essential oils is complicated due to their volatility, insolubility in water, and complex chemical structure (Calsamiglia *et al.*, 2007). The antibacterial effects of *Cuminum cyminum* Boiss. essential oil on different microorganisms depends on the concentration and composition of nutrients, storage temperature, and nature of the organism's metabolites (Mahmoudi, 2013). The survival rate of *Lactobacillus acidophilus* in bioyogurt containing different concentrations of the essential oils, *Mentha piperita* and *Ziziphoraclinopodioides*, was significantly reduced 7 days of storage time at 4°C. One of the main criteria for selecting probiotic bacteria is resistance to NaCl and bile salts (Sarabi Jamab and Niazmand, 2009). According to the obtained results, treatments under stress with 0.15% BS were to be effective in the food at end of the 21st day. However, the survival rate of *L. casei* in BS stress treatment was significantly decreased from the 21st to the 28th day of storage time. It was less than the acceptable limit ($P < 0.05$). Probiotic bacteria have different mechanisms of protection against stress, one of which is the bile hydrolysis system. The resistance of some strains to bile salts is associated with the bile salt hydrolysis activity. Therefore, the hydrolysis of the bile salts will reduce their toxicity and side effects (Sahadeva *et al.*, 2011). According to Taranto *et al.* (2006), *Lactobacillus delbrucium* subsp. *Bulgaricus* treated with different concentrations of thiorodoxylate (one of the bile salts) showed different levels of activity of the hydrolysis system (Taranto *et al.*, 2006). The researchers reported that in some bacterial cells, the bile hydrolysis system's activity was significantly stronger than others, which resulted in cells showing greater resistance to higher concentrations and longer exposure times to these bile salts (Taranto *et al.*, 2006). When probiotic bacteria are exposed to bile salts, cellular homeostasis disorders occur. Destruction of lipid membranes and cell

membrane proteins leads to bacteria's death (Sahadeva *et al*., 2011). The survival rate of *L. casei* with considering salt stress was within acceptable limit until the end of 21 days of storage, and these findings are following other research that studied the viability of probiotic strain more than 2% of concentration (Fortin *et al.*, 2011). Other researchers reported that probiotic strains' viability decreased in samples with high concentrations of salt (4%) (Hekmat *et al*., 2009).

The suitable pH for commercial yogurt is 4.5. pH enhanced shelf life of the yogurt maintained mild taste and optimum appearance. Undesirable pH (less than 4), which had been resulted by *Lactobacillus bulgaricus,* produced large amounts of lactic acid, acetaldehyde, and byproducts from proteolytic activity (Mahmoudi *et al*., 2014). The main reason for the lower pH of CEO, BS, and NC salts compared to the control sample was due to the presence of some phenolic compounds in the *C. cyminum* Boiss essential oil, which may have inhibitory effects on the growth of *L. casei* and changed pH values (Mahmoudi, 2013). Salt also affected pH and acidity values by influencing the growth of microorganisms, which may cause lactic acid production during storage time (Fortin *et al*., 2013). Some studies reported that probiotic yogurt's pH decreased during storage time (Mahmoudi, 2013 ; Azizkhani and Pasaeimehr, 2018).

The acidification degree of probiotic yogurt is a crucial process control model that directly impacts the gel intensity and the commercially available fermentation period (Alizadeh Khaledabad *et al*., 2020). The production of organic acids by lactic acid bacteria was the main reason for increased acidity during storage time (Mahmoudi *et al*., 2014). Salt also influenced the acidity of samples because it impacted the growth of microorganisms, which influenced lactic acid production during storage time (Fortin *et al*., 2013). The consumption of lactose by lactic acid bacteria led to lactic acid production, and then the acidity of samples increased. The production of lactic acid in yogurt is the main factor in producing a unique flavor. Due to casein instability, conversion of the colloidal calcium phosphate complex to soluble calcium phosphate, calcium excretion, and casein coagulation take place at pH 4_4.6 (Ramasubramanian *et al*., 2008).

Significant factors such as heterogeneity, high acidity, storage, breakage of protein strand, and structural rearrangement that will induce yogurt's whey to leakage are recognized as substantial defects (Yildiz and Ozcan, 2020). The main reason for increased syneresis in probiotic yogurt during storage might be the activation of microorganisms of starter culture and their effects on long-chain biopolymers, which could be an important factor in reducing the softness and enhancing the syneresis of the yogurt during storage (Kailasapathy, 2006). At the same time, Akgun (2018) found that the syneresis rate in probiotic yogurt samples increased during storage at 4°C. The consistency of yogurt increased by stabilizers, an increase in the amount of milk casein concentration, and a reduction of acidification rate (Everett and Mcleod, 2005). Samples with stress under essential oil had a lower syneresis percentage than others. The main reason for lower syneresis was acidification of yogurt containing herbal essential oils, which might cause high-strength gels, low permeability, a fine protein mesh, and higher water uptake. Consequently, the syneresis of probiotic yogurt was decreased (Ozer *et al*., 2007).

In the overall acceptability of food products by consumers, sensory characteristics represent a vital role. Researches affirm that flavor is the first criterion for food acceptance, followed by health considerations as the second rule (Alizadeh Khaledabad *et al*., 2020). The reduction of flavor scores in all samples during storage might be related to increasing acidity and reduced starter bacteria activity, which induced flavor components (Yangilar and Yildiz, 2018). The percentage of syneresis causes a change in the firmness of the sample. The firmness of yogurt decreased with increasing of syneresis. Indeed, the percentage of syneresis was inversely correlated to yogurt's firmness (Ayar and Gurlin, 2014). Treatments that contained more probiotic counts had better flavor and texture than treatments with less probiotic counts, so the overall acceptance scores of yogurts with more probiotic counts were higher than the yogurt with less probiotic counts (under stress). Proteolytic strains of *Lactobacillus* could produce taste through carbohydrate metabolism, proteolysis, and low lipolysis processes. Enzymes of *Lactobacillus* hydrolyzed casein and produced large and medium bioactive peptides. Proteolytic enzymes may subsequently degrade these peptides from starter bacteria, non-acidic lactic acid probiotic bacteria to small peptides and free amino acids, which are the major contributors to taste in dairy products (Everett and Mcleod, 2005; Grom *et al*, 2020). All in all, the highest and lowest sensory scores were attributed to control and CEONCBS at 28 days of storage time, respectively. According to Silva *et al*. (2018), salt is also expressed as a factor affecting the formation and development of aroma compounds and impacts the dairy product's sensory properties. Won-Young *et al*. (2020) described that olive leaf extract to yogurt reduces the yogurt's sensory score.

According to the results, the pH and population of *L. casei* decreased for all the treatments during the storage time, while their acidity and percentage of syneresis increased. The enumeration of *L. casei* was in the range of the recommended amount (10^6_10^7 CFU mL^{-1}) for probiotic yogurt with stresses less than MIC during 28

days of storage. Therefore, it is possible to produce novel functional products such as probiotic yogurt containing herbal essential oil. The usage of combined *C. cyminum* Boiss, NaCl, and bile salts in cool conditions and MIC up to 50% during 21 days of storage is recommended for probiotic yogurt containing *L. casei*.

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Ultrasound-assisted extraction of Gac (*Momordica cochinchinensis* Spreng.) leaves: effect of maturity stage on phytochemicals and carbohydrate-hydrolyzing enzymes inhibitory activity

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Abstract

Although phytochemical contents of Gac fruit have been extensively analyzed, information about the bioactive compounds and valorization of Gac leaves is limited. In this study, Gac (*Momordica cochinchinensis* Spreng.) leaves at different maturity stages (young: YL, mature: ML and old: OL leaves) were extracted during a 20 min of 150-W sonication process. Color, phytochemicals, antioxidant activity, and inhibitory effects against carbohydrate-hydrolyzing enzymes were assessed by colorimetric, high-performance liquid chromatography, and spectrophotometric methods, respectively. Results indicated a decrease in L^* (lightness) and an increase in a^* (greenness–redness) during maturation of leaves. The YL extract had the highest contents of phytochemicals with 4897.01 (mg gallic acid equivalent [GAE] per 100 gram dried weight [DW]), total phenolics, 592.81 (mg quercetin [QE]/100 g DW), total flavonoids, 34.77% α-amylase inhibitory activity, and 40.21% α-glucosidase inhibitory activity. Myricetin (43%), vitexin (22%), and esculetin (11%) were the major bioactive compounds detected in YL extract. Also, the superoxide dismutase (SOD)-like capacity of the extract decreased from 11,599.96 to 3,999.63 U/g DW during the transformation of YL to OL. Extract of Gac leaves was found to be a potential ingredient for food preservation and supplementation that could reduce postprandial hyperglycemia.

Keywords: carbohydrate-hydrolyzing enzymes inhibitory activity; emerging processing technologies; Gac (*Momordica cochinchinensis* Spreng.) leaves; hyperglycemia treatment; ultrasonic-assisted extraction

Introduction

Ultrasound-assisted extraction is an innovative technique used widely for extracting bioactive compounds from plant materials (Munekata *et al.*, 2020). Through cavitation phenomena of bubbles, ultrasound-assisted extraction can increase the permeability of plant cell walls, enhance the contact surface area between solvent and samples, and release more phenolic compounds (Moreira *et al.*, 2019). This simple and low-cost technique has been suggested to be an alternative to the conventional extraction method, as it can save extraction time and enhance extraction efficiency (Guglielmetti *et al.*, 2017; İşçimen *et al.*, 2018; Karadag *et al.*, 2019; Surin *et al.*, 2020). This technology is used to extract Gac peel and other plant material (Chuyen *et al.*, 2020).

Starch hydrolysis because of the activities of α-amylase in the salivary pancreas and glands combined with α-glucosidase in the small intestine is the main reason of blood glucose. Inhibiting the functioning of these enzymes could reduce postprandial hyperglycemia. Hence, many

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of the medicines prescribed for type II diabetes contain bioactive compounds with carbohydrate-hydrolyzing enzymes inhibitory activity (Adekola *et al*., 2017; Özcan, 2020). Usually, synthetic medicines taken for diabetes (e.g., voglibose, acarbose and miglitol) are good competitive inhibitors of α-glucosidase and α-amylase, hence inhibit patient's ability to break down complex carbohydrates into glucose. However, some side effects, including diarrhea, flatulence and abdominal discomfort, have been reported for the long-term prescription of these medicines (Mogole *et al*., 2020). Besides, there is a trend for replacing synthetic medicines with natural ones among the health-conscious consumers. Therefore, there is a growing market for natural medicines such as bioactive compounds extracted from plants. Moreover, some plant extracts have been suggested as a suitable source of carbohydrate hydrolase inhibitors with probably limited or no side effects (Veiga *et al*., 2020). For example, *Dioscorea polystachya* and *Morus Alba* have been demonstrated for their high α-amylase and α-glucosidase inhibitory potential, respectively; and the blends of pineapple, apple and ginger as a beverage can be used as a dietary supplement to prevent diabetes mellitus because of their hypoglycemic effects (Ademosun *et al*., 2020; Ng and See, 2019). The food and pharmaceutical industries are exploring the possibility of developing new anti-diabetics from other plant materials to satisfy market demand.

Belonging to the Cucurbitaceae family, Gac (*Momordica Cochinchinensis* Spreng.) is a perennial plant distributed widely in many regions of the world, including Northeastern Australia as well as South-East and South Asia (Chuyen *et al*., 2014). Gac fruit was used as a natural colorant in cuisines and was believed to improve human vision. Previous studies have demonstrated that high contents of carotenoids, lycopene and medicinal components (e.g., saponins and triterpenoids) are found in fruit, aril and seeds of Gac, respectively (Abdulqader *et al*., 2018; Chuyen *et al*., 2014; Kha *et al*., 2013; Yu *et al*., 2017). Besides, Gac leaves were consumed in traditional medicine for various purposes such as curing fever, back pain, inflammation, wart and hemorrhoids. However, information about its bioactive components and biological effects is limited. A previous study had explored carbohydrolytic enzyme inhibitory, anti-inflammatory and antioxidant abilities of other species of *Momordica*, that is, *M. dioica*, *M. charantia*, *M. charantia* var. *muricata* (Nagarani *et al*., 2014). However, to the best of our knowledge, there is no published paper in the scientific literature that has examined the bioactive content and possible biological effects of Gac leaves (*M. cochinchinensis* Spreng.). The objectives of this research were to analyze the phytochemicals available in Gac leaves and to explore their best maturity stages that could yield an extract with the highest α-amylase and α-glucosidase inhibitory activities.

Materials and Methods

Plant materials

The fresh Gac leaves were collected in Pingtung county, Taiwan in November 2019. The identity of the plant species (*M. cochinchinensis* Spreng.) was confirmed as described by experts in the literature. These leaves were divided into three different stages of maturity (YL: young; ML: mature and OL: old leaves) according to visual color and size as described in the literature (Angmo *et al*., 2019). The average width and length of leaves were measured using a ruler. Besides, the color values of leaves (L^*: lightness; a^*: greenness–redness and b^*: blueness–yellowness) were measured using a colorimeter (ZE 2000, Nippon Denshoku, Japan). Then, leaves were dried at 45°C for 48 h using the oven drying method (OV-100, Precision oven, HiPoint, Taiwan) until the final water activity of samples reached 0.32 (Roukas and Kotzekidou, 2020). Dried leaves were ground into powder using a pulverizing machine (RT-N08, RT, Taiwan), sieved by a 0.2–0.5-mm mesh screen to maintain constant particle size, packaged in airtight polyethylene films (0.04-mm thickness) and kept at –4°C until analysis (schematic representation is presented in Figure 1).

Chemicals

Standards of phenolic and flavonoid compounds: 4-nitrophenyl α-D-galactopyranoside (PNPG), 3,5-dinitrosalicylic acid (DNS), α-amylase from *Bacillus sp.*, intestinal acetone powder from rat, Folin & Ciocalteu's phenol reagent, L-methionine, riboflavin and nitrotetrazolium blue chloride (NBT) were purchased from Sigma-Aldrich, USA. Aluminum chloride hex hydrate, dimethyl sulfoxide (DMSO), potassium acetate, potassium hydrogen phosphate, potassium dihydrogen phosphate, potassium sodium tartrate-4-hydrate, sodium acetate, sodium carbonate, sodium hydroxide and sodium hydrogen phosphate were purchased from J.T. Baker, Avantor, PA, USA. Methanol was obtained from Aencore Chemical Pvt. Ltd., Surrey Hills, Australia.

Extraction procedure

A mixture of Gac leaves powder (1 g) and 50% aqueous ethanol (40 mL) was extracted using a 150-W ultrasonic bath (DC150, Delta, Taiwan) for 20 min based on preliminary studies. The temperature was monitored by a laboratory thermometer and adjusted at 25°C. These extraction conditions were defined according to a previous study with some modifications (Tian *et al*., 2019). The extract was then centrifuged at 10,000 rpm for 20 min, filtered with 90-mm filter paper and kept at -20°C until further analysis.

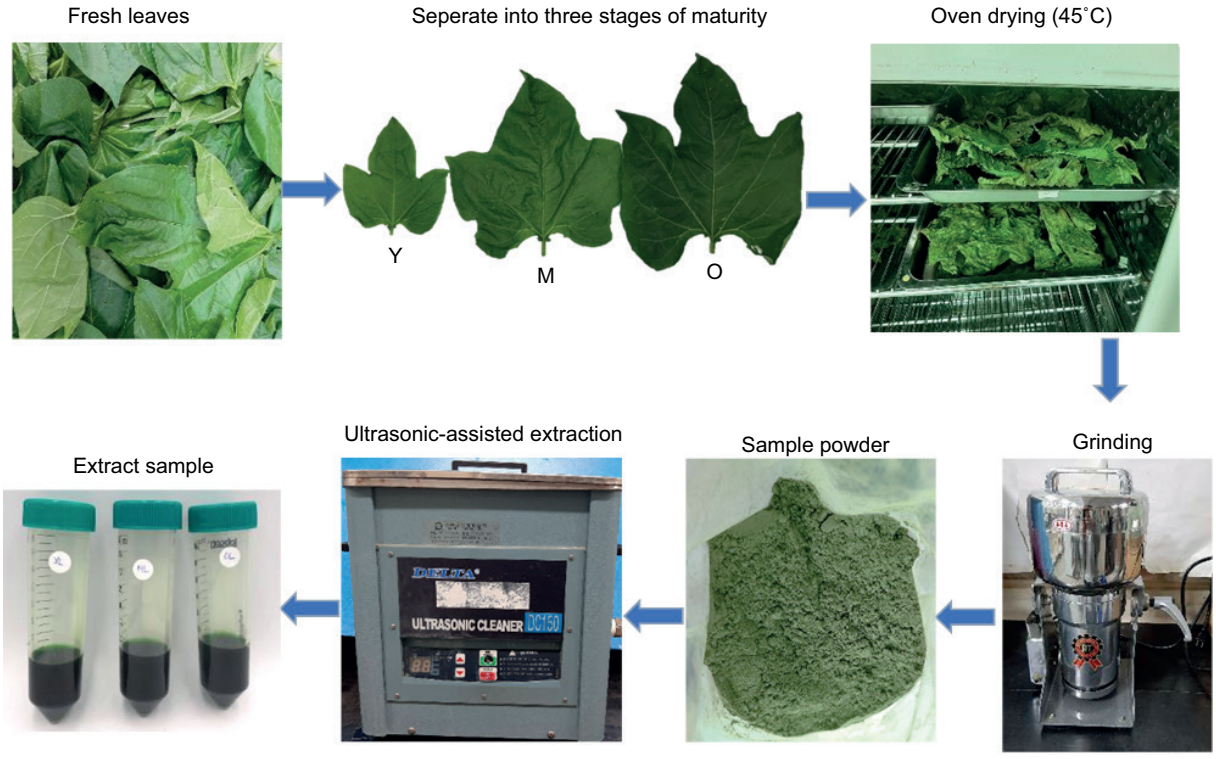

Figure 1. Schematic representation of the protocol employed to prepare Gac leaves extract. YL: young leaf; ML: mature leaf; OL: old leaf.

Chemical analysis of extracts

Determination of total phenolics content

Total phenolics content (TPC) was evaluated according to the Folin–Ciocalteu assay with minor modifications (Singleton and Rossi, 1965). A mixture of leaves extract (0.1 mL), 50% Folin–Ciocalteu reagent (0.1 mL) and 2% sodium carbonate (2 mL) was incubated at about 25°C in a dark place for 30 min. The absorbance of reactive mixture was recorded with an ultraviolet spectrophotometer (U-200 Spectrophotometer, Hitachi, Japan) at 760-nm wavelength. The TPC was measured using gallic acid calibration curve and presented as milligram gallic acid equivalent (GAE) per 100 g dried weight (DW) of sample (mg GAE/100 g DW).

Determination of total flavonoids content

Total flavonoids content (TFC) of Gac leaves was determined using a colorimetric method of aluminum chloride ($AlCl_3$) with slight modifications (Nguyen *et al.*, 2020). An aliquot of 250 µL of extracts was mixed well with 750 µL of 95% ethanol, 50 µL of 10% aluminum chloride, 50 µL of 1 M potassium acetate and 1.4 mL of distilled water in a test tube. The mixture was left for 30 min at room temperature in dark before recording at 415 nm using an ultraviolet spectrophotometer. The TFC was represented as milligram quercetin equivalent per 100 g dried weight sample (mg QE/100 g DW) through a QE calibration curve.

Identification of phenolic and flavonoid compounds using high performance liquid chromatography

The sample extract was filtered using a 0.45-µm syringe filter (PTFE013N045I, PureTech Syringe Filter, Taiwan) before injecting it into the high-performance liquid chromatography (HPLC) system (L-7100, Hitachi, Japan). The injected extract (20 µL) was then separated chromatographically on a Mightysil RP-18 column (250 mm × 4.6 mm, 5 µm) (Dobrinčić *et al.*, 2020). The mobile phases were 0.25% formic acid, 2% methanol in ultrapure water (solvent B) and 100% methanol grade (solvent C) with a flow rate of 1 mL/min. The chromatographic peaks in extracts were identified and measured by collating their UV-visible spectra (280 nm) and retention period with those of external standards. The content of each polyphenol was presented as milligram per 100 g of dried weight sample (mg/100 g DW).

Determination of α-amylase inhibitory activity

α-Amylase inhibitory activity was determined based on a previous report with slight modifications (Shori, 2020). Briefly, the solvent of extract was first removed by a vacuum evaporator, the residue was then dissolved by 2-mL 50% DMSO. Next, a mixture of this DMSO extract (100 µL) and 0.5 U/mL α-amylase solution (100 µL) was transferred in a tube and incubated at 37°C for 5 min. Next, 100 µL of starch solution (1%) was added and re-incubated for 5 min at 37°C. The reaction ended using

1 mL of DNS color reagent, then incubated for 5 min in a boiling water bath and cooled down. The absorbance was read at a wavelength of 540 nm. The background (replacing the starch by pH 6.9 buffer) and control (50% DMSO instead of sample extract) were also prepared by the same procedure. The α-amylase inhibitory activity was calculated according to Equation 1:

$$\alpha\text{-amylase IA (\%)} = \frac{A_C - (A_S - A_{BG})}{A_C} \times 100\% \quad (1)$$

where IA is inhibitory activity inhibitory activity, A_C is the control absorbance, A_S is the sample extract absorbance and A_{BG} is the background absorbance.

Determination of α-glucosidase inhibitory activity

α-Glucosidase inhibitory activity was determined according to a previous method with partial modifications (dos Santos Pereira *et al.*, 2020). Before pre-incubated for 5 min at 37°C, a mixture of DMSO extract (5 µL) and α-glucosidase solution (10 µL) was put into a 96-well microplate. Then, 0.01-M p-nitrophenyl α-D-galactopyranoside (10 µL) was added in each well and incubated for 20 min at 37°C. The mixture reaction was stopped by adding 200-µL 0.1-M sodium carbonate. The absorbance was recorded at a wavelength of 405 nm using a microplate reader (AMR-100, Clubio, Taiwan). Control without sample, and background without PNPG were also determined. Ability to inhibit α-glucosidase was calculated according to Equation 2:

$$\alpha\text{-glucosidase IA (\%)} = \frac{A_C - (A_S - A_{BG})}{A_C} \times 100\%, \quad (2)$$

where IA is inhibitory activity inhibitory activity, A_C is the control absorbance, A_S is the sample extract absorbance and A_{BG} is the background absorbance.

Determination of superoxide dismutase-like activity

Superoxide dismutase (SOD)-like capacity was evaluated following the previous procedure with slight modifications (Cheng *et al.*, 2015). A volume of 2.95 mL of 1.67×10^{-4} M NBT and 0.01-M L-methionine was added into each tube containing 50 µL of extract sample; 3 µL of 2.4×10^{-6} M riboflavin was added before transferring the test tubes into a light box and incubated for 15 min. The blue formazan formed was read at 560 nm. The control (buffer instead of sample) and background (riboflavin replacement buffer) were also determined. The SOD-like activity (percentage of scavenging ability) was first defined by Equation 3, then measured through the SOD standard curve and represented as unit per gram dried weight sample (U/g DW).

$$\text{Scavenging ability (\%)} = \frac{A_C - (A_S - A_{BG})}{A_C} \times 100\%, \quad (3)$$

where A_C is the control absorbance, A_S is the sample extract absorbance and A_{BG} is the background absorbance.

Besides, the relationship between maturity level of Gac leaves and the SOD-like activity of the extract was explored using different equations to develop a mathematical model for the prediction of antioxidant activity of extract as a function of degree of maturity.

Statistical analysis

The results were presented as mean ± standard deviation (SD) (n = 3). The significant difference between samples was compared by Duncan's multiple-range tests ($P < 0.05$) by one-way analysis of variance (ANOVA) using IBM SPSS statistics version 22. The correlation between phenolic compounds and the ability to inhibit carbohydrate hydrolyzed enzymes was determined using Pearson's correlation analysis with two-tailed test.

Results and Discussion

Physical properties of Gac leaves at different maturity stages

Average size

At different maturity stages of Gac leaves, the average size (width and length) was measured as represented in Table 1.

Table 1. Average size and color values of Gac leaves at different maturity stages.

	Average size		Color values		
	Width (cm)	Length (cm)	L*	a*	b*
YL	7.07 ± 0.51^{c}	9.63 ± 0.70^{c}	44.89 ± 0.08^{a}	–7.44 ± 0.06^{c}	18.08 ± 0.09^{b}
ML	14.67 ± 1.06^{b}	15.40 ± 0.70^{b}	42.76 ± 0.26^{b}	–6.58 ± 0.10^{b}	18.37 ± 0.13^{a}
OL	16.37 ± 0.45^{a}	17.57 ± 0.31^{a}	37.95 ± 0.24^{c}	–6.44 ± 0.02^{a}	16.14 ± 0.09^{c}

The values are presented as mean ± standard deviation (SD) (n = 3). $^{a–c}$Values in columns with different letters indicate the significant difference ($P < 0.05$). YL: young leaf; ML: mature leaf; OL: old leaf. L*: lightness; a*: greenness–redness; b*: blueness-yellowness.

It was observed that OL had the highest values of width and length (16.37 and 17.57 cm, respectively) as compared to those of ML and YL. This suggests that the leaf size increases with development of plant and reaches the maximum limit at old stage. Similar results were demonstrated at three different maturity stages of beet leaves (Angmo *et al*., 2019).

Color values

The color values of Gac leaves at different maturity stages were evaluated using L^*, a^* and b^* parameters as presented in Table 1. With significant difference in samples, following color value ranges were obtained: lightness L^* (37.95–44.89), yellowness b^* (16.14–18.08) and greenness a^* (-7.44–-6.44). The decrease in L^* and increase in a^* from YL to OL indicated that the green of Gac leaves was becoming darker and browner with leaf maturation. This suggested that the plant growth could affect pigment components and color parameters in different parts of the plant. Similar observations were reported in a previous study (Patsilinakos *et al*., 2018).

Total phenolics content and total flavonoids content

Total phenolics content and total flavonoids content of Gac leaves at three maturing stages extracted by ultrasound-assisted extraction treatment are presented in Table 2. According to the results, the extract obtained from YL had the highest TPC and TFC values (4897.01 mg GAE/100 g DW and 592.81 mg QE/100 g DW, respectively) followed by that of ML and OL. This observation indicated that the secondary metabolites (phenolics and flavonoids) are probably reduced by oxidative stress during plant growth and aging. Previous studies have established a similar trend in *Coffee arabica L.* leaves, the highest content of total phenolics was found in the extract of young leaves (Ngamsuk *et al*., 2019). However, the TFC expressed a slight difference in trend: it increased significantly ($P < 0.05$) from mature to old stage after decreasing from young to mature stage, that is, some new flavonoid compounds were synthesized in smaller quantities in old stage.

Phytochemicals in different maturing stages

The phytochemicals obtained from YL, ML and OL extracts are listed in Table 3. In YL extract, myricetin, vitexin and esculetin accounted for nearly 43%, 22% and 11%, respectively, of all the individual compounds detected, which were approximately 4.15, 10.62 and 2.36 times higher than that in OL extract (Figure 2). This indicated that these compounds were reduced with the aging of plant. Similarly, chlorogenic acid and vanillic acid, respectively, decreased from 339.74 and 228.56 mg/100 g DW in YL extract to 56.53 and 11.63 mg/100 g DW in OL extract. In addition, catechin, caffeic acid and quercetin reduced by leaf senescence couldn't be detected in OL extract. This observation suggested that because of the aging process, there were notable differences in the amounts of components at different maturing stages of Gac leaves, and this was demonstrated in the guava leaf extract (Angmo *et al*., 2019). Results demonstrated that extract of Gac leaves contained several phenolic and flavonoid compounds that are found in the leaves of *Momordica* species (Nagarani *et al*., 2014). For example, gallic acid, chlorogenic acid, ferulic acid, quercetin, ellagic acid and catechin were identified in these *Momordica* leaves extract; the major components of *M. charantia* var *muricata* and *M. charantia* leaves were chlorogenic acid and quercetin, while *M. dioica* had rutin and ellagic acid.

Some compounds (including catechin, vanillic acid, epicatechin, chlorogenic acid and myricetin), in a previous study, have established inhibitory ability against α-amylase and α-glucosidase thru IC_{50} value with the following respective values: catechin, 8.41 mM and 13.4 μM; vanillic acid, 27.89 mM and 277.38 μM; epicatechin, 7.34 mM and 11.75 μM; chlorogenic acid, 11.57 mM and 231.80 μM; and myricetin, 1.19 mM and 2.73 μM (Tan *et al*., 2017). Containing the highest levels of these compounds in the extract, YL has clearly demonstrated a strong inhibitory ability toward α-amylase and α-glucosidase. In addition, OL demonstrated a significant increase in ellagic acid and morin with 2.36 and 1.88 times higher than that in YL, respectively; and 3.06 and

Table 2. Phytochemicals content and carbohydrate hydrolyzing enzymes inhibitory activity at different maturity stages.

Maturing stage	TPC (mg GAE/100 g DW)	TFC (mg QE/100 g DW)	α-amylase inhibitory activity (%)	α-glucosidase inhibitory activity (%)	SOD-like activity (Scavenging ability %)
YL	4897.01 ± 98.74^a	592.81 ± 18.87^a	34.77 ± 1.71^a	40.21 ± 1.55^a	24.29 ± 1.00^a
ML	4318.08 ± 261.32^b	365.69 ± 14.67^c	32.18 ± 1.14^b	29.17 ± 2.34^b	16.27 ± 1.21^b
OL	3329.28 ± 223.70^c	487.73 ± 18.64^b	29.98 ± 0.55^b	41.82 ± 0.29^a	10.15 ± 1.71^c

The results are presented as mean ± standard deviation (SD) ($n = 3$). $^{a–c}$In the same column, different letters express remarkable difference between samples ($P < 0.05$), Duncan's test. YL: young leaf; ML: mature leaf; OL: old leaf; TPC: total phenolic content; TFC: total flavonoid content; GAE: gallic acid equivalent; DW: dry weight; QE: quercetin.

Table 3. Concentrations of total phenolic and flavonoid compounds at different maturity stages.

Compounds	Chemical formula	OL (mg/100 g DW)	ML (mg/100 g DW)	YL (mg/100 g DW)
Esculetin	$C_9H_6O_4$	227.41 ± 39.28^{cB}	320.02 ± 61.72^{bB}	537.29 ± 46.95^{cA}
Catechin	$C_{15}H_{14}O_6$	ND	25.08 ± 3.64^{eB}	66.14 ± 3.07^{ghA}
Chlorogenic acid	$C_{16}H_{18}O_9$	56.53 ± 5.07^{fgC}	151.55 ± 21.57^{dB}	339.74 ± 41.25^{dA}
Vanillic acid	$C_8H_8O_4$	11.63 ± 0.67^{hC}	39.22 ± 9.50^{dB}	228.56 ± 11.85^{eA}
Caffeic acid	$C_9H_8O_4$	ND	1.42 ± 0.16^{eB}	2.24 ± 0.50^{hA}
Epicatechin	$C_{15}H_{14}O_6$	28.34 ± 1.99^{ghB}	23.25 ± 4.09^{eB}	46.06 ± 5.00^{ghA}
***p*-coumaric acid**	$C_9H_8O_3$	22.26 ± 2.85^{ghB}	24.29 ± 2.45^{eB}	51.97 ± 1.82^{ghA}
Sinapic acid	$C_{11}H_{12}O_5$	10.77 ± 0.92^{hC}	31.93 ± 3.26^{eB}	88.16 ± 11.01^{fgA}
Ferulic acid	$C_{10}H_{10}O_4$	10.61 ± 1.75^{hC}	26.23 ± 2.00^{eB}	62.56 ± 4.11^{ghA}
Vitexin	$C_{21}H_{20}O_{10}$	104.91 ± 23.36^{deC}	247.20 ± 17.31^{cB}	1114.31 ± 135.69^{bA}
Ethyl 3,4-dihydroxybenzoate	$C_9H_{10}O_4$	71.91 ± 11.33^{ef}	ND	ND
Rutin	$C_{27}H_{30}O_{16}$	16.70 ± 1.90^{hA}	5.17 ± 0.40^{eB}	1.90 ± 0.35^{hC}
Resveratrol	$C_{14}H_{12}O_3$	ND	ND	1.74 ± 0.36^{h}
Ellagic acid	$C_{14}H_6O_8$	364.71 ± 48.92^{bA}	118.85 ± 9.96^{dC}	154.51 ± 4.99^{fB}
Myricetin	$C_{15}H_{10}O_8$	530.73 ± 58.48^{aC}	968.44 ± 95.50^{aB}	2203.48 ± 118.71^{aA}
Morin	$C_{15}H_{10}O_7$	138.26 ± 19.50^{dA}	32.45 ± 6.98^{eC}	73.64 ± 4.17^{ghB}
Cinnamic acid	$C_9H_8O_2$	4.38 ± 0.57^{hC}	14.26 ± 2.61^{eB}	17.98 ± 0.46^{ghA}
Quercetin	$C_{15}H_{10}O_7$	ND	11.19 ± 2.49^{eB}	52.53 ± 3.62^{ghA}
Hesperidin	$C_{28}H_{34}O_{15}$	0.63 ± 0.16^{h}	ND	ND

The values are presented as mean ± standard deviation (SD) (n = 3). $^{a–h}$Values in columns with different lowercase letters indicate the significant difference ($P < 0.05$). $^{A–C}$Values in rows with different uppercase letters indicate the significant difference ($P < 0.05$). ND: not detected. YL: young leaf; ML: mature leaf; OL: old leaf; DW: dry weight.

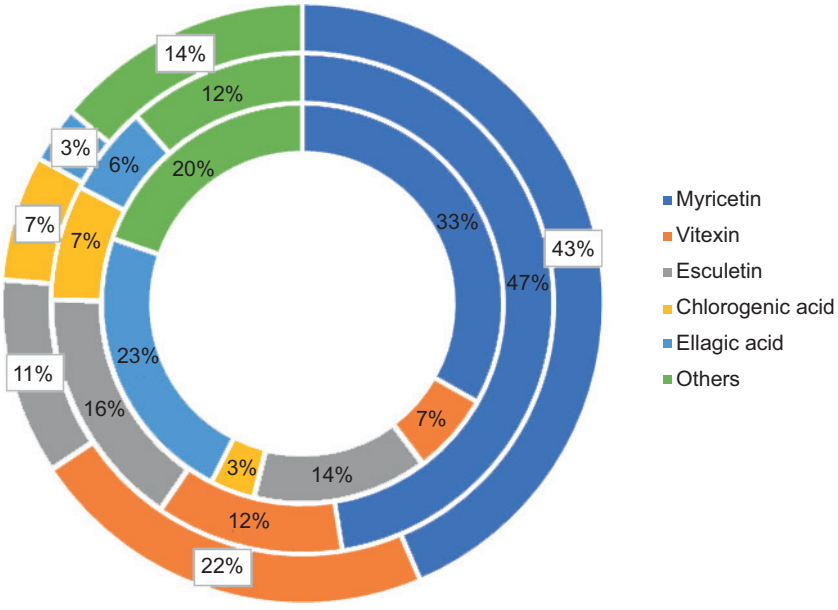

Figure 2. Main components of Gac leaves extract as affected by maturity stages. YL (young leaf), ML (mature leaf), and OL (old leaf) are presented in the inner, middle, and outer graphs, respectively.

4.26 times higher than that in ML, respectively. With a low IC_{50} value (morin: 32 μM, ellagic acid: 18.4 μg/mL) (Assefa *et al*., 2020; Proença *et al*., 2017), the increase of these two compounds may explain a higher inhibition of α-glucosidase in OL extract. According to the results, YL samples present the best maturing stage of Gac leaves for obtaining the highest concentration of bioactive compounds and the strongest inhibition of carbohydrate-hydrolyzing enzymes for further experiments.

Effects on carbohydrate-hydrolyzing enzymes' inhibitory activity

For the starch hydrolysis enzymes' inhibitory activity, the effects of maturing stages of Gac leaves presented the same tendency with the content of phytochemicals (Table 2). The higher α-amylase and α-glucosidase inhibitory abilities belonged to YL extract (34.77 and 40.20%, respectively), which is probably because of higher concentrations of most phenolic and flavonoid components in YL. It has been explained previously, how flavonoid compounds could contribute in lowering the blood sugar and what are the mechanisms involved in their antidiabetic properties (AL-Ishaq *et al*., 2019). The results demonstrated that Gac leaves contain bioactive compounds that could lower the blood sugar, and the concentration of these compounds varies depending on the maturity stage of Gac leaves. This indicates the importance of effective compounds in lowering the blood sugar level, and this study established that such an extract and its effective components could be established as possible ingredients for developing nutritional supplements.

The results of the present study were similar to those reported for extracts obtained from the leaves of *Momordica charantia* Linn and *Momordica charanti* var. *muricata* (with an approximate α-amylase inhibition of 45% and α-glucosidase inhibition of 40%, respectively) (Nagarani *et al*., 2014). Besides, previous research done on Ceylon cinnamon leaves demonstrated a similar trend, that is, higher concentrations of polyphenolic compounds were correlated with stronger anti-amylase activity (Abeysekera *et al*., 2019). However, the anti-glucosidase activity obtained from YL and OL extracts was almost the same in spite of significant difference in TPC and TFC, suggesting that several new compounds (e.g., ethyl 3,4-dihydroxybenzoate and hesperidin) were synthesized in the old stage of Gac leaves.

Effects on SOD scavenging activity

Superoxide dismutase, an important antioxidant enzyme in animals and plants, is a free radical scavenger that transformed reactive oxygen species formed during

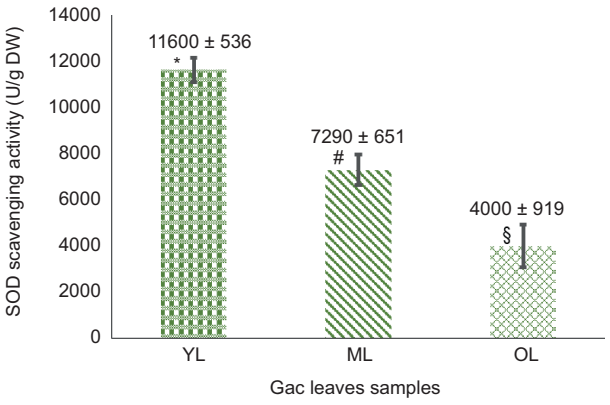

Figure 3. Superoxide dismutase (SOD) scavenging activity of the extract as affected by maturation degree of Gac leaves. YL: young leaf; ML: mature leaf; OL: old leaf.

reduction and oxidation into H_2O_2 and O_2 (Cheng *et al*., 2015; Stephenie *et al*., 2020). SOD activity at different maturity stages of Gac leaves is presented in Table 2. The data ranged from 10.15% to 24.29% of free radical scavenging activity of SOD (equivalent to 3999.63–11599.96 U/g DW), which presented the same result with *M. charantia* Linn. leaf extract (22%) but lower than that of *M. charantia* var. *muricata* and *M. dioica* leaf extracts, that is, 35% and 51%, respectively (Nagarani *et al*., 2014). Besides, the highest SOD level was achieved from YL extract (11599.96 U/g DW), followed by ML and OL (7289.70 and 3999.63 U/g DW, respectively) (Figure 3) ($P < 0.05$). This demonstrates a similar trend with the content of phytochemicals and carbohydrate hydrolyzing enzyme inhibitory activity. The high correlation between them has been proved in a previous study (Shimada *et al*., 2020). For example, a previous study explained the association between anti-glucosidase ability and SOD-like activity (correlation coefficient: 0.72), and between total soluble phenolic content and SOD-like activity (correlation coefficient: 0.71).

According to Figure 3, the SOD scavenging activity of Gac leaves extract depended on the degree of maturity, which declined with the aging of leaves. A polynomial model was developed, which well predicted ($R^2 = 1$) the changes in the SOD scavenging activity of Gac leaf extract (Equation 4):

$$y = 675.87x^2 - 6337.9x + 17, \quad (4)$$

where y is the SOD scavenging activity of the extract in U/g DW and x^2 is the degree of maturity of Gac leaves (YL = 1, ML = 2 and OL = 3).

Conclusion

For the first time, bioactive compounds of Gac leaves were extracted and analyzed whereas previous research

had primarily focused on the fruit of this plant. The Gac leaves extract was found to be rich in phenolics and flavonoids. Relatively high concentrations of compounds, such as myricetin, vitexin, esculetin, chlorogenic acid and vanillic acid, suggested that this plant extract is a source of valuable bioactive compounds. It was demonstrated that the degrees of maturity can significantly affect the chemical composition and carbohydrate-hydrolyzing enzymes inhibitory activity of Gac leaves. Young leaves were found to be superior to others in terms of quantity and quality of phytochemicals because of the highest concentration of most compounds obtained, and they possess the strongest ability in inhibiting α-amylase and α-glucosidase. Therefore, Gac leaves have the potential to be used as an ingredient of functional foods, nutritional supplements and pharmaceuticals because of the benefits such as lowering hyperglycemia and antioxidant properties. Additional pharmacological research is needed for its use in the treatment of diabetes.

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DNAFoil, a novel technology for the rapid detection of food pathogens: Preliminary validation on *Salmonella* and *Listeria monocytogenes*

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ORIGINAL ARTICLE

Abstract

Over the past decades, several tools have been developed for food pathogen detection, including proteomics, metabolomics, immunological, biosensor, and nucleic acid-based approaches. Although these techniques are reliable and precise, they are time-consuming, technically challenging, and costly. Hence, it is necessary to develop rapid techniques for food pathogen detection, which can be performed at the household level. DNAFoil mechanism is a portable, completely self-administered, on-site DNA test that does not need expensive instruments or settings to confirm food pathogen detection in as little as 30 min. DNAFoil was used successfully for detecting food contamination and adulteration with pork derivatives (down to 0.1%) and vegetal components (down to 0.01%), respectively. In this study, initial validation experiments of DNAFoil were investigated to detect *Listeria monocytogenes* and *Salmonella* contamination. To confirm the specificity of the proposed method toward *Salmonella*, 18 different *Salmonella* strains, 6 non-*Salmonella* bacteria, and 2 fungi were investigated; also, in the case of *Listeria monocytogenes*, five bacterial strains, two fungi, and *Listeria monocytogenes* were investigated. The results stated that the Swiss Decode *Salmonella* and *L. monocytogenes* solutions can detect as few as 1 and 10 copies of DNA per microliter, respectively. The results also showed that the accuracy of our method ranges between 92 and 100%, while the precision value ranged between 88 and 100%. In terms of quality control applicability, DNAFoil *Salmonella* and *Listeria monocytogenes* reactions could be visually detected with the naked eye using a lateral flow strip, which could be used for in-place quality control during manufacturing and also can be used for more lab tests. In terms of cost, DNAFoil is usually much cheaper than the traditional detection methods. Therefore, DNAFoil is proposed as a promising and universal detection technology for food pathogens.

Keywords: DNAFoil technology; food pathogen detection techniques; food safety; foodborne diseases; health and economics threats

Introduction

It is well-known that food safety is affected by many factors and variables, including, for example, globalization of food trade, population increase in the world, change in the lifestyle of consumers, climate changes, and the accompanying rapid changes in food systems. International trade means that unsafe food can be distributed widely (Pinu, 2016; WHO, 2020). Recently, many food-borne disease outbreaks in the world, the most

prominent of which were due to several microbial species, for example, *Listeria monocytogenes*, *Escherichia coli*, *Campylobacter jejuni*, *Salmonella* sp., *Shigella* sp., have proven that food safety is under severe threat from food pathogens (Bintsis, 2017; Chlebicz and Śliżewska, 2018; Faour-Klingbeil and Todd, 2020). Unsafe food containing food pathogens, that is, bacteria, viruses, parasites, or fungi, can cause different diseases ranging from diarrhea to cancers (Dwivedi and Jaykus, 2011; Food Safety Education Program, 2016; WHO, 2020).

Centers for Disease Control and Prevention (CDC) estimates that each year 48 million people get sick from foodborne diseases, 128,000 are hospitalized, and 3000 die. Foodborne pathogens cause diseases and deaths in all populations, particularly in groups at risk such as infants, children, elderly, and immunocompromised persons (CDC, 2020; FDA, 2021; WHO, 2020, 2021). The most common microorganisms responsible for the major foodborne illnesses are shown in Table 1.

In addition to the severe health risks caused by foodborne illnesses, they may also threaten international trade and cause significant economic losses. This has been confirmed by the reports received from the USDA's Economic Research Service. These reports indicated that foodborne illnesses cost the United States more than $15.6 billion. *Salmonella* tops the list of foodborne pathogens, with a treatment cost equivalent to $3.6 billion, followed by *Listeria monocytogenes*, which equals $2.8 billion, and then *Escherichia coli* with a value of $271 million (USDA ERS, 2014). This is in addition to the cost of recalls of the products as well (Tyco Integrated Security, 2012).

Hence, there is an urgent need to develop simple, sensitive, specific, robust, reliable, inexpensive, and rapid techniques for food pathogen detection, can perform at the household level and ensure food safety. These requirements comply with new, portable, completely self-administered, on-site DNA test technology called "DNAFoil technology," which does not need expensive equipment or laboratory settings to get the final results in as little as 30 min (El Sheikha, 2019). Additionally, DNAFoil technique has proven to be effective in:

- Detecting food contamination through its ability to detect pork contamination in beef as lower as 0.1% (Meat and Livestock Australia Limited "MLA", 2018);
- Detecting of food adulteration through its ability to detect the adulteration of milk products by vegetal materials as lower as 0.01% (Aronoff *et al*., 2018).

Through five steps (Figure 1), it can be clearly understood as to how this technique works. Sample preparation

Table 1. The most common microorganisms responsible for the major foodborne illnesses.

Foodborne illness or toxin	Associated microorganism	Health risks	Most population group(s) affected	Reference
Listeriosis	*Listeria monocytogenes*	Meningitis, mild illness in pregnant women, in babies (miscarriage, stillbirth, premature birth, potentially fatal infection after birth)	Pregnant women, newborns, the elderly, immuno-compromised individuals	Buchanan *et al*. (2017), Mayo Clinic (2020a)
Salmonellosis	*Salmonella* spp.	Typhoid fever, inflammatory bowel disease, stomach or bowel disorders	All groups	Bintsis (2017), Mayo Clinic (2019)
Shigellosis	*Shigella* spp.	Dehydration, seizures, rectal prolapse, hemolytic uremic syndrome, toxic megacolon, reactive arthritis, bloodstream infections (bacteremia)	Malnourished children, immuno-compromised individuals, the elderly	NCBI (2017), Mayo Clinic (2020b)
Campylobacteriosis	*Campylobacter* spp.	Mild to severe diarrhea, bloody diarrhea, stomach pain, cramps, nausea and/or vomiting, fever, muscle pain	All groups	Bintsis (2017), Ontario Ministry of Health and Long-Term Care (2020)
Botulism	*Clostridium botulinum*	Breathing problems, trouble swallowing, muscle weakness, slurred speech, headache, nausea	All groups	Bintsis (2017), Rasetti-Escargueil (2020)
Toxoplasmosis	*Toxoplasma gondii*	Headache, seizures, lung problems, severe eye infections, e.g., inflammation of retina, enlarged liver and spleen	All groups, especially babies, immuno-compromised individuals	EFSA and ECDC (2016), Mayo Clinic (2020c)
Yersiniosis	*Yersinia* spp.	Fever, abdominal pain, diarrhea (which is often bloody)	All groups, especially children, adults	EFSA and ECDC (2016), Ontario Ministry of Health and Long-Term Care (2018)
Amoebiasis	*Entamoeba histolytica*	Bowel perforation, gastrointestinal bleeding, stricture formation, intussusception, peritonitis, empyema	All groups	NCBI (2016), Park (2015)

without the need to be pre-enriched before analysis is considered the main obstacle in most methods, but the enrichment remains essential for the revival of stressed or injured cells (Cossarizza *et al*., 2017; Valderrama *et al*., 2016). But, through the DNAFoil mechanism, the sample preparation and DNA extraction stages were completed in a single step without the need to use spin-columns and centrifuges. For the amplification stage, cross-contamination is one of the difficulties faced by the commercially available kits used to detect food pathogens, that is, *Salmonella* and *Listeria monocytogenes* (Baraketi *et al*., 2018). In contrast to what happened using the DNAFoil technique, it is obvious that the DNA target amplification is done in one pot master mix without requiring trained staff, using thermos-cycling and cold chain. For the final stage (DNA detection stage), there are several problems generated from DNA electrophoresis and staining such as it being time-consuming, gel preparation, smearing, mutagenicity, toxicity, lower efficiency, etc. (Drabik *et al*., 2016; Hall, 2020). DNAFoil as a detection method during the final stage provides the test strip material, which allows for transport by a capillary force of the target DNA through the detection surface, allowing the target to hybridize specifically to their complementary capture sequences (target DNA fragments are captured in a band). Conjugation of micrometer-sized beads to DNA permits the results to be visualized by the naked eye (visible color reaction), enabling immediate, simple to interpret, cost-efficient, and on-site detection, while eliminating the need for advanced expensive instrumentation (El Sheikha, 2019).

Hence, the aim of this study was to investigate initial validation experiments of the DNAFoil technique to detect food pathogens, that is, *Salmonella* and *Listeria monocytogenes*.

Materials and Methods

Reference materials

Crude bacterial DNA extracts were purchased from the Culture Collection of Switzerland (CCOS), and experiments were conducted at the Swiss Decode labs in Renens, Switzerland. Live strains tested were procured from the Pasteur Institute (France). The strains tested were divided into two groups one for inclusivity and the other for exclusivity toward *Salmonella* and *Listeria monocytogenes* as follows. Firstly, the strains were tested for inclusivity and exclusivity toward *Salmonella*: (i) For inclusivity (*Salmonella Montevideo*, *Salmonella Heidelberg*, *Salmonella mbandaka*, *Salmonella enteritidis*, *Salmonella agona*, *Salmonella Indiana*, *Salmonella infantis*, *Salmonella arizonae* IIIa, *Salmonella senftenberg*, *Salmonella cerro*, *Salmonella Virchow*, *Salmonella anatum*, *Salmonella Newport*, *Salmonella thyphimurium*, *Salmonella arizonae* IIIb, *Salmonella saintpaul*, *Salmonella hadar*, *Salmonella enteritidis abony*); (ii) For exclusivity (*Listeria monocytogenes*, *Bacillus subtilis*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Aspergillus brasiliensis*, *Candida albicans*). Secondly, the strains tested for inclusivity and exclusivity toward *Listeria monocytogenes*: (i) For inclusivity (*Listeria monocytogenes*); (ii) For exclusivity (*Bacillus subtilis*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Aspergillus brasiliensis*, *Candida albicans*). All experiments involving living strains were conducted under BSL2 conditions at the independent microbiology lab in Couternon, France.

Sample preparation, DNA extraction, and amplification stages

DNAFoil mechanism is depicted in Figure 1. Steps 1 and 2 show the DNA extraction, lysing, neutralizing, and stabilizing processes of 200 µL of culture using a barrel without the need to use spin-columns and centrifuges. Briefly, bacteria cells are broken by an alkaline solution that contains chaotropic salts. This allows DNA to be released in the solution. The alkaline pH of the solution is not compatible with downstream DNA amplification; therefore, a neutralization/stabilization step is added, and it consists of buffering the pH with a second solution, which also provides monovalent salts that facilitate the DNA amplification reaction. Amplification stage of DNA target is started via step 3; one drop of extracted DNA is transferred to the reaction tube and is incubated in a water bath at 65°C. Then, using the specific primers and enzymes, the DNA targets will amplify and make multiple copies without using thermos-cycling and cold chain.

End-point assays

After 30 min of incubation at 65°C, DNAFoil strips were dipped into the reaction tube (detection step; 4th step). Migration by lateral-flow caused a positive band's appearance due to colloidal gold concentrating on the DNA capture line without using electrophoresis and staining (detection step; 5th step).

Real-time confirmatory assays

For real-time assays, 1 µL of extract was combined with 24 µL of *Salmonella* and *Listeria monocytogenes* reactions mix and incubated at 65°C. Fluorescence (FAM channel) was monitored every 20 s, and the fluorescence signal was plotted over time.

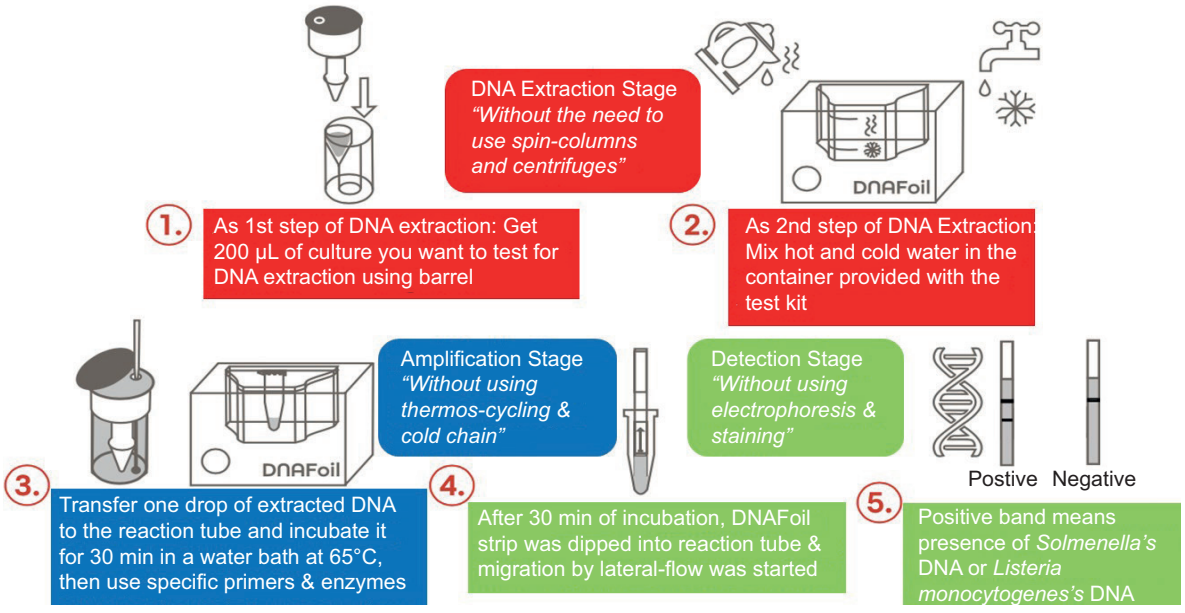

Figure 1. Procedural diagram for the mechanism of food pathogen detection using DNAFoil technology. Source: Adapted from El Sheikha (2019). Reproduced with permission of Elsevier.

Statistical analysis

All data were presented as the mean value ± standard deviation (SD) of independent experiments on various days.

Performance metrics

Accuracy (%)

Results from the experimental specificity (Figure 3A and B) were used to calculate the method accuracy using the following equations:

$$\text{Accuracy (\%) at 10 min} = \frac{(TP+TN)}{(TP+TN+FP+FN)} \times 100 \quad (1)$$

$$\text{Accuracy}(\%) \text{ at 15 min} = \frac{(TP+TN)}{(TP+TN+FP+FN)} \times 100 \quad (2)$$

$$\text{Accuracy}(\%) \text{ at 20 min} = \frac{(TP+TN)}{(TP+TN+FP+FN)} \times 100 \quad (3)$$

where TP, FN, FP, and TN represent the number of true positives, false negatives, false positives, and true negatives, respectively.

Precision (%)

Results from the experimental specificity (Figure 3A and B) were used to calculate the method accuracy using the following equations:

$$\text{Precision}(\%) \text{ at 10 min} = \frac{TP}{(TP+FP)} \times 100 \quad (4)$$

$$\text{Precision}(\%) \text{ at 15 min} = \frac{TP}{(TP+FP)} \times 100 \quad (5)$$

$$\text{Precision}(\%) \text{ at 20 min} = \frac{TP}{(TP+FP)} \times 100 \quad (6)$$

where TP, FN, FP, and TN represent the number of true positives, false negatives, false positives, and true negatives, respectively.

Results

Inclusivity and exclusivity

To confirm the specificity of our method toward *Salmonella*, 18 different *Salmonella* strains, 6 non-*Salmonella* bacteria, and 2 fungi were investigated; also, in the case of *Listeria monocytogenes*, five bacterial strains, two fungi, and *Listeria monocytogenes* were investigated.

Independent microbiology lab prepared cultures containing 10^8 CFU/mL. Swiss Decode analyzed the broth media in a blinded manner. The amplification time for each strain is reported in Figure 2A and 2B.

The standard amplification time used for our DNAFoil kit is 30 min. Samples for which the amplification signal was not detected after 30 min were considered as negative (no presence of *Salmonella* or *Listeria monocytogenes*).

Swiss Decode *Salmonella* solution positively identified the 18 *Salmonella* strains after 10 to 13 min (Figure 2A).

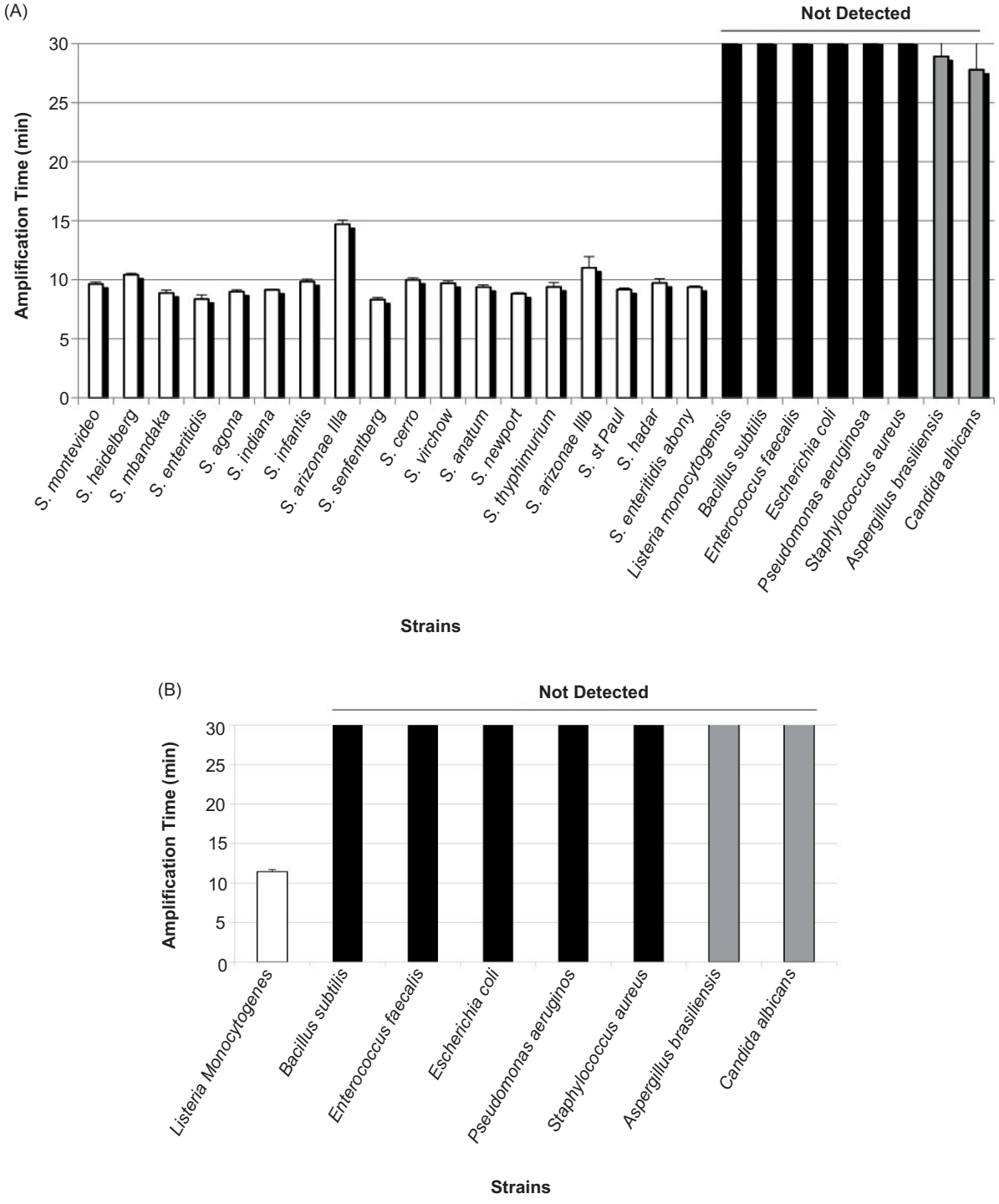

Figure 2. Amplification time—selectivity experiment. (A) Different cultures of *Salmonella* (in white), non-*Salmonella* bacteria (in black), and fungus (in gray) were prepared at an independent microbiology lab. (B) *Listeria monocytogenes* (in white), different bacterial cultures (in black), and fungus (in gray) were prepared at an independent microbiology lab. Broth media (200 µL) was withdrawn from cultures containing 10^8 CFU/mL. Bacteria were lysed according to the standard DNAFoil method. DNA detection was performed with a real-time assay according to the Swiss Decode protocol. Data represent mean ± SD, n = 3.

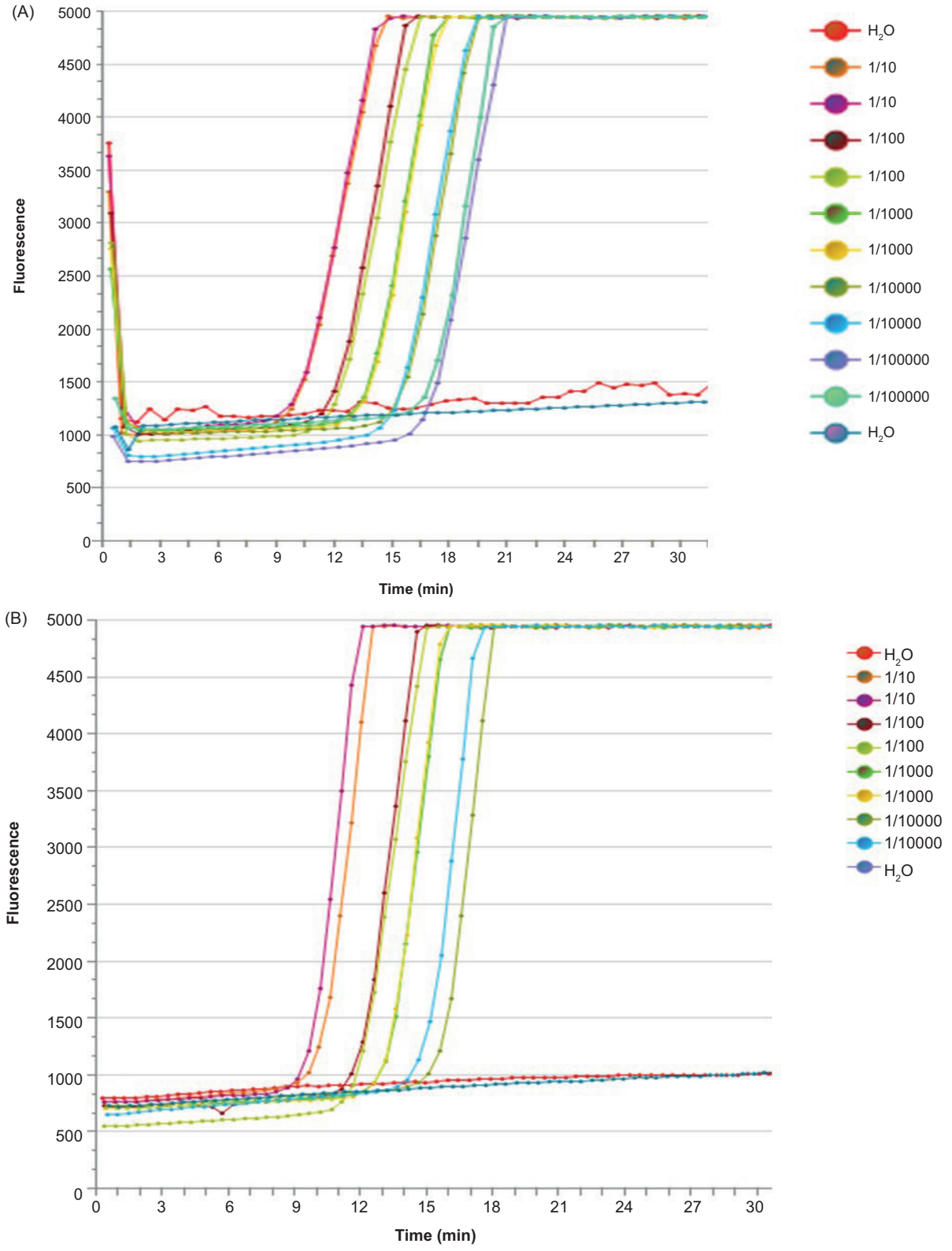

Figure 3. Amplification time-sensitivity experiment. Ten times serial dilution of crude DNA extracts from *Salmonella enterica* and *Listeria monocytogenes* were diluted in 10 mM TRIS pH 8.0 or DNAFoil lysis buffer. One microliter of the lysis solution was taken to run a real-time assay. (A) We can observe that the time needed for the detection of *Salmonella* increases as the amount of DNA present in the sample decreases. However, the detection time is well below the 30 min used as our standard amplification time. (B) We can observe that the time needed for the detection of *Listeria monocytogenes* increases as the amount of DNA present in the sample decreases. However, the detection time is well below the 30 min used as our standard amplification time.

Other non-*Salmonella* strain samples were still negative after 30 min. Our method also allowed *Salmonella*'s identification in broth media samples where *Salmonella* was mixed with several other non-*Salmonella* strains (data not shown).

Regarding the *Listeria monocytogenes*, Swiss Decode *L. monocytogenes* solution positively identified the *Listeria monocytogenes* strains after 11 min (Figure 2B). The other strain samples were still negative after 30 min.

Limit of detection (LOD) with serial dilutions

To determine the sensitivity of our method, a serial dilution of crude bacterial DNA extracts was analyzed. Crude extracts for *Salmonella Enterica* subspc. *enterica* and *Listeria monocytogenes* were obtained from CCOS. Crude extracts containing 10^5 CFU/µL were serially diluted 1:10 either in 10 mM TRIS pH 8.0 or in our DNAFoil lysis buffer. The reactions were analyzed in duplicate by real-time assays (Figure 3A and 3B). The results were similar for both dilution methods (data not shown).

Accuracy and precision (%)

Accuracy and precision (%) are calculated to measure the performance of our method to identify *Salmonella* and *Listeria monocytogenes*. These results are shown in Figure 3A and 3B. The results also showed that the accuracy of our method ranges between 92 and 100%, while the precision value ranged between 88 and 100%.

Point-of-need detection with lateral flow

As real-time thermocyclers may not be present at the point-of-need (i.e., factory), we verified if the DNAFoil *Salmonella* and *Listeria monocytogenes* reactions could be visually detected with the naked eye using a lateral flow strip. Serial dilutions of *Salmonella enterica* and *Listeria monocytogenes* extracts were prepared and amplified as before. After 30 min of amplification at 65°C, the results were confirmed with DNAFoil strips (Figure 4A and 4B).

Discussion

The detection of foodborne pathogens has historically been culture-, or conventional-, or cultural-, or gold-standard-based methods, which were used since the inception of microbiological sampling (Adzitey and Huda, 2010, 2011; Bhunia, 2014). These methods mainly involve enrichment (pre-enrichment and/or selective

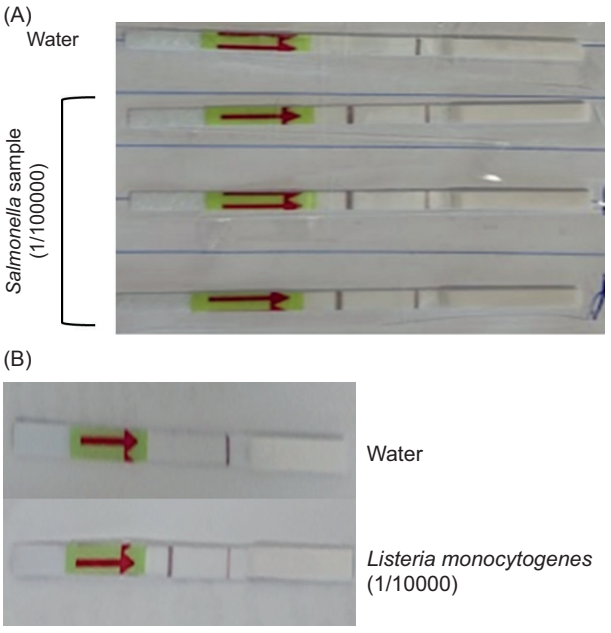

Figure 4. Strips results. (A) Amplified samples from *Salmonella* dilution 1/100,000 were applied on DNAFoil strips (triplicate). A few minutes later, we could see bands appearing. The band on the left confirmed the presence of *Salmonella*, whereas the band on the right is the positive control of the strips. (B) Amplified samples from *Listeria monocytogenes* dilution 1/10,000 were applied on DNAFoil strips. Four minutes later, we could see bands appearing. The band on the left confirmed the presence of *Listeria monocytogenes*, whereas the band on the right is the positive control of the strips.

enrichment) followed by biochemical tests (metabolic fingerprinting), molecular tests (typically PCR [polymerase chain reaction]), or mass spectrometry (Adzitey *et al*., 2011; Corry *et al*., 2003; Ellis *et al*., 2019) to confirm that the isolate is indeed the pathogen of interest. The gold-standard-based methods have the advantages of being inexpensive, detecting only viable pathogens, and yielding isolates that can further be studied (Adzitey and Huda, 2011; Engberg *et al*., 2000). However, they are cumbersome, relatively slow, and less efficient (Foddai and Grant, 2020; Jasson *et al*., 2010; Keramas *et al*., 2004; Li and Zhu, 2017; Myint *et al*., 2006). Regarding the biochemical and mass spectrometry methods, they are rapid, sensitive, and accurate techniques that involve the analysis of entire microbial cells or their extracts (Beale *et al*., 2014; Cevallos-Cevallos *et al*., 2011; Singh *et al*., 2011; Singhal *et al*., 2015; Toscano *et al*., 2018; Wu *et al*., 2016; Yang *et al*., 2015). However, they are labor-intensive, costly, and the reliance on existing spectral databases of the mass fingerprints of known microbes makes mass spectrometry techniques incapable of identifying new species (Anderson *et al*., 2012; El Sheikha and Hu, 2020; Jadhav *et al*., 2018; Mirmajlessi *et al*., 2015; Reta *et al*., 2020). Molecular techniques have the advantage of being

rapid, less laborious, more sensitive, specific, and efficient, compared to the conventional method (Keramas *et al*., 2004; Magistrado *et al*., 2001). Nonetheless, certain components/compounds in foods such as fats, lipids, and salts, enrichment media, or DNA extraction solution can inhibit the sensitivity of PCR-based methods (El Sheikha, 2010; Rossen *et al*., 1992; Wilson, 1997).

To overcome the limitations of traditional methods used for pathogen detection from the side and from the other side to meet industrial and commercial food needs, there is an urgent need for fast, sensitive, accurate, and more efficient detection methods in terms of saving time, labor, and preventing human errors (Baraketi *et al*., 2018; Law *et al*., 2015; Mandal *et al*., 2011; Rajapaksha *et al*., 2019).

Hence, the importance of answering the principal question, namely, why is DNAFoil technology proposed to detect food pathogens? This question is the hypothesis on which the research idea was based, and which the results of this study approved and provided the answers as follows:

DNAFoil is a fast, accurate, precise, sensitive, and reliable technique

As a new technology that needs assessment, the "real-time" amplification technology (real-time PCR) is used to evaluate the efficacy and accuracy of DNAFoil technology (El Sheikha, 2019). Aronoff *et al*. (2018) reported that the efficiency of DNAFoil kit used to detect the vegetal material in milk products (DNAFoil UniPlant) was confirmed using real-time PCR assays. The same authors concluded that the DNAFoil UniPlant kit provides a quick and reliable method to validate product content with less than 1% adulteration of any product, confirming identity and purity in 30 min, without lab equipment, technicians, or scientists. The final report provided by Meat and Livestock Australia Limited (MLA) (2018) illustrated that the DNAFoil kit is able to detect pork contamination in beef as lower as 0.1%.

The present study illustrated that the DNAFoil is a fast-detection technique of *Salmonella* and *Listeria monocytogenes* that can get the final results in as little as 30 min. In addition, the results of this study stated that the Swiss Decode *Salmonella* and *L. monocytogenes* solutions can detect as few as 1 and 10 copies of DNA per microliter, respectively. However, the commercially available kits used to detect food pathogens, that is Salmonella and Listeria monocytogenes, which are based on nucleic acid for detection, are characterized by reliability, high specificity, and sensitivity; they are limited by the difficulties of:

- differentiating the viable cells from nonculturable cells;
- the primers' design.

Moreover, these kits require trained staff to avoid cross-contamination (Baraketi *et al*., 2018). Table 2 evaluates the commercial kits used to detect *Salmonella* and *Listeria monocytogenes*. In terms of performance metrics (accuracy, precision %), the results show promising performance, to be used for *Salmonella* and *Listeria monocytogenes* detection.

The DNAFoil technology is efficient in terms of cost and quality control applicability

In terms of cost. DNAFoil is available to the partners (academic and industry) that accede to the Early Access Program. The fee to enter the Early Access Program is

Table 2. Commercially available kits based on nucleic acid methods for the detection of foodborne pathogens.*

Pathogen	Commercially available kits	Sensitivity	Sample matrix	Company
Salmonella sp.	BAX® System Standard PCR assays for *Salmonella*	10^4 CFU/mL, after enrichment	Poultry, dairy, fruits, vegetables, bakery products, pet food, environmental samples	HYGIENA
	BAX® System Real-time PCR assay for *Salmonella*	10^4 CFU/mL, after enrichment	Meat, poultry, dairy, fruits, vegetables, bakery products, pet food, environmental samples	HYGIENA
	GeneQuence® for *Salmonella*	1–5 CFU/25 g	Food and environmental samples	HYGIENA
Listeria monocytogenes	BAX® System PCR Assay for *L. monocytogenes*	10^5 CFU/mL, after enrichment	Variety of food types	HYGIENA
	BAX® System PCR Assay for *L. monocytogenes* 24E	10^4 CFU/mL, after enrichment	Dairy, meat, fish, vegetables, environmentals	HYGIENA
	BAX® System Real-Time PCR Assay for *L. monocytogenes*	10^4 CFU/mL, after enrichment	Dairy, ready-to-eat meat, seafood, vegetables, environmental samples	HYGIENA
	GeneQuence® for *L. monocytogenes*	1–5 CFU/26 g	Food and environmental samples	NEOGEN

*Source: Baraketi *et al*. (2018). Licensed under Creative Commons Attribution 3.0. PCR, polymerase chain reaction.

€ 990 and the program makes access free of charge for a kit of 5 tests. The final cost per test is negotiable, and it is usually much cheaper than the traditional detection methods (Aronoff *et al*., 2018; Lüdin *et al*., 2018).

In terms of quality control applicability. The DNAFoil output gives a (±) answer, for example, while using DNAFoil Pork Test Kit, a positive result indicates the presence of porcine DNA in the sample tested. This is enough for an inspector to take instant action. The strips with the results can be stored as evidence to prove that adequate controls are in place during manufacturing. In the case of litigations, the Swiss Federal Lab concluded that DNA can be easily extracted from the strip, and such DNA can be used for more lab tests (Lüdin e*t al*., 2018).

Conclusions, Remarks, and Future Trends

In this study, the developed DNAFoil *Salmonella* and *Listeria monocytogenes* reactions correctly identify a wide range of *Salmonella* strains and also *Listeria monocytogenes*, among other bacterial strains. These reactions are specific and sensitive, with a virtual limit of 1 and 10 CFU detection per reaction for *Salmonella* and *Listeria monocytogenes*, respectively. In terms of accuracy and precision, the results show promising performance in which the accuracy ranged between 92 and 100% and the precision ranged between 88 and 100%. Real-time PCR may not be present at the point-of-need (i.e., factory); therefore, the DNAFoil *Salmonella* and *Listeria monocytogenes* reactions could be visually detected with the naked eye using a lateral flow strip. The strips with the results can be stored for quality control in place during manufacturing and also can be used for more lab tests. The results of the present study revealed and confirmed several advantages that considered DNAFoil fact sheet as follows: the DNAFoil is easy to use and does not require specialized skills to perform testing; the DNAFoil is standalone and does not require laboratory equipment other than a source of boiling water. In the absence of boiling water, the kit is compatible with a standard thermoblock; the DNAFoil detects specifically each target without any cross-reaction; the final results can be interpreted with the naked eye with a lateral flow dipstick without the need for thermocyclers, electrophoresis, and staining.

Food pathogen detection approaches have become ever more substantial for all of the food chain components, that is, consumers, producers, and legislators (El Sheikha, 2015; Hoffmann and Scallan, 2017). The embodiments of food pathogen risks in food products continue to evolve in different forms that have resulted in tremendous improvements in analytical methodologies to detect the food pathogens (Hemalata and Virupakshaiah, 2016). Although DNA-based techniques have proven to be the best detection tools in food pathogen detection, at the industrial level its practical application has to go a long way (El Sheikha *et al*., 2018; Zhao *et al*., 2014). Hence, the demand for a novel, rapid, easy, potent, and universal technology for food pathogen detection is still an urgent need. Therefore, it is hoped that the DNAFoil technology could be a powerful tool that meets all of the requirements for food pathogen detection and its applications either at the household or industrial level.

As a future trend, more applications are recommended for DNAFoil technology as a food pathogen detection tool on different food matrices. Moreover, the DNAFoil test kits for *Salmonella* and *Listeria monocytogenes* are qualified for further validation using ISO16140.

Compliance with Ethical Standards

Acknowledgments

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Conflict of Interest

The author declares that there is no conflict of interest.

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Natural protective agents and their applications as bio-preservatives in the food industry: An overview of current and future applications

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Abstract

Today, the usage of natural additives in the food matrix has increased. Natural antimicrobial compounds include peptides, enzymes, bacteriocins, bacteriophages, plant extracts, essential oils, and fermented compounds that can be used as alternatives to chemical antimicrobials. Plant extracts and essential oils contain terpenes, flavonoids, aldehydes, and phenolic compounds that cause antimicrobial and antioxidant activity. The synergistic activity of compounds synthesized from lactic acid bacteria (LAB) prevents the growth of bacteria and fungi. In addition to removing mycotoxins, LAB compounds have antioxidant and anticancer potentials and increase food safety and nutritional value. One of these antimicrobial molecules is bacteriocin, which is made by various microorganisms. Nisin is one of these bioactive peptides that are used widely in food bio-preservation. Antimicrobial peptides can be used alone or along with other compounds to enhance food security. This article reviews natural preservatives and their applications in food products.

Keywords: bioactive compounds; antioxidants; protective culture; Antimicrobial peptides; bacteriocin; essential oils

Introduction

Microorganisms and lipid oxidation are important problems in food safety (Aziz and Karboune, 2018). Bacteria, yeasts, and molds are microorganisms that spoil food products (Figure 1) (Gonelimali *et al*., 2018). Many strains of *Aspergillus* and *Penicillium* produce mycotoxins that cause disease (Varsha and Nampoothiri, 2016). In general, the food industry relies on chemicals for food preservation. Food additives include the following: preservatives (antimicrobials, antioxidants, and anti-browning agents), nutritional additives, coloring agents (azo compounds, xanthan, chinophthalon derivatives, indigo, and triarylmethane), flavors (sweeteners and flavor enhancers), and textural ingredients (stabilizers and emulsifiers) (Carocho *et al*., 2018).

Usually, chemical preservatives are applied to prevent lipid oxidation and microorganisms growing in the food industry. The use of nitrite and sulfur dioxide (chemical preservatives) can have side effects on foods and human health. Owing to the extensive use of these substances, bacteria have become resistant, and consumers are looking for natural products with nonchemical preservatives, so there is a need to find natural preservatives (Amiri *et al*., 2021a; Mahmud and Khan, 2018; Pisoschi *et al*., 2018; Rai *et al*., 2016). With regard to these needs, bio-preservation, super chilling, and active packaging were

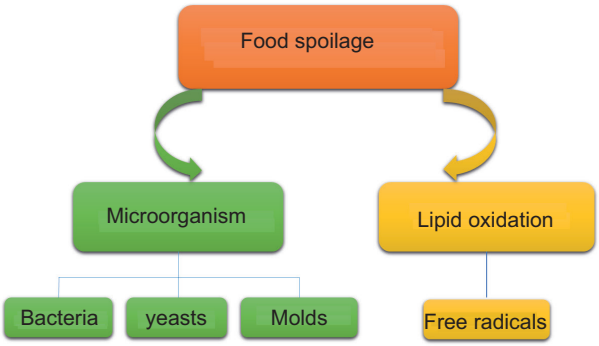

Figure 1. The cause of food spoilage.

considered (Aziz and Karboune, 2018; Tumbarski *et al.*, 2020).

Most natural antimicrobial compounds include extracts (grape seed, tea, strawberry, cranberry, and blueberry), spices (garlic, nutmeg, cinnamon, clove, black pepper, and ginger) herbs (basil, oregano, rosemary, marjoram, and sage), enzymes (lysozyme, lactoferrin), bacteriocins (BACs) (nisin and natamycin), peptides, organic acids (propionic, citric acid, and sorbic), vitamins (α-tocopherol and ascorbic acid), and natural polymers (chitosan) (Table 1). They are added directly to foods or put into packaging because the latter is beneficial (Aziz and Karboune, 2018; Mahmud and Khan, 2018; Bai-Ngew *et al.*, 2021; Sohrabpour *et al.*, 2021). All herbal essential oils (EOs) and extracts have an inhibitory effect against viruses, bacteria, fungi, oxidative corruption, and insects (Xing *et al.*, 2012). Various herbal and spice antimicrobial agents are used to eliminate foodborne microorganisms such as *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, and *Salmonella,* and to enhance the quality of different foods. The most common sources of EOs of clove, cinnamon, oregano, citrus, garlic, coriander, parsley, lemongrass, sage, rosemary, and vanillin are antimicrobial and antioxidant agents that are used in bioactive packaging of foods (Gonelimali *et al.*, 2018; Mahmud and Khan, 2018).

Bio-preservation is applying bacteria or their bioactive metabolites to inhibit foodborne pathogens (Amiri *et al.*, 2021b; Azizi *et al.*, 2021; Gonelimali *et al.*, 2018; Tumbarski *et al.*, 2020). The most crucial advantage of antimicrobial peptides is that they do not alter food quality and are harmless (Rai *et al.*, 2016). Lactic acid bacteria (LAB) is used as a protective culture in dairy foods due to the production of lactic acid, hydrogen peroxide, fatty acids, acetaldehyde, and BACs that inhibit the growth of microorganisms (Amiri *et al.*, 2020a, 2020b, 2020c;

Table 1. Natural antimicrobials and antioxidants in the food industry.

Natural preservatives				
Natural Antimicrobial			**Natural antioxidant**	
Plant based	*Essential oils*	Cinnamon, Oregano, Clove, Garlic, Rosemary, Parsley, Coriander, Lemongrass, Rosemary, Allspice, Litsea Cubeba, Sage, purple, and bronze Muscadine seeds	*Pigments*	Anthocyanins, Anthocyanins
	Spices	black, white, cayenne Peppers, Mustard, Chillies, Paprika, Coriander, Clove, Cumin, Dill fennel, Nutmeg, Mace, Cinnamon, Garlic, Ginger	*Spices*	Cinnamon, Cloves, Nutmeg, Ginger, Black pepper, Garlic, Onion, Cumin, Turmeric
	Herbs	Thyme, Basil, Bay leaf, Marjoram, Shallot, Onion, Garlic, Oregano, Rosemary, Sage	*Herbs*	Rosemary, Oregano, Marjoram, Sage, Basil, Thyme
	Extracts	Grape seed, Tea, Strawberry, Cranberry, Blueberry	*Extracts*	Tea, Grape seed, Cranberry, Blueberry, Strawberry, Pomegranate, Citrus fruits
	peptides	Lipid transfer protein 2, Snakin1, Kalata B1, Thionin, Defensins	*Vitamins*	α-Tocopherol, Ascorbic acid
Animal based	*Enzymes*	Lysozyme, Lactoperoxidase,		
	Peptides	Pleurocidin, Protamine, Magainins, Sarco toxin, Hymenoptaecin, Attacin, Diptericin, Coleoptericin, Lactoferricin, kappacin, k-casecidin, S1- S2- A-B Caseins		
	Natural polymers	Chitosan, Poly-L-Lysine		
Microbial based	*Enzymes*	Glucose oxidase		
	Bacteriophages	ListexTMP100, EcoshieldTM, SalmofreshTM		
	Organic acids	Propionic, Citric acid, Sorbic, Lactic acid		
	Bacteriocins	Nisin, Natamycin, Enterocin, Pediocin, Reuterin,		

Rai *et al*., 2016; Tumbarski *et al*., 2020). BACs are ribosomally synthesized proteins or peptides from certain bacteria. BACs are efficient against antibiotic-resistant pathogens such as *S. aureus* and *Enterococcus faecalis* without showing toxicity (Ahmad *et al*., 2017; Amiri *et al*., 2021c; Mahmud and Khan, 2018). LAB produces a diversity of antifungal compounds and may be used as a bio-protective without changing the organoleptic characteristics. LAB has been found naturally in various foods for centuries and has been used in fermented foods without side effects. It is known as GRAS (generally recognized as safe) (Amiri *et al*., 2019a; Favaro *et al*., 2015; Maleki *et al*., 2020; Rezazadeh-Bari *et al*., 2019; Varsha and Nampoothiri, 2016). The Food and Drug Administration of USA (FDA) accepted LAB as a safe agent for fermented foods (Upendra *et al*., 2016). It is heat resistant and has a tremendous antimicrobial effect against Gram-positive (G^+) and Gram-negative (G^-) pathogens after pasteurization and also does not alter the taste, aroma, or texture and physical, chemical, or biological properties of foods (Figure 2) (Rai *et al*., 2016).

Natural Antimicrobial Agents

Plant-based antimicrobial agents

Essential oils

Essential oils (EOs) are natural and volatile compounds determined by odor and taste (Mohajeri *et al*., 2021; Ghamari *et al*., 2021; Regnault-Roger *et al*., 2012). EOs are include terpenes, alcohols, acids, esters, epoxides, aldehydes, ketones, amines, and sulfides. They are synthesized in the cytoplasm and plastids of plant cells through malonic acid, mevalonic acid, and Methyl-D-erythritol-4-phosphate pathway. Terpenes are hydrocarbons and consist of various isoprene units. At the same time, terpenoids are made from the biochemical alteration of terpenes by enzymes that add oxygen molecules and transfer methyl. Terpenoids have high antimicrobial activity (Pisoschi *et al*., 2018). The constituents in EOs are the following groups: terpenes and terpenoids that are more effective to G^+ bacteria (Mahmud and Khan, 2018). G^- bacteria's resistance is due to the presence of an outer membrane of lipopolysaccharide around the cell wall (Tongnuanchan and Benjakul, 2014). However, there are reports that EOs of cinnamon, oregano, clove, garlic, rosemary, parsley, coriander, lemongrass, sage, purple, and bronze muscadine seeds are effective on both types of bacteria, many of which are carvacrol, thymol, eugenol, and citral (Aziz and Karboune, 2018; Irkin and Esmer, 2015). EOs of cinnamon, oregano, clove, garlic, coriander, parsley, lemongrass, rosemary, and sage have good effects. EOs with great amounts of eugenol (allspice, clove, and cinnamon), trans-cinnamaldehyde (cinnamon), and citral (litsea cubeba, lemon myrtle, and lime) have strong effects (Dussault *et al*., 2014).

The oregano EO has irreversible damage (in 1 min) against *E. coli* O157: H7 cells (Pisoschi *et al*., 2018). The role of oregano, thyme, and savory as an antimicrobial agent is because of phenolic compounds caracole, thymol ρ-cumene, and y-terpinene (Aziz and Karboune, 2018; Dussault *et al*., 2014). The existence of a hydroxyl group in the structure of phenolic compounds and its position is the reason for the antimicrobial effect. The antimicrobial effect of sage is caused by terpene thujone. Rosemary EO has an inhibitory effect against G^- bacteria (*E. coli, Klebsiella pneumonia*) and G^+ bacteria (*Bacillus subtilis, S. aureus*) (Aziz and Karboune, 2018; Tongnuanchan and Benjakul, 2014). This is because of terpene groups such as borneol, camphor, 1, 8-cineole, a-pinene, camphone,

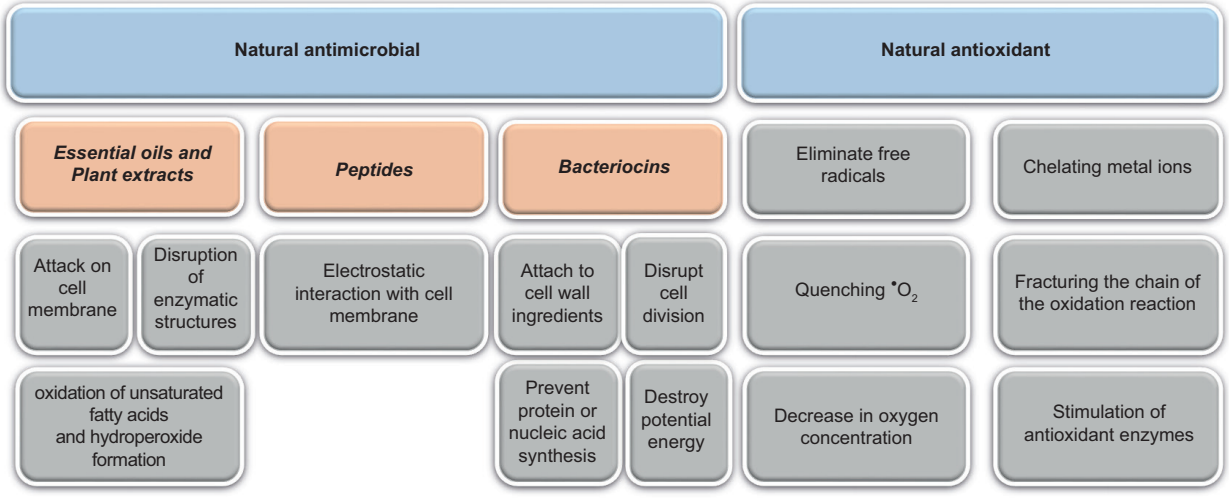

Figure 2. Mechanisms of action of natural protective agents.

verbenonone, and bornyl acetate. The antimicrobial activity of garlic and onion is due to allicin and mustard caused by allyl and isothiocyanates. Allyl isothiocyanate is effective against fungi and G^- bacteria (Aziz and Karboune, 2018). Ginger, oregano, rosemary, sage, thyme, and peppermint are significant sources of EOs and include alcohols, aldehydes, phenylpropanoids, terpenes, and ketones that are antioxidants. In addition to antioxidant activities, EOs can provide antibacterial activity (*S. aureus*, *E. faecalis*, *E. coli*, *Clostridium perfringens*, and *Clostridium sporogenes*) in meat foodstuffs (Mahmud and Khan, 2018). Marei *et al*. (2012) studied 12 monoterpenes' antifungal activity against *Rhizoctonia solani*, *Fusarium oxysporum*, and *Penicillium*, and *digitatum* and *Aspergillus niger* thymol were the good antifungal compounds comparable to fungicide and carbendazim. Allyl isothiocyanate, cinnamon oleoresin, cinnamon Chinese cassia, and oregano Chinese cinnamon EOs have antimicrobial activity against *E. coli* O157:H7, *S. aureus*, *B. cereus*, *L. monocytogenes*, and *Salmonella enterica* serovar *typhimurium*. The coriander seed oil has antimicrobial efficacy against G^+ and G^- bacteria, some yeasts, dermatophytes, and molds (Silva and Domingues, 2017). Low pH, temperature, and oxygen content can complete EOs' performance (Aziz and Karboune, 2018; Dussault *et al*., 2014). These compounds are GRAS and can be used as food additives (Mahmud and Khan, 2018; Bai-Ngew *et al*., 2021).

Mechanisms of action

Plant material affects microorganisms through various mechanisms, such as attacking the cell membrane, disrupting enzymatic structures, compromising bacterial genetic material, and fatty acid hydroperoxide formation by oxygenation of unsaturated fatty acids (Tajkarimi *et al*., 2010). The function of phenolic compounds of EOs likely depends on alteration of bacterial cell permeability, damage to the cytoplasmic membrane, disruption of the cellular production system of cellular energy (ATP), proton motility, and cell death that occurs by damaging the cytoplasmic membrane (Mahmud and Khan, 2018).

Herbs and spices

Herbs are obtained from the same plant's leaves, but spices are obtained from different parts of the plant. Herbs and spices are divided into the following categories: hot spices (peppers), mild flavor spices (paprika and coriander), aromatic spices (clove, cumin, dill fennel, nutmeg, mace, and cinnamon), and aromatic herbs and vegetables (thyme, basil, marjoram, shallot, onion, and garlic). Phytochemicals are bioactive compounds that contain flavonoids and other phenolic ingredients, carotenoids, plant sterols, glucosinolates, and other sulfur compounds. Extracts and herbs act against G^+ bacteria. Also, the shelf life can increase using bioactive ingredients such as phenol, alcohol, aldehyde, ketone, esters, and hydrocarbons (cinnamon, cloves, garlic, mustard, and onions) (Embuscado, 2015; Mahmud and Khan, 2018). Cinnamon has antibacterial activity against both types of bacteria, such as *B. cereus*, *L. monocytogenes, S. aureus*, *E. coli*, and *Salmonella anatum* (Shan *et al*., 2007).

Plant extracts

Grape seed and green tea extracts

Grape seeds contain high amounts of phenolic compounds, including catechin, epicatechin, epicatechin-3-O-gallate, dimeric, and trimeric tetrameric procyanidins. Green tea leaves have epicatechin, epicatechin gallate, epigallocatechin, teaflavin gallate, teaflavin monogallate A, and B teaflavin digallate. Extracts of grape and green tea postpone growing of microorganisms in raw beef (Banon *et al*., 2007). Tea phenols have antimicrobial properties against both types of bacteria. The antimicrobial effect of arrowroot tea is related to catechins. Green tea has antibacterial activity against pathogens (Kim and Fung, 2004). It has been reported that grape seed extract decreased *E. coli* O157: H7 and *S. Typhimurium*, and postponed the growth of *L. monocytogenes* and *Aeromonas hydrophila* in cooked meat (Aziz and Karboune, 2018). Antibacterial compounds of coffee are caffeic acid, chlorogenic acid, and protocatechuic acid. The growth of *E. coli* O157: H7 is inhibited by trimethylated alkaloid caffeine (Ibrahim *et al*., 2006).

Cranberry extracts

Phenolic compounds, such as low molecular weight phenolic acids, condensed tannins, proanthocyanidins, and flavonoids (anthocyanins, especially proanthocyanidins), which are composed of tetramers of type A and pentamer type A, are the main factors of antimicrobial activity in cranberry (Khaneghah *et al*., 2018). Extract of cranberry fruit at 15% concentration inhibited *A. hydrophila*, *B. cereus*, *Enterobacter aerogenes*, *E. coli*, *K. pneumonia*, *Proteus vulgaris*, *P. aeruginosa*, *S. typhimurium, S. aureus,* and *Yersinia enterocolitica* (Sagdic *et al*., 2006).

Mechanisms of action

An accurate way has not yet been elucidated. Nevertheless, plant substances can be affected by attacks on the cell membrane's phospholipid bilayers, disruption of enzyme activity, endangering the genetic material of bacteria, and oxidation of unsaturated fatty acids (Tajkarimi *et al*., 2010). The inhibitory activity of aromatic and phenolic compounds is due to affecting the cytoplasmic membrane's structure and function. The outer membranes of *E. coli* and *S. typhimurium* was dissipated after exposure to carvacrol and thymol. The antimicrobial effect of nonphenolic isothiocyanates is due to the denaturized of extracellular enzymes. Terpenes can disrupt and affect the bacterial cell wall's fat composition

and ultimately cause bacterium death (Aziz and Karboune, 2018). Carvacrol can split G^- bacteria's outer membrane diffusing lipopolysaccharides and enhancing the permeability to ATP. Its activity against G^+ bacteria is because of its interplay through the bacterial membrane, which changes H^+ and K^+ cations (Tongnuanchan and Benjakul, 2014).

Animal-based antimicrobial agents

Enzymes

Lysozyme

It is an enzyme and natural antimicrobial agent that hydrolyzes the-beta 1, 4 glycosidic bonds between N-acetylmuramic acid and N-acetyl glucosamine existing in peptidoglycan. G^+ bacteria are sensitive to lysozyme since 90% of these bacteria's cell wall is composed of peptidoglycans. Therefore, it is a natural antimicrobial agent (Irkin and Esmer, 2015). G^- bacteria are resistant to lysozyme because of the lipopolysaccharide layer, which prevents lysozyme from approaching the peptidoglycan layer. However, this enzyme can also affect G^- bacteria if membrane destabilizing factors, for example, detergents and chelating agents, are present. Lysozyme has a high resistance to a wide range of temperatures and pH, which enables its usage in edible active films (Bayarri *et al*., 2014). Inovapure is a commercial lysozyme studied in model studies, either alone or with other compounds, against *L. monocytogenes*, *Clostridium botulinum*, *Campylobacter jejuni*, *Pseudomonas* spp., *Salmonella enteritidis*, *Clostridium thermosaccharolyticum*, *Bacillus stearothermophilus*, and *Clostridium tyrobutyricum*. Lysozyme derived from egg white has been allowed by Health Canada to be used in hard cheeses to inhibit the gas blowing of *C. tyrobutyricum* (Aziz and Karboune, 2018; Baines and Seal, 2012). Lysozyme has also been assayed in eggs, milk, beef, and part in edible films and coatings, along with other antimicrobial materials (Bayarri *et al*., 2014).

Lacto-peroxidase system

One of the natural antimicrobial systems is the lactoperoxidase system obtained from milk, saliva, and tears. It consists of lactoperoxidase, thiocyanate, and hydrogen peroxide. Lacto-peroxidase accelerates the oxidation of thiocyanate ions using H_2O_2. Afterward, hypothyroidism and acid hypothyroidism show inhibitory effects on microorganisms through the oxidation of sulfhydryl groups to enzymatic systems and proteins. It shows antimicrobial activity against bacterial and fungal species (Campos *et al*., 2011). The general application of lactoperoxidase is to preserve raw milk (especially if the refrigerator is not available). Because thiocyanate in milk is not enough to stimulate the lactoperoxidase system's activity, thiocyanate should be added. Its use in juice storage and food coating is suggested (Marcio Carocho *et al*., 2018). Considerations of using the lactoperoxidase system in packaging films depend on the cost and the antimicrobial activity of the lactoperoxidase system (thiocyanate and H_2O_2). If H_2O_2 levels are exceeded in food products, it may raise toxicological concerns (Aziz and Karboune, 2018).

Antimicrobial peptides

Eukaryotic peptides

Animal peptides. Mammalian antimicrobial peptides are secreted into mucosal, epithelial, and paneth cells. Mammalian leukocytes are a rich origin of cationic antimicrobial peptides that prevent bacterial infection. Several of them can rapidly destroy the lipid layer of the cell membranes of microorganisms. In addition to inhibition of both types of bacteria, they also have antifungal and antiviral activity. These are considered a suitable option for antibiotic resistance (Aires *et al*., 2009; Tiwari *et al*., 2009). Pleurocidin and protamine isolated from fish have antimicrobial activity on G^- bacteria such as *Vibrio parahemolyticus*, *L. monocytogenes*, *E. coli* O157:H7, *Saccharomyces cerevisiae*, and *Penicillium expansum*. Nevertheless, magnesium and calcium prevent their efficacy (Burrowes *et al*., 2004). Magainins from amphibians have shown extensive activity against both types of bacteria and are generally used to preserve meat and cheese (Rai *et al*., 2016).

Insect's peptides. Antimicrobial peptides such as sarco toxin IIA, hymenoptaecin, attacin, diptericin, and coleoptericin act against *Micrococcus luteus*, *Aerococcus viridians*, *Bacillus megaterium*, *B. subtilis, Bacillus thuringiensis*, and *S. aureus* secreted by insects (Wang *et al*., 2016).

Plant peptides. These peptides are cysteine-rich (molecular weight 2–9 kDa), such as lipid transfer protein 2, Snakin1, Kalata B1, thionin, and potato defensins. They have antimicrobial effects against *L. monocytogens*, *S. Typhimurium*, and *E. coli* O157:H7. In particular, defensins have antimicrobial potential against microorganisms (Rai *et al*., 2016; Tiwari *et al*., 2009).

Milk peptides. Lactoferricin (an 80 kDa iron-binding glycoprotein), kappacin, and k-casecidin are antimicrobial peptides of milk proteins. Lactoferrin (as a GRAS) is an iron-binding glycoprotein with antimicrobial effect on both types of bacteria, such as *Salmonella* and *E. coli*, and fungi. In the past, it was used in baby formulas and to treat beef carcasses, but currently, lactoferrin approved in the USA for use on beef (Aziz and Karboune, 2018; Baines and Seal, 2012; Rai *et al*., 2016; Tiwari *et al*., 2009). Casocidin is a byproduct of the casein hydrolysis and has an antibacterial effect against *Staphylococcus* spp.,

Sarcina spp., *B. subtilis*, *Diplococcus pneumoniae*, and *Streptococcus pyogenes*. Casein A and casein B inhibit the growth of *Enterobacter sakazakii*. The peptides containing an S2-casein, an S1-casein, and k-casein have good inhibitory effect on *E. coli* and *B. subtilis* (Elbarbary *et al*., 2012).

Mechanisms of action

Antimicrobial peptides act through an electrostatic interaction with the membrane and have a permeability function. They can enter the membrane and eventually disrupt it (Rai *et al*., 2016). Several mechanisms are responsible for antibacterial activity of lactoferrin: prevention of growing by isolating iron from pathogens, the potential of cations in the surface of lactoferrin for a direct reaction with lipid A, and modifying the permeability of the outer membrane and causing the release of lipopolysaccharide (Jenssen and Hancock, 2009).

Chitosan

Chitosan is natural cationic linear polysaccharides made up of (1, 4) linked 2-amino-deoxy-β-D-glucan. Sources of chitosan are shrimp shells, fungi, and green algae (Dutta *et al*., 2009; Irkin and Esmer, 2015). It is effective against most microorganisms (both types of bacteria, yeasts, and molds). Due to the positive charge on C_2 of glucosamine monomer at pH below 6, chitosan is soluble than chitin and has higher antimicrobial effect (Irkin and Esmer, 2015). The positively charged amino group of chitosan can interact with the negatively charged cell membrane, causing leakage of intracellular compounds of microbes. Chitosan can inhibit toxin synthesize and bacterial growth by chelating the trace metals. It can activate some protection systems in the host tissue and prevent several enzymes' activity. Also, it can penetrate the nucleus of microorganisms and interfere in the translation process. The inhibitory mechanism of chitosan is diverse in G^+ and G^- bacteria; antimicrobial effect on *E. coli* increases with decreasing molecular weight of chitosan, while it has the opposite effect on *S. aureus* (Aziz and Karboune, 2018; Pisoschi *et al*., 2018).

Microbial-based antimicrobial agents

Glucose oxidase

Glucose oxidase is synthesized by *A. niger* and *Penicillium* spp. Glucose oxidase is an oxidoreductase that catalyzes the oxidation of D-glucose to H_2O_2 and D-glucono-δ-lactone. D-glucono-δ- lactone reacts with water to form D-gluconic acid. The antimicrobial role of glucose oxidase is because of the cytotoxicity of H_2O_2, lowering of pH by the formation of D-gluconic acid. Hydrogen peroxide levels may also exceed the acceptable levels by the FDA and cause toxic problems. Long-dated disposal of foods to H_2O_2 can enhance lipid oxidation and lead to laxity. H_2O_2 can be removed from foods using catalase, which changes it to water and oxygen. Microbial glucose oxidase is allowed to remove oxygen and preserve taste and aroma in bottled beverages and is used as an additive in bakery products, flour, yolk, and white egg. D-gluconic acid, a catalytic product of glucose oxidase, is secure, and the WHO does not have a regulation for it (Aziz and Karboune, 2018).

Bacteriophages

Bacteriophages are viruses that attack bacteria. Lithium bacteriophages disrupt the metabolism of bacteria that results in bacterial death. Bacteriophages have been shown to be innocuous to mammalian cells, so they can be used for biological control of pathogens in foods (Carvalho *et al*., 2017). Bacteriophages are suitable for use in carcasses, vegetables, and fruits to increase various food products' shelf life. In 2006, ListexTMP100 and LMP102 bacteriophages were accepted by the FDA to control *L. monocytogenes* contamination, and in 2010, approved the use of ListexTMP100 against *L. monocytogenes* in meat, fish and poultry, vegetable, and some dairy products (Chibeu *et al*., 2013). In 2014, Canadian Health accepted the use of EcoshieldTM in meat to control *E. coli* O157: H7 and SalmofreshTM to control *Salmonella* growth in all foods (Aziz and Karboune, 2018). Bacteriophages have been investigated as antimicrobial agents in food packaging by immobilization on cellulose membrane or by encapsulating phages in alginate beads (Lone *et al*., 2016). Also, chitosan film contains liposome-encapsulated beef meat storage (Cui *et al*., 2017).

Fermented ingredients

Fermentation ingredients can be prepared by propionic acid bacteria and LAB from raw materials such as milk, sugar, or plant materials. The preservative role of LAB is because of producing organic acids (acetic, lactic, propionic, and formic acids) diacetyl, acetylene, hydrogen peroxide, fatty acids, antifungal agents (phenyl-lactate, hydroxyphenyl-lactate, propionate, cyclic dipeptides, and 3-hydroxy fatty acids), and the BACs (reuterin, reutericyclin, nisin, pediocin, lacticin, and enterocin). LABs are used as antimicrobials and sugars, sweeteners, polymers, disinfectants, aromatic compounds, and various enzymes (Favaro *et al*., 2015).

LAB and bio-preservation

The biologically active ingredients in kimchi, such as benzyl isothiocyanate, indole, thiocyanate, and cystosterol, are antibiotics, anticancer, and immune stimulation. LAB was used to prevent *L. monocytogenes* and *S. enteritidis* in poultry and inhibition of *S. typhimurium*, *E. coli*, and *L. monocytogenes* in lettuce and apples. *Enterococcus* and *Pediococcus* were used to vacuum-package cold-smoked salmon (Varsha and Nampoothiri, 2016). MicrogardTM

is a commercial milk supply that is fermented by dairy microorganisms. MicrogardTM could be effective against G^- bacteria (*Yersinia*, *Salmonella*, and *Pseudomonas*) and some fungi but ineffective against the G^+ bacteria (*L. monocytogenes*, *S. aureus*, and *B. cereus*). FDA has approved *propionibacterium* metabolites as GRAS. The MicrogardTM products are used in dairy desserts, cheese, yogurt, pasta, sauces, and others (Aziz and Karboune, 2018; Favaro *et al*., 2015). LAB produces antifungal compounds, for example, hydroxyl fatty acids, organic acids, low molecular weight bioactive ingredients, and proteinaceous components. Some species of *Enterococci*, *Lactobacilli*, *Pediococci*, and *Lactococci* are also known as antifungal agents (Favaro *et al*., 2015; Gholam-Zhiyan *et al*., 2021). The antifungal effect of *Lactococcus lactis* on *Aspergillus*, *Penicillium*, *Mucor*, and *Rhizopus* has been reported (Oranusi *et al*., 2013). Also, *Lactobacillus acidophilus* and *Bifidobacterium bifidum* were efficient in preventing *A. niger* from growing on the surface of brined white cheese (Moghanjougi *et al*., 2020). Use of *L. plantarum* on *Botrytis cinerea*, *Glomerella cingulate*, *Phytophthora drechsleri*, *P. citrinum*, and *F. oxysporum* and application of *Lactobacillus* and *Weisella* cultures on *A. niger*, *Candida albicans*, *Aspergillus tubingensis*, and *P. crustosum* have been reported (Varsha and Nampoothiri, 2016). Feedtech Silage F3000 is a commercial compound of antifungal bacteria (*L. plantarum* Milab 393, *P. acidilactici*, *E. faecium*, and *L. lactis*) that improves growth beneficial bacteria and prevents yeast fungi and *Clostridia*. DuraFresh (Kerry) is also a commercial preservative derived from the fermentation of LABs that inhibits G^- bacteria, yeast, and mold. ALTA 2341 is another fermented product. Therefore, the use of LAB in food has existed for a long time, and recent studies indicate their potential as an alternative to chemical preservatives (Varsha and Nampoothiri, 2016).

Bacteriocins

BACs are ribosomal antimicrobial proteins that usually have antimicrobial activity against similar genetic strains. The beneficial properties of BACs are stability to high temperature and low pH and sensibility to proteolytic enzymes. Their application can decrease the intensity of heat treatment and the use of chemical preservatives. Also, BACs and other methods as a hurdle can be helpful (Favaro *et al*., 2015). BACs are used in food products because they are harmless, do not change foods' nutritional characteristics, are efficient in low concentration, and active in refrigerator storage, and, therefore, an ideal bio-preservation (Mahmud and Khan, 2018; Milicevic *et al*., 2021). BACs isolated from G^+ bacteria are classified into the following groups: Class I, Class II, Class III, and Class IV. Class I are divided into (A, B, and C) subgroups: Type A corresponds to linear peptides and is further subdivided into two subgroups including AI subtype contains nisin like (nisin A, nisin U, streptin), and subtype AII contains SA-FF22 like (SA-FF22, lacticin 481, salvaricin A, sublancin 168). Type B are spherical peptides such as meracidin and cinnamycin belong to globular peptides, and type C are a few details BACs such as lactin, 3147 and cytolysin, that made by more than one part and biological activities are needed. Class II BACs are divided into (IIa, IIb, and IIc) subgroups: type IIa is related to pediocin (pediocin Pa-1, carnobacteriocin B2, listerocin 743A, and ubericin A), type IIb is related to multifactorial BACs (lactococcin G, thermophilin 13, lactacin F, and lactocin 705), and Type IIc are sundry such as sakacins Q, T, and X, and aureocin A53. Class III contains lysins (class IIIa) and nonlytic BACs (class IIIb). Class IV contains circulating inhibitory peptides such as enterocin AS-48 (Favaro *et al*., 2015; Milicevic *et al*., 2021).

Nisin

Nisin is produced by fermenting milk modified by the strains of *L. lactis*. Nisin is a Canadian Food and Drug and Health License and is accepted as a preservative in more than 48 countries. Nisin is efficient on most G^+ bacteria such as *L. monocytogenes* and *S. aureus*, and Ethylenediaminetetraacetic acid (EDTA) can also inhibit G^- bacteria (Aziz and Karboune, 2018; Campos *et al*., 2011; Pisoschi *et al*., 2018).

Enterocin

Enterocin AS-48 is one of the cyclic BACs that exhibit significant stability in pH and heat. The effectiveness of AS-48 for the control of *staphylococci* in culture has been represented. Also, chemical preservatives (sodium nitrite, sodium lactate, and sodium chloride), clusters, and temperate heat are in synergy with AS-48 on *B. cereus, S. aureus, Salmonella choleraesuis*, and *E. coli* O157:H7 (Favaro *et al*., 2015). Enterocin AS-48 has the ability to eliminate the parasitic protozoan *Trypanosoma brucei*, and Enterocin V effectively decreases *C. albicans* (Martínez-García *et al*., 2018).

Pediocin

Pediocins are produced by *P. acidilactici* and *P. pentosaceus*. They are thermal resistant and can work in a wide range of pH. Pediocins have an antimicrobial effect against *L. monocytogenes*, *E. faecalis*, *S. aureus*, *C. perfringens*, and *Oenococcus oeni*. Pediocin can be used in dairy products or as a film in crushed ham and vegetables (Díez *et al*., 2012; Juneja *et al*., 2012).

Natamycin

Natamycin is a polyene macrolide that is synthesized by *streptomyces* species with an antifungal activity that has almost no effect on bacteria, protozoa, and viruses. Natamycin is associated with ergosterol, so it does not develop resistance in fungi. It has been used as a free, encapsulated, or film-forming agent to control yeast growth in cheese, salami sausages, and beverages (Pisoschi *et al*., 2018).

Reuterin

Reuterin (b-hydroxypropionaldehyde) is a BAC produced by *L. reuteri* that affects both types of bacteria (*L. monocytogenes*, *S. aureus*, *S. typhimurium*, and *E. coli*). It is soluble in water and stable to temperature and proteolytic and lipolytic enzymes. It is characterized by resistance in a wide range of pH. Reuterin is used in combination with *lactobacillus* in glycerol as starter cultures in cheese or as pure extract at the level of sausages and salmon or inside cheese (Gyawali and Ibrahim, 2014; Montiel *et al*., 2014).

Mechanisms of action

BACs affect the basic functions of living cells such as transcription, translation, replication, and cell wall biosynthesis, but most of the time, destroy the potential energy of sensitive cells by creating channels or pores in the membrane (Milicevic *et al*., 2021). BACs perform their function on bacteria (both types of bacteria) by disrupting cell division and preventing protein or nucleic acid synthesis, using various mechanisms. BACs can attach to cell wall ingredients using specific or nonspecific receptors, such as the lipid-binding site or the molecular surface, leading to the formation of pores or direct cell lysis and ultimately to death through the loss of the proton motor bacterial system. Nisin is a more effective combination with EDTA attacking the cytoplasmic membrane of G^- bacteria due to the chelating effect of EDTA on cell wall components. By inhibiting the cell wall, mercasidine eliminates G^+ bacteria. Colicin eliminates G^- bacteria's membrane pore-forming mechanism (Ahmad *et al*., 2017; Mahmud and Khan, 2018).

Poly-L-Lysine

Poly-L-Lysine is a lysine homopolymer as a GRAS that is used as a staple in foods. Large quantities produce a bitter flavor. It has high antimicrobial power, so it is used in small quantities. It also acts better with other antimicrobial agents. It is used in rice, noodles, soups, salads, cakes, custard, and for external protection of fish and sushi (Baines and Seal, 2012).

Natural Antioxidant Agents

Oxidation of lipids is the most important cause for the lower quality of foods (Prakash *et al*., 2012). Antioxidants inhibit oxidation by preventing the making of free radicals or by stopping their release—large amounts of synthetic antioxidants (BHA or BHT) may be carcinogenic in some animals. Therefore, natural antioxidants are a good option (Ahmad *et al*., 2015). Foods that use antioxidants include meat, dairy, oil, fried or extruded foods, and sauces (Baines and Seal, 2012). All polyphenol derivatives (phenolic-hydroxybenzoic acids or hydroxyquinamic acids, flavonoids including anthocyanins, tannins, lignins, acetylbases, and coumarins) due to high antioxidant ability and health properties can be used as bioactive substances in foods (Savaş *et al*., 2020; Carocho *et al*., 2018).

Plant-based antioxidant agents

Vitamins (ascorbic acid and α-tocopherol), spices (cinnamon, cloves, nutmeg, ginger, black pepper, and garlic), herbs (rosemary, oregano, marjoram, sage, and basil), and plant extracts (tea, grape seed, cranberry, blueberry, and strawberry) contain antioxidant ingredients (Ahmad *et al*., 2015; Amiri *et al*., 2019b). The antioxidant role of plant extracts is because they contain phenolic acids (gallic, protocatechuic, caffeic, and rosmarinic acids), phenolic diterpenes (carnosol, carnosic acid, rosmanol, and rosmadial), flavonoids (quercetin, catechin, naringenin, and kaempferol), and volatile oils (eugenol, carvacrol, thymol, and menthol). Plant pigments such as anthocyanins and anthocyanins also have antioxidant roles. Catechins, epicatechins, phenolic acids, proanthocyanidins, and resveratol confer antioxidant effect in tea and grape seed extract (Aziz and Karboune, 2018; Gonelimali *et al*., 2018). Ferric acid, a hydroxyaminic acid, is used as an antioxidant and other preservatives in edible films and gels (Kumar and Pruthi, 2014). Catechin is a 3-ol flavon that has antioxidant activity. Ascorbic acid can be used as an oxygen scavengers to stabilize lipids and oils in foods, regenerating phenolic oxidants and oxidized tocopherols. Carotenoids also have antioxidant effects but are sensitive to light oxidation and, therefore, less used. Lycopene is the primary carotenoid in tomatoes. Beta-carotene is used as a singlet oxygen quencher in cooked foods, eggs, and dairy products. As a maker of vitamin E, tocopherols are powerful antioxidants that are used individually or in combination with ascorbic acid (Carocho *et al*., 2018).

Herb extracts

Rosemary

The antioxidant role of rosemary is found in phenolic diterpens (carnosic, carnosol, rosmanol, rosmadial, 1, 2-methoxycarnosic acid, and epi- and iso-rosmanol) and phenolic acids such as rosmarinic and caffeic acid (Brewer, 2011). Carnosic acid, a hydroxybenzoic acid derivative found in rosemary extract, has the highest antioxidant effect and is used in oils, sauces, bakeries, meat, and fish (Birtić *et al*., 2015). Rosemary extract E 392 is available on the European Food Additives list (EU Regulation 1129/2011). Rosemary extracts for food preservation alone or with other antioxidants, such as nisin, polyphenols, BHA, and BHT, are examples of the application of natural antioxidants (Carocho *et al*., 2018).

Oregano and Sage

Oregano extract has large amounts of phenolic acids (especially rosmarinic acid), phenolic carboxylic acids, apigenin, dihydroquercetin, and glycoside antioxidant agents that help eliminate superoxide anion radicals (Brewer, 2011). Oregano EO especially reduces lipid and protein oxidation and improves the color of chicken meat (Al-Hijazeen *et al*., 2016).

Marjoram

Marjoram EO contains high amounts of rosmarinic acid, carnosol, terpinen-4-ol, cis-sabinene hydrate, p-cumene, and ϒ-terpinene (Brewer, 2011).

Basil and Thyme

The main constituents of basil aromas include 3,7-dimethyl-1,6 octadien 3-ol, 1 methoxy 4(2-propenyl) benzene, methyl cinnamate, 4 allyl 2 methoxyphenol, and 1,8 cineole. Eugenol, thymol, carvacrol, and 4 allyl phenol are antioxidant agents comparable to BHT and α-tocopherol (Aziz and Karboune, 2018).

Spice extracts

Cinnamon

Cinnamon contains the highest amount of polyphenolic compound with the highest antioxidant and antiradical activity. Eugenol and cinnamaldehyde are the most important elements known in cinnamon leaf oil and oleoresin in cinnamon bark. Vanillic, caffeic, gallic, photochatechuic, p-hydroxybenzoic, p-coumaric, ferulic acids, and phydroxybenzaldehyde are also antioxidant components of cinnamon (Brewer, 2011; Bai-Ngew *et al*., 2021).

Garlic and Onions

Garlic and myrrh contain the major antioxidants of flavonoids (flavones and quercetins) and the sulfuric components (allyl-cysteine, diallyl sulfide, and allyl trisulfide) (Brewer, 2011). In addition, it has antioxidant roles *in vitro* and *in vivo* due to its antimicrobial efficacy. Allicin is one of the major constituents of thiosulfinates in garlic that has a specific garlic odor. When the garlic crumbles, allicin is converted to alliin by alliinase. Onion extracts have higher free radical removal than garlic due to their higher total phenolic content (Aziz and Karboune, 2018). Fresh or oil extract and dried powder of garlic can prevent lipid oxidation and increase sausage persistence (Sallam *et al*., 2004).

Other spices

Fresh and dried ginger contains a high concentration of volatile oils of camphene, p-cineole, α-terpineol, zingiberene, and pentadecanoic acid. Ginger extract exhibits approximately equal antioxidant activity to BHA and BHT. The main constituents of cumin are cuminal, ϒ-terpinene, and pinocarveol. The effect of cumin EO on reducing Fe^{3+} ions is better than fresh and dried ginger. Turmeric is mainly composed of curcumin, dimethoxycurcumin, bis-dimethoxycurcumin, and 2,5-xylenol, which is associated with vitamin E and BHT in the removal of free radical. α- and β-turmerone, curlone, and α-terpineol are the most important turmeric oil compounds with antioxidant activity. Black pepper, nutmeg, and cloves also have antioxidant activities (Brewer, 2011; Gonelimali *et al*., 2018).

Tea and fruit extracts

Tea and grape seed extracts

Green tea extract has the highest total phenolic content, 94% of which contains flavonoids. Oolong tea has about 18% total phenols and 4.4% flavonoids. In black tea, teaflavins and thearubigins are the predominant ones. The high antioxidant role of tea is due to flavonoids, tannins, and vitamins. Grape seed extract contains catechin and epicatechin. The total phenolic amounts depend on the grape varieties, climatic conditions, degree of maturity, extraction, and solvents (Brewer, 2011).

Pomegranate extracts

The pomegranate peel contains a good amount of tannins, anthocyanins, and flavonoids. Pomegranate juice has three times more antioxidant effects than green tea and red wine (Ahmad *et al*., 2015).

Other fruit extracts

Cranberries have one of the highest levels of total phenol and antioxidant properties among fruits, and citrus fruits have antioxidant efficacy (Ahmad *et al*., 2015). Citrus fruits contain flavonoids, especially glycosylated flavanones, and polymethoxy flavones. Citrus juice has been reported at about 5–10% to decrease extra nitrite content and degree of lipid oxidation in tending sausages (Aziz and Karboune, 2018).

Mechanism of action

Some of the factors that contribute to the oxidation of lipids are oxygen and metal ions, moisture, heat, and light. EOs have different mechanisms, including inhibition of chain initiation and inhibition of hydrogen accumulation, free radical scavengers, termination of peroxides, singlet oxygen formation quenchering, and binding of metal ions (Mahmud and Khan, 2018). Most antioxidants of spices and plants react with free radicals when autoxidation begins, while others form complexes with metal ions. Eighty-five percent EOs are phenolic compounds such as carvacrol, eugenol, and thymol, which are effective

as primary or fragile chain antioxidants and free radical scavengers (Hyldgaard *et al*., 2012).

Antioxidants are important components that inhibit autoxidation by preventing the making of free radicals and/or scavenge free radicals by the following mechanisms: Eliminating those that begin to peroxidize, chelating metal ions, quenching $^{\cdot}O_2$, which prevent the making of peroxides, fracturing the chain of the oxidation reaction, decreasing oxygen concentration, or stimulating antioxidant enzymes. Antioxidants that are able to disrupt free radical chain reactions are most effective. In general, they have aromatic phenolic rings with hydroxyl groups and can donate $H^{\bullet}$ to free radicals that convert themselves to radicals. Phenolic acids trap free radicals, and flavonoids can quench free radicals and chelate metals (Brewer, 2011).

Food Applications

Natural antimicrobial compounds can be used as preservatives in foods. There are two major issues for the application of plant compounds in foods, one being the odor caused by the high concentrations of these substances and the other being the cost. The safety of natural antimicrobials for use in foods is paramount, so toxicity tests must be done before application.

Meat products

Natural antibacterial components, such as spice and plant extracts, EOs, organic acids, salts, and BACs, are used to modify the shelf life of meat. To improve sausages' retention, lemon and thymol are used with modified atmospheric packaging (MAP). A combination of bay EO and oxygen-free MAP showed that it controls *L. monocytogenes* and *E. coli* growing and increases chicken meat's retention time. One study showed that separate extracts of clove, rosemary, cassia bark, and liquorice extracts alone have potent antimicrobial efficacy. The mixture of rosemary and liquorice extracts showed a good repressor effect on *L. monocytogenes*, *E. coli*, *Pseudomonas fluorescens*, and *Lactobacillus sake* in freshly packed pork slices with atmospheric packaging and vacuum ham slices (Mahmud and Khan, 2018). Nisin, Pediocin PA-1, Enterocin AS-48, Enterocins A and B, sakacin, and leucocin A can be used for bio-preservation of meat products and increase their shelf life. Pediocin is a good bio-preservative in meat products. The antimicrobial efficacy of the mixture of lysozyme, nisin, and EDTA against *L. monocytogenes* was observed in packed ostrich slices. Immersing meat products in thyme and oregano oil (0.1 and 0.3% concentrations) improves shelf life (Gonelimali *et al*., 2018; Rai *et al*., 2016). Massani *et al*. (2014) showed that active polymers including *Lactobacillus curvatus* CRL705 BACs reduced *L. monocytogenes* levels in Wiener sausages. Bukvički *et al*. (2014) studied the efficacy of *Satureja horvatii* (containing EOs of pcymene, thymol, and thymol methyl ether) on pork meat. In addition to inhibiting *L. monocytogenes*, these compounds also improved meat color and taste during storage.

Fish products

G^- bacteria spoil fish and fish products. *Clostridium* spp. and *L. monocytogens* are responsible for spoilage in vacuum-packed fish. Application of thyme EO and odor leaves increased the shelf life of fish. The combination of MAP and thyme EO increased the retention time of Mediterranean fish fillets. Oregano oil has a strong effect against *Photobacterium phosphoreum* in codfish fillets than salmon. EOs of *Aloysia sellowii* were effective on both types of bacteria and two yeasts in brine shrimp. Coating with EOs is a good way to increase the product quality of fish. Also, application of chitosan coated with cinnamon EOs prolongs salmon fillet and increases texture, odor, and color. Nisin can be used to store fish and other seafood. The combination of nisin with MicroGARD is the best way to preserve fish and prevent aerobic microorganisms' growth (Mahmud and Khan, 2018; Rai *et al*., 2016).

Vegetables and fruits

Some of the methods used to maintain fruit quality and fresh vegetables are immersion, coating, and spraying. Alginate coating with EOs (lemongrass, cinnamon EOs, citral, and cinnamaldehyde) reduced *E. coli* O157:H7 in Fuji apple slices and increased their shelf life. Antimicrobial activity of propionic, maleic, acetic, lactic, and citric acid was also demonstrated in red apple and lettuce against *E. coli* O157:H7, *S. typhimurium*, and *L. monocytogenes*. Garlic oil encapsulates in β-cyclodextrin has influenced the microbial and sensory property of fresh-cut tomatoes. Fruit juices are susceptible to yeasts such as *Pichia anomala*, *S. cerevisiae*, and *Schizosaccharomyces pombe*. Standard processes such as heat treatment, aseptic packaging, or the use of weak acids prevent yeast. EOs are acidic to prevent yeast growth. Lemon EO added to apple juice prolongs the time of "open" storage at room temperature and gives a fresh and pleasant taste (Mahmud and Khan, 2018). Lu *et al*. (2014) used a washing solution containing thymol that reduced *salmonella* and total aerobic bacteria in grape tomato without altering its quality.

Enterococci could be applied as starter cultures or cocultures to inhibit microbial contamination. BAC-producing LAB are screened in olives, sourdough, miso, sauerkrauts, refrigerated pickles, and mungbean sprouts. Nisin is used to prevent the growth of *C. thermosacchrolyticum* and

Geobacillus stearothermophilus in canned food (canned peas, carrots, and potatoes), fruit juice, and vegetables. Enterocin AS-48 is utilized in fruit juice and canned vegetables to prevent contamination (Rai *et al*., 2016).

Cereal products

Chitosan-coated bread improves bread quality by reducing microbial growth and delaying oxidation. The use of citrus oils also affects the sensory characteristics of the bread and inhibits microbial growth. Natural components, for example, anise, black cumin, rosemary, and sage, indicated antimicrobial and antioxidant activity in bakery products. EOs of basil, oregano, and thyme have antibacterial activity against *B. cereus* in rice-based foods (Mahmud and Khan, 2018). The antifungal activity of hydrolyzed goat whey as a bio-preservation agent against *Penicillium* spp was effective in pita bread (Luz *et al*., 2020).

Dairy products

Microorganisms, especially fungi, contaminate dairy products. The effect of natural ingredients has been documented either alone or with other technics such as spraying, immersion, or dusting on milk or cheese directly or in packaging materials. It has been found that mango seed extract reduces bacterial count and inhibits coliforms' growth, has a significant antimicrobial effect on *E. coli*, and increases the longevity of pasteurized milk. Lysozyme and EDTA in MAP-packaged burrata cheese resulted in increased cheese retention time, particularly at high lysozyme amounts. Enterocin CCM4231 and EJ97 are applied in soy milk and zucchini puree. Entrosine As-48 is used in mangoes, milk, and cheese. Nisin in sodium caseinate films has been used in Babybel mini red cheese (Mahmud and Khan, 2018). Nisin is very effective with other antimicrobial ingredients such as thymol and lysozyme. Lipid nanoparticles with nisin have an antibacterial effect on food pathogens. Pediocin ACH has shown an antimicrobial effect on *L. monocytogens*, *E. coli*, and *S. aureus*. Oregano and Thyme with MAP have been used in Feta cheese against *E. coli* O157:H7 and *L. monocytogenes*. The addition of eucalyptus oil and lemongrass had a high effect on West African soft cheese's nutritional, sensory, and microbial characteristics (Mahmud and Khan, 2018; Rai *et al*., 2016).

Conclusions

Given the resistance of microorganisms to antibiotics and consumers' tendency to eat healthy foods, the use of bio-preservatives is a reasonable approach. Some chemical additives may alter specific nutrient properties. For example, sulfites destroy vitamin B1 or nitrate was reduced to nitrite in the meat which converts to carcinogenic nitrosamines. Natural compounds such as EOs of cinnamon, cloves, lemongrass, and their active ingredients are GRAS. The antimicrobial and antioxidant effects of plant extracts and EOs owe to have phenolic compounds such as terpenes and flavonoids. However, despite the tremendous potential, flavor problems and toxicity can limit their use. To alleviate these problems and improve effectiveness, encapsulation of EOs and active compounds and coatings containing these compounds are the best options. Due to the potency of enzymes to produce antimicrobial compounds and their capability to separate some bacteria's outer membrane, they can be known as natural antimicrobials compounds. However, their use in foods needs to be further studied. Bacteriophages are used as an auxiliary process to control some pathogens but do not increase products' shelf life. BACs are antimicrobial or peptide proteins that are ribosomally encoded and widely used in food production. BACs can be used as bio-preservatives because they are safe. The use of antimicrobial peptides in the form of nanoparticles can be very effective. For example, antimicrobial peptide coatings with metal nanoparticles will be beneficial in removing food contamination. The application of films and coatings, including natural agents, is expanding due to their biodegradability and potency to enhance food safety, quality, and shelf life. This study has shown that EOs, BACs, enzymes, organic acids, and coatings can change chemical additives, but more research is needed.

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Incorporation of essential oils in Iranian traditional animal oil: an assessment of physicochemical and sensory assessment

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Abstract

This study aimed to incorporate the herbal essential oils (*Ziziphora tenuior* L. (*Z. tenuior*), *Ferulago angulata* (*F. angulata*), and *Bunium persicum* (*B. persicum*)) all in three levels (5, 7, and 10% w/w) and tertiary butyl hydroquinone (100, 200, and 300 ppm) to evaluate the oxidation stability of Iranian animal oil (IAO). *Z. tenuior*, *F. angulata*, and *B. persicum* (5, 7, and 10% w/w) and tertiary butyl hydroquinone (100, 200, and 300 ppm) were added to IAO. The physicochemical properties and color analysis, and sensory characteristics (odor, taste, rancidity, and overall acceptability) of the treatments were investigated on days 0, 7, 14, 21, and 28. The results showed that increasing the amounts of *Z. tenuior*, *F. angulata*, and *B. persicum* was associated with reducing acid, peroxide, and thiobarbituric acid values. It also lowered brightness and yellowness while the oxidative stability of the IAO significantly was increasing. It was concluded that the incorporation of *B. persicum* had the highest effectiveness regarding the proposed criteria with the least effect on sensory properties.

Keywords: bioactive compounds; herbal antioxidant; oil stability; natural preservative; color analysis

Introduction

Oxidation in food, especially in fats and oils, is a destructive process with adverse effects on their nutritional values and chemical composition (Henry and Heppell, 2002). It can also have undesirable effects on the color, taste, and texture of foods, while degrading the essential vitamins and fatty acids or creating toxic compounds (Stoilova *et al*., 2007; Wang *et al*., 2008). Antioxidants are natural or synthetic substances added to food products to prevent or delay the damage caused by oxygen (Sharififar *et al*., 2010). Although compounds such as tocopherols are naturally available in some oils, synthetic antioxidants with a phenolic structure are commonly used to prevent the oxidation of oils (Agregán *et al*., 2019; Mousavi Khaneghah *et al*., 2016).

Synthetic antioxidants, such as tertiary butyl hydroquinone (TBHQ), are inexpensive, easily accessible, and have high efficacy in inhibiting oxidation. TBHQ at 75 ppm is used in the oil industry in many countries, including Iran. It affects the free radicals of lipids in oxidative chain

reactions. Its phenolic group stabilizes free fatty radicals by using hydrogen and thus preventing the oxidation of fats (Wang *et al*., 2008).

However, some concerns have been raised regarding the mutagenicity and the role of synthetic antioxidants in the disruption of liver enzymes' activity as well as the development of diseases such as cancer and cardiovascular disease (Iqbal and Bhanger, 2007; Tavakoli *et al*., 2018). As a result, replacement of these compounds with natural antioxidants of plant origin has been considered. Antioxidant properties of plant extracts mainly depend on their phenolic compound content (Ahmed *et al*., 2019; Burt, 2004). In this regard, herbs and their derivatives (essential oils and extracts) with potent antioxidant properties are widely used to prevent the oxidative degradation of food (Hashemi *et al*., 2017; Tiwari *et al*., 2009).

"Kermanshahi Roghan," known as the "Roghan-*e*-Heyvani," is an Iranian animal oil (IAO) and a yogurt by-product, like ghee and yayik butter commonly used in India and Turkey, respectively (Mostafaie *et al*., 2018). It is a traditional anhydrous milk fat product with a golden yellow color because of carotenoids. As an expensive dairy product, IAO is one of the most favorable oils consumed in many regions of Iran (Salarabadi *et al*., 2015). Produced from cow, sheep, or goat's milk, IAO is exported to many countries worldwide (Chalabi *et al*., 2018). The fatty acid composition of IAO (99% fat and < 0.2% moisture) is different from that of butter, as the lipids are extracted directly from milk. Therefore, it has lower long-chain fatty acids and cholesterol (Najafi *et al*., 2011).

Z. tenuior, *Ferulago angulata* (Chavill), and *Bunium persicum* (Black Caraway) belong to *Lamiaceae*, *Apiaceae*, and *Chattrian*, respectively. *Z. tenuior* (Bakhtiar *et al*., 2021) and *F. angulata* (Ghasemi Pirbalouti *et al*., 2016) are widely distributed in Iran. Also, *B. persicum* grows in areas with a Mediterranean climate such as Central and Western Asia (e.g., Iran) (Hassanzadazar *et al*., 2018). Due to their phenolic and flavonoid compounds, these plants have antioxidant and antibacterial activities, and gastrointestinal transit accelerating properties (Amiri and Joharchi, 2016; Dakah *et al*., 2019; Ehsani *et al*., 2016; Hazrati *et al*., 2019; Mahboubi, 2019).

Considering the increasing awareness among consumers regarding the harmfulness of synthetic additives in food, as well as studies on the use of herbal extracts to increase the shelf life of vegetative oils, the present study is designed to compare the oxidation of IAO treated with *Z. tenuior*, *F. angulata*, *B. persicum*, with that of synthetic antioxidant TBHQ.

Materials and Methods

Extraction of essential oil

Fresh IAO, free of any antioxidants, was purchased from a local market. The dried leaves of *Z. tenuior, F. angulata*, and *B. persicum* were obtained from the Iranian Institute of Medicinal Plants, Karaj, Iran. The essential oils were extracted from these leaves using a Clevenger-type apparatus (Biogenic, Brasilia, Brazil), and based on the steam distillation method of Gharibzahedi *et al*. (2015). The brief protocol is as follows:

One-hundred grams of the leaves were placed in the distillation flask, and the oil was extracted from the vegetal substrate at 5-, 15-, 30-, 60-, and 100-min intervals. In order to completely isolate the water from the essential oil, 0.5 g of sodium sulfate was added to the separation column each time. The extracted essential oils were stored at 4 ± 1°C until experiment time. TBHQ and other chemicals were purchased from Merck Co. (Darmstadt, Germany).

Preparation of the treatments

Different concentrations of the essential oils and TBHQ were added to 100 g of IAO based on the values indicated in Table 1 and mixed using RH Basic 2 magnetic stirrer (Staufen, Staufen Germany). StaufenThe IAO samples were evaluated on days 0, 7, 14, 21, and 28 of storage. All tests were performed in three replications.

Table 1. Iranian animal oil treatments with incorporation of different essential oils and TBHQ[a].

Treatments	*Z. tenuior* (%)	*F. angulata* (%)	*B. persicum* (%)	TBHQ (ppm)
T1	5	–	–	–
T2	7	–	–	–
T3	10	–	–	–
T4	–	5	–	–
T5	–	7	–	–
T6	–	10	–	–
T7	–	–	5	–
T8	–	–	7	–
T9	–	–	10	–
T10[b]	–	–	–	100
T11[b]	–	–	–	200
T12[b]	–	–	–	300

[a]TBHQ: Tertiary butyl hydroquinone, artificial antioxidant.
[b]T10–T12 treatments were used as control.

IAO analysis

IAO with a peroxide value of less than 5 meq O^2/kg was purchased from the Pegah Golpayegan Dairy Company (Golpayegan, Iran). It was kept at 4°C during the whole study, as recommended by the producer.

Acid value

The AV was defined as the amount (mg) of potassium hydroxide (KOH) required to neutralize the free fatty acids in 1 g of IAO samples dissolved in a mixture of ethanol. The titration method was used to determine the AV according to ISO 660 (ISO, 2009) using the following equations:

$$\text{Acid value (mL KOH/g IAO)} = \frac{56.1 \times N \times V}{M} \times 100 \quad (1)$$

N: KOH normality; V: Consumed KOH volume (mL); M: IAO weight (g); 56.1 = KOH molecular weight (g/mol).

Peroxide value

IAO was dissolved in a solution of isooctane and glacial acetic acid and then mixed with potassium iodide. Free iodine via peroxides was measured (ISO 3960, ISO, 2017) using iodometry in the presence of starch and sodium thiosulfate solution. PV was determined through sodium thiosulfate titration and was calculated using the following equation:

$$\text{Peroxide Value (meq } O^2\text{/kg IAO)} = \frac{V \times N}{M} \times 1000 \quad (2)$$

V: Consumed thiocyanate volume; N: Thiosulfate normality; M: IAO weight.

Thiobarbituric acid value

Thiobarbituric acid (TBA) value shows the amount of malondialdehyde (MDA) present in each 100 g of oil. MDA, commonly used as an oxidation marker, is one of the most abundant aldehydes generated during secondary lipid peroxidation in foods (Reitznerová *et al*., 2017). This method allows direct measurement of the TBA levels in fats and oils without the MDA's need for prior isolation. MDA reacts with TBA and forms a color complex with absorption maxima at a wavelength of 530 nm (AOCS, 2017).

$$\text{TBA (mg MDA/kg IAO)} = \frac{50 \times (A - B)}{M} \quad (3)$$

A: Absorption of IAO sample; B: Absorption of TBA as the blank sample; M: IAO weight

Oxidative stability

The ability of the oil against oxidation is known as Os and is expressed as h. The test was performed based on the Rancimat technique using a Metrohm 743 Rancimat (FanAzma Gostar, Alborz, Iran) at 110°C and 20 l/h airflows (ISO 6886, ISO, 2016). Oxidation resistance ends when a rapid increase is seen in the specific conductance due to the decomposition of the carboxylic acids resulting from lipid oxidation and their absorption in deionized water.

Color analysis

The color evaluation was performed using Hunter lab colorimeter (Hunter lab D25-DP9000, Germany) with black and white calibration plates. L* (lightness) from black (0) to 100 (white), a* (green-red), and b* (blue-yellow) was calculated from –120 to 120 (Guo *et al.*, 2016).

Sensory evaluation

The sensory characteristics of the treatments such as odor, taste, rancidity, and overall acceptability of coded IAO samples during 28 days of storage were evaluated based on a 5-point hedonic scale from 1 (dislike very much) to 5 (like very much) (Mohammadi *et al*., 2011).

Statistical analysis

A randomized complete block design was applied. The data were analyzed by analysis of variance (ANOVA) using SAS 9 (SAS Institute Inc., Cary, NC, USA) followed by Duncan's multiple range test (Koushki *et al*., 2011). Values were reported as mean ± standard deviation (SD) of six repetitions for each treatment. P values < 0.05 were considered statistically significant for all comparisons (Amiri-Rigi *et al*., 2011).

Results and Discussion

AV, PV, TBA values and Os analysis of different IAO treatments

AV value depends on the type and the amount of the applied essential oils. In other words, the difference in the AV is due to the difference in the antioxidant content of the active compounds as well as their ability to trap water. According to Figure 1A, there was a significant difference between the AV values of the controls (T10–T12) and that of the treatments containing essential oils (P < 0.05). A decreasing trend was observed in AV values with increasing concentrations of essential oils. Treatments containing *B. persicum* and *Z. tenuior* were reported to have significantly lower AV values than those containing *F. angulata*. This is mainly because the high polyphenolic, flavonoid, and antioxidant content of treatments containing *B. persicum* deactivates free radicals derived from the environmental oxidation process (Hassanzadazar *et al*., 2018). AV indicates the progress of the oxidation process, and therefore its value decreases with the increasing flavonoid content of the oil. This is mainly because of the increased concentration of applied essential oils.

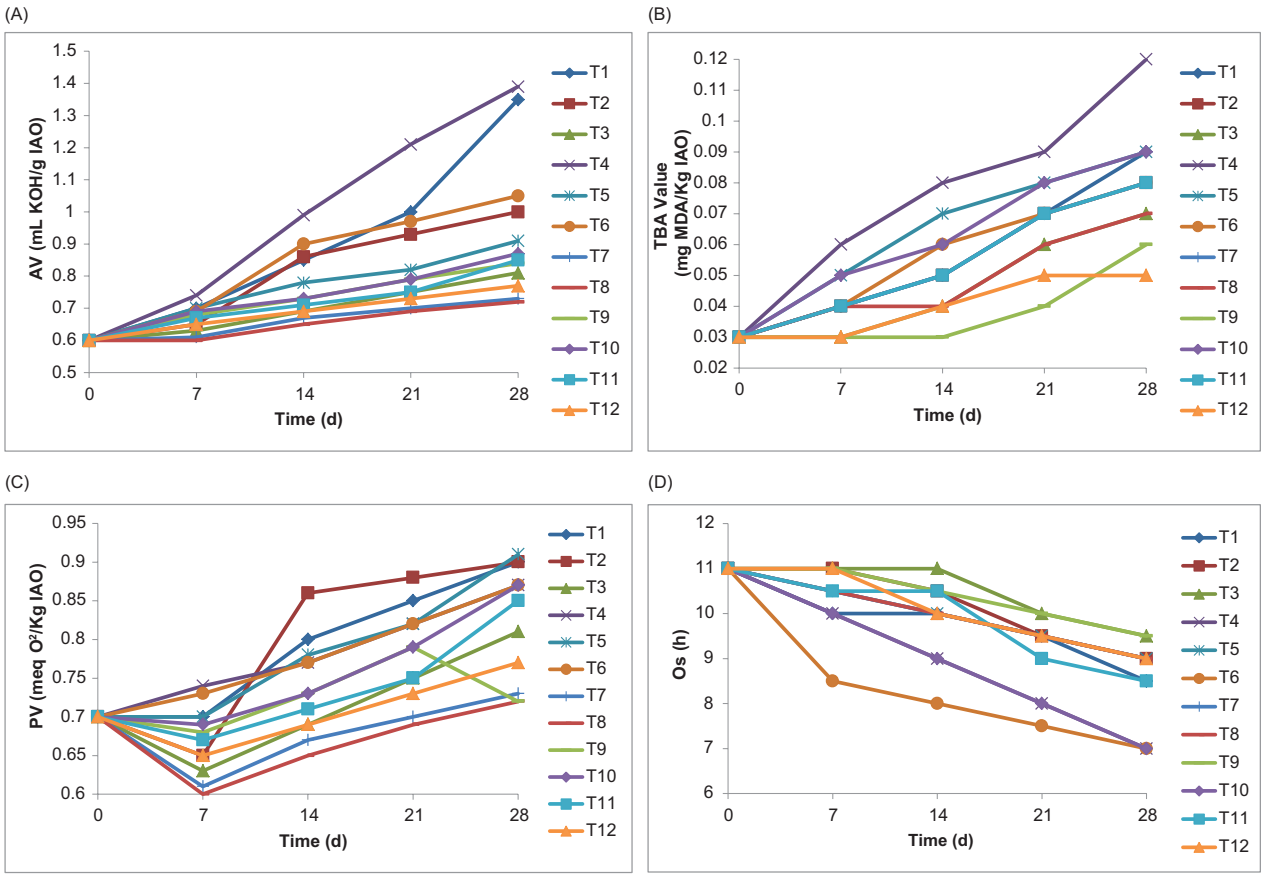

Figure 1. Acid value (AV) (A), peroxide value (PV) (B), Thiobarbituric acid (TBA) value (C), and oxidative stability (Os) (D) of Iranian animal oil treated with herbal essential oils during storage time.
Treatments (T1–T12) are described in Table 1.

There was an overall increase in the AV of IAO treatments with increased storage time that negatively affects the oil quality. This can be secondary to the increase of IAO's free fatty acid content during this time, either because of the hydrolysis of triglycerides or their formation as an end product of the oxidation process (Guo *et al.*, 2016). Moreover, the increased exposure of oil to oxygen, light, and acidic compounds as the oxidation process progresses could also be responsible for the significant increase noted in the AV values. For instance, the increase noted in AV of IAO treatments containing *B. persicum* and *Z. tenuior* was lower than that of controls containing TBHQ. Treatments containing 10% *B. persicum* and *Z. tenuior* had the lowest AV enhancement (28 days) ($P < 0.05$).

On the other hand, the AV of treatments containing *F. angulata* increased significantly during storage but was negatively correlated with the amount of *F. angulata* ($P < 0.05$). Compared with *B. persicum* and *Z. tenuior*, *F. angulata* is high in aromatic compounds but low in polyphenolic ones. In line with our findings, Najafi *et al.* (2011) showed similar results while studying the antioxidant effect of olive leaf and its extract on soybean oil stability (Najafi *et al.*, 2011).

Unsaturated fatty acids such as oleic and linoleic acids with double bonds in their chemical structure are highly susceptible to oxidation-accelerating factors such as heat, light, oxygen, and lipoxygenase activity (Pradhananga and Manandhar, 2018). Hydroperoxides are the primary products of the oxidation reactions and are thus used to evaluate the oxidation reactions' progression (Alizadeh *et al.*, 2016). IAO is very susceptible to oxidative corruption. According to Figure 1B, the use of essential oils changed the PV of the treated IAO significantly compared with the TBHQ-containing controls ($P < 0.05$). The treatments containing *B. persicum* had the lowest PV. This can be due to the presence of active compounds such as limonene and Trans-Carveol and limonene derivatives (Mohammadi *et al.*, 2021). PV of all treatments increased with longer storage time, depending on the type and the amount of applied essential oil. Among the applied essential oils, *F. angulata* had the least effect on PV values. In other words, there was no significant difference between the PV of the *F. angulata*–treated IAO and the control containing 100 ppm TBHQ. The least amount of change in PV values was reported in IAO containing 10% *B. persicum* after 28 days of storage ($P < 0.05$). Darughe *et al.* (2012) also found that the antioxidant compounds

in *B. persicum* significantly inhibited any increase in PV values of the cake during storage (Darughe *et al*., 2012).

PV breaks down after reaching a certain level and the by-product compounds are formed (Pradhananga and Manandhar, 2018). For this reason, TBA value, alongside PV, was also investigated. According to Figure 1C, there was a significant difference between the TBA values of different IAO treatments based on the type and the amount of applied essential oil ($P < 0.05$). TBA followed an increasing trend during the 28 days of storage. The treatment containing 5% *F. angulata* had the highest TBA value, whereas treatments with *B. persicum* had the lowest increase in TBA value during storage ($P < 0.05$). This can be due to the increase in the latter's phenolic and limonene compounds that inhibited oxidation more effectively (Mohammadi *et al*., 2021). The high aromatic compound content of *F. angulata*, on the other hand, was not as effective in inhibiting the oxidation process. The increase noted in the TBA values of the IAO treatments indicates the formation of products such as aldehydes and ketones that have an adverse effect on the organoleptic properties of the oil (Serfert *et al*., 2010). In corroboration with our results, Hassanzadazar *et al*. (2018) also concluded that *B. persicum* could be used as a natural alternative to synthetic antioxidants based on the results of PV and TBA values of their investigation (Hassanzadazar *et al*., 2018).

Figure 1D illustrates a significant difference between the treatment's oxidation resistance time, and the controls ($P < 0.05$). Treatments containing 10% of each essential oil had the highest Os compared with those with 5% and 7% of the corresponding essential oils. The Os rate in treatments containing *B. persicum*, regardless of their concentration, was higher than that of IAO samples treated with *F. angulata* and *Z. tenuior*. The lowest Os was reported in the IAO samples treated with *F. angulata*. Also, it was observed that the treatments with *B. persicum* had an Os equivalent to TBHQ. While increasing the storage time was associated with a significant decrease in the Os values, increasing the concentration of essential oils had the opposite effect ($P < 0.05$). The former could be secondary to the partial inactivation of phenolic compounds as well as an increase in the levels of secondary hydroperoxide compounds due to storage-related oxidation (Hashemi *et al*., 2016). Our results were also supported by the research on rosemary extract's antioxidant effects on soybean oil (Casarotti and Jorge, 2014).

Color analysis of IAO treatments

Color is one of the apparent qualitative properties of the food products with a substantial impact on their marketability. This points out the importance of evaluating IAO's color indices treated with herbal essential oils as part of the quality control study. In this study, a* value did not change significantly due to the absence of red pigment and lycopene compounds in *Z. tenuior*, *F. angulata*, and *B. persicum*. L* and b* values of treated IAO samples during the 28 days of storage are shown in Figure 2 (A,B). Figure 2A illustrates that IAO samples treated with *Z. tenuior* and *F. angulata* had lower L* values than the control and *B. persicum*–treated ones. In addition, treatment with higher concentrations of *F. angulata* and *Z. tenuior* resulted in a lower L* value than those containing 5% essential oils. Therefore, it could be concluded that the L* value is significantly but inversely correlated with the concentration of the essential oils ($P < 0.05$). This could be due to the significant but negative effects of the chlorophyll content and pigment content of herbal essential oils on the light reflection, and thus the L* value. Compared with *B. persicum*–treated IAO samples, the control IAO samples were not reported to have significantly different L* values.

The L* values of treatments decreased significantly with increasing storage time ($P < 0.05$). This reduction, which is believed to be secondary to oxidative and lipolytic corrosion, was not significant in IAO samples treated with

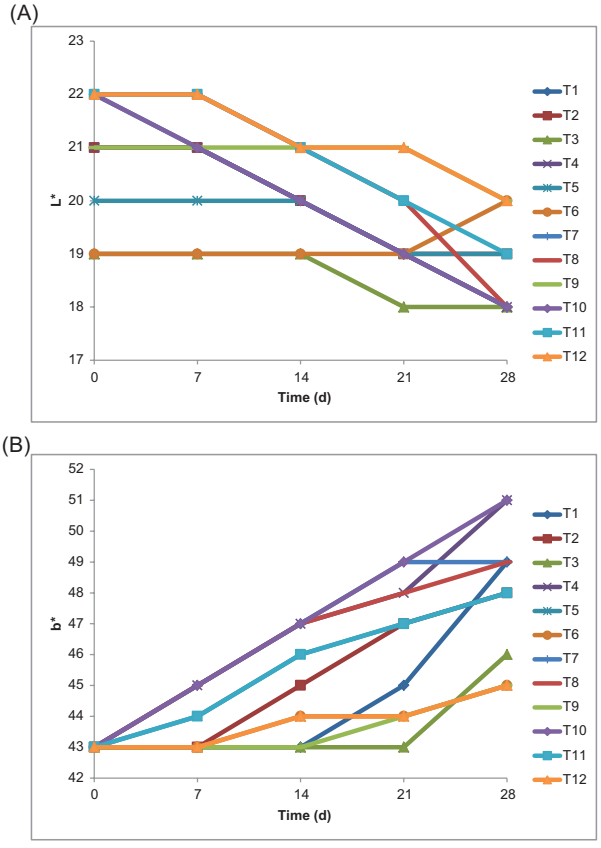

Figure 2. L* value (A) and b* value (B) of Iranian animal oil treated with herbal essential oils during storage time. Treatments (T1–T12) are described in Table 1.

essential oils at concentrations higher than 5%. These findings are in line with that of Guo *et al.* (2016), who reported that the rosemary ethanol extract had a significant effect on the L* value of treated palm oil samples (Guo *et al.*, 2016). They also reported that an increase in rosemary ethanol extract could decrease the L* value of palm oil treatments. Similarly, Hozhabri *et al.* (2014) investigated the effect of fish oil and *Z. tenuior* on the quality and oxidation rate of the Mahabadi goat meat. They showed that the addition of *Z. tenuior* could significantly reduce L* value (Hozhabri *et al.*, 2014).

Figure 2B shows significant differences observed between the b* values of various treatments ($P < 0.05$). The highest b* value was observed in the treatment containing 5% *F. angulata* essential oil, whereas the lowest values were found in those with 10% of *B. persicum*, *F. angulata*, and *Z. tenuior* ($P < 0.05$). An increasing trend in b* values was reported in the treated IAO samples with increased storage time; the increase, however, was lower in the IAO samples containing 10% of essential oils and 300 ppm TBHQ. Similar results were reported in previous research into the antioxidant effects of rosemary extract on soybean oil (Casarotti and Jorge, 2014).

Sensory evaluation of IAO treatments

According to Figure 3A, a significant decrease in taste scores of treatments increased the essential oil concentration. One of the reasons for this may be the dominance of the plant's taste over IAO's special taste. Guo *et al.* (2016) also found that rosemary ethanolic extract in high amounts in palm oil caused a vegetable taste in palm oil. With increasing storage time, there was a decreasing trend in the taste scores of the treatments. Treatments containing higher than 5% essential oils had lower taste scores on day 0, but they kept their scores steadily during storage time. Treatments with *F. angulata* and then *Z. tenuior* showed the lowest scores, but the treatments containing *B. persicum* had the same score as the control treatment with 300 ppm TBHQ. Even treatments with 200 and 100 ppm TBHQ had lower taste scores than those with *B. persicum* during storage until the end of day 28 ($P < 0.05$).

According to Figure 3B, increasing essential oil concentration led to a significant decrease in the odor scores in IAO treatments. Treatments with *B. persicum* had the least effect on the odor characteristic because the aromatic compounds in *Z. tenuior* and *F. angulata* are

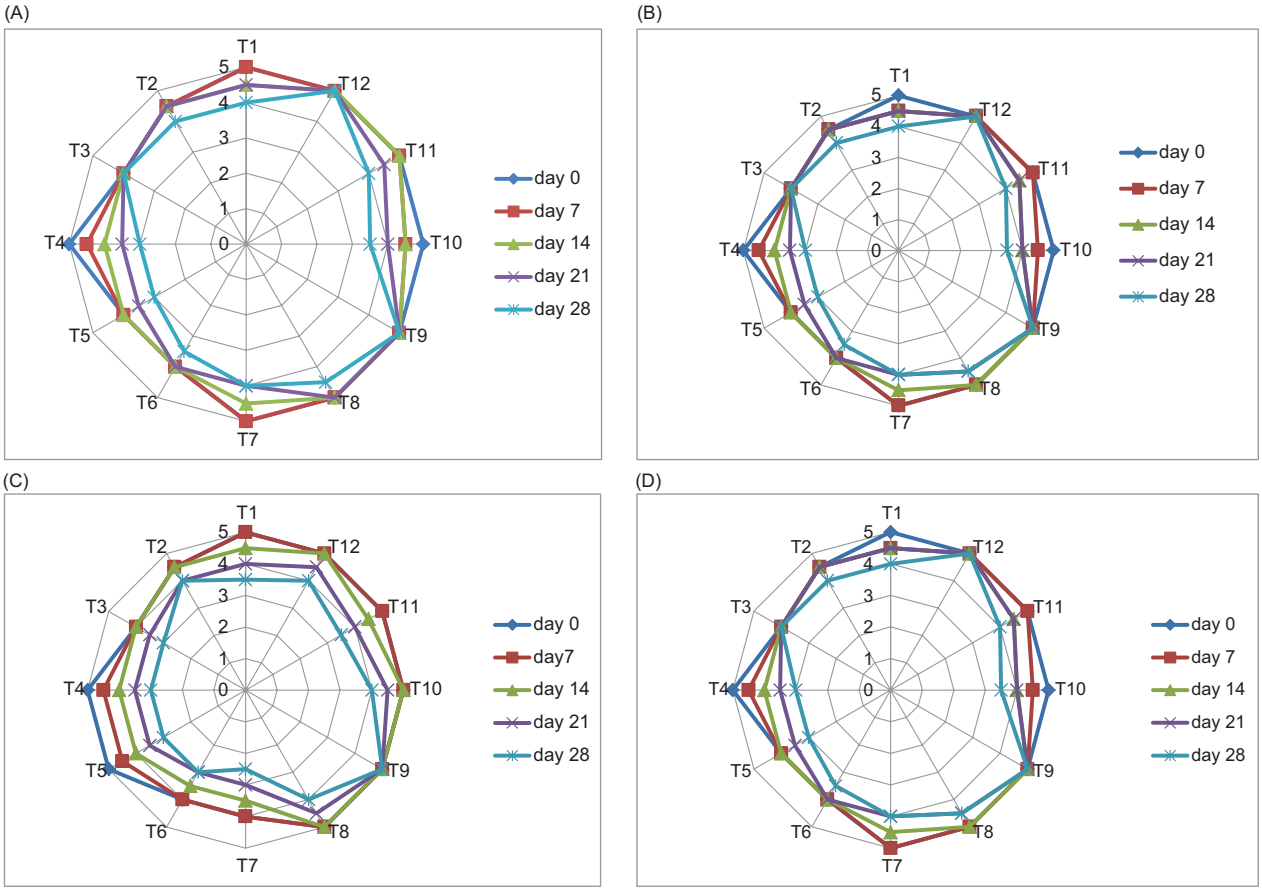

Figure 3. Taste scores (A), Odor scores (B), Rancidity scores (C), and Overall acceptability (D) of Iranian animal oil treated with herbal essential oils during storage time.
Treatments (T1–T12) are described in Table 1.

higher than the *B. persicum*, which reduces IAO's odor. Also, during the storage period, the flavor and odor scores of all treatments significantly decreased ($P < 0.05$). This decrease in the treatments containing *F. angulata* was more than in the other treatments.

According to Figure 3C, minimum scores of rancidity belonged to treatments containing *F. angulata* and then to *Z. tenuior*; the highest scores belonged to treatments with 300 ppm TBHQ and 10% *B. persicum*. The results showed that the effect of *F. angulata* on the rancidity character of IAO was lower than the effect of *B. persicum* and *Z. tenuior*, which is due to the difference in the chemical content of these essential oils. All the essential oils extracted from three medicinal herbs have high amounts of bioactive compounds. Mohammadi reported that the main chemical constituents in *Z. tenuior*, *F. angulata*, and *B. persicum* essential oils were pulegone (12.77%), ferulagon (14.97%), and (+)-*trans*-carveol (57.70%), respectively. However, higher total phenolic content, total flavonoid content, total carotenoid, and total chlorophyll in *B. persicum* essential oil were found compared to *Z. tenuior* and *F. angulata* essential oils (Mohammadi *et al*., 2021).

Also, increasing the essential oil concentration led to an increase in rancidity scores of treatments. During the storage period, the reduction of rancidity scores was lower in treatments with 10% essential oils. This can be due to the prevention of oxidation by essential oils during storage. Treatments containing 10% *B. persicum* had higher rancidity scores on the 28th day in comparison to other treatments.

According to Figure 3D, treatments containing *B. persicum* and TBHQ had the highest overall acceptability among the other treatments. The overall acceptability of treatment containing 5% *F. angulata* was significantly reduced because of the sensitivity of the *F. angulata* aroma and aromatic compounds of the *F. angulata*. Overall acceptability of all treatments decreased by increasing storage time, and increased by increasing essential oil content. Only the treatments containing 10% *B. persicum* and 300 ppm TBHQ maintained their overall acceptability until the end of the 28th day of storage time. Mehraban Sangatash *et al*. (2006) investigated the effect of *Ziziphora Clinopodioides* essential oil and extract on the activity of yogurt starter bacteria and found that the use of high concentration of essential oil reduced the final overall acceptability of yogurt treatments, which is consistent with the results of the present study.

Conclusions

It is concluded that increasing concentrations of *Z. tenuior*, *B. persicum*, and *F. angulata* are associated with a significant decrease in AV, PV, TBA value, L*, and b* of the treated IAO. The addition of herbal antioxidants, regardless of their concentrations, also improved the Os of the treated IAO; their effects though started to decrease after 28 days of storage. Moreover, by increasing the essential oils' content, taste and odor of IAO decreased, while rancidity and overall acceptability were increased. The bioactive effects of the applied essential oils was found to be in the order *B. persicum* > *Z. tenuior* > *F. angulata*. The addition of 10% *B. persicum* to the IAO was found to be an applicable and safe replacement for TBHQ. Our results are beneficial for developing strategies for producing edible oils and lipid-rich foods containing natural antioxidants with appropriate oxidative stability and pleasing sensory characterizations. In general, more study should be done to evaluate the antimicrobial effects of edible oils and emulsions enriched with the studied essential oils.

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Conflict of Interest

The authors of this manuscript wish to declare no conflict of interest associated with the submission and its content.

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Biodecontamination of milk and dairy products by probiotics: Boon for bane

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Abstract

In recent decades, "contamination of the environment, food, and feed by different contaminants such as heavy metals and toxins is increasing due to industrial life." Commercial milk and milk products can be contaminated with heavy metals and mycotoxins. Biosorption is a low-cost method and has good potential for decontamination. In dairy products, "various starters, especially probiotics, can be used as biosorbants, while microorganisms are able to bind to heavy metals and toxins and decrease their bioavailability and hazards in the human body." In this article, the key role of dairy starters and probiotics in the decontamination of toxins and heavy metals, and the best probiotics for decontamination of aflatoxins and heavy metals has been reviewed. After a quick glance at introducing dairy products and the main risks in association with the intake of some hazardous materials from dairy products, the application of biological systems is mentioned. Then, the article is focused on the role of beneficial microorganisms as the last chance to decrease the risk of exposure to toxins and heavy metals in dairy products. This review can be helpful for biotechnologists and scientists who have challenges about the existence of heavy metals and toxins in milk and dairy products, and help them to find the best method to decrease the content of the usual contaminants.

Keywords: aflatoxins; biosorption; decontamination; heavy metals; dairy products

Introduction

The World Health Organization (WHO) defines food safety as, "Approaches and methods for certifying the manufacture, maintenance, distribution and utilization of food happen in an assured system." However, some people defined safe food as food without any contamination (El Sheikha, 2015).

Heavy metals naturally exist in the environment. Industrial activities can increase their content in air and soil, leading to phytotoxicity of plants (Asati *et al.*, 2016; Yang *et al.*, 2018). Milk and dairy products have an important role in the human food chain, especially children's food; so, contamination of dairy products by toxins and heavy metals is one of the most important issues that can negatively impact consumers' health. Milk and dairy

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products can be contaminated with heavy metals under certain conditions through contamination of water and animal feed with environmental contaminants such as metal and cement smelters, sewage effluents, and industrial waste. Heavy metals' accumulation in milk can easily enter the human body and be dangerous for consumer's health (Abedi *et al.*, 2020). Dairy product contamination (heavy metal and aflatoxin) is very common all over the world (Ziarati *et al.*, 2018).

Heavy metals' toxicity occurs in levels of about 1.0–10 mg/L; however, lead and cadmium could have a toxic effect in 1–100 µg/L (Alkorta *et al.*, 2004). For example, different levels of exposure to cadmium could cause renal dysfunction, hepatic injury, and lung damage (Miura *et al.*, 2017; Naidoo *et al.*, 2019; Zhang *et al.*, 2014). Arsenic poisoning can cause death through disorder in essential metabolic enzymes (Khairul *et al.*, 2017). Maximum permissible limits of heavy metal contents in milk (considered by International Dairy Federation) are 2.6 µg/kg for cadmium, 10 µg/kg for Copper, 20 µg/kg for lead, and 328 µg/kg for zinc (Malhat *et al.*, 2012).

Aflatoxins directly (through eating contaminated food) and indirectly (primary contaminated products such as milk of contaminated livestock) can enter into the human body by the use of contaminated dairy products. Aflatoxins can cause negative effects on human health, such as liver or kidney cancer and chronic intoxications (Karazhiyan *et al.*, 2016). The most common aflatoxin in dairy products is aflatoxin M_1 (AFM_1). AFM_1 is a metabolite of aflatoxin B_1 (AFB_1) after ingestion of contaminated feed (AFB_1) by livestock. About 0.3 to 6.2% of AFB_1 (Abdelmotilib *et al.*, 2018) can be bio-transformed into AFM_1 (4-hydroxy- AFB_1) and can excrete into milk and urine (Iha *et al.*, 2013; Karazhiyan *et al.*, 2016). AFM_1 is carcinogenic and toxicogenic, and can resist pasteurization and sterilization processes (Gonçalves *et al.*, 2020). AFM_1 compared with AFB_1 is approximately 10 times less mutagenic, genotoxic, and toxigenic. Its carcinogenic effects are displayed in different kinds of species (Elsanhoty *et al.*, 2014). AFM_1 can also cause gene mutation, DNA damage, cell transformation in mammalian cells, and chromosomal anomalies. Food and Drug Administration (FDA) and the European Commission recommended that the maximum permissible limits of AFM_1 in milk are 0.5 µg/kg and 0.05 µg/kg, respectively (Commission, 2006; FDA, 2019)

It is reported that mycotoxins in milk and dairy products, which can be produced by different kinds of fungi are: Aflatoxins (by *Aspergillus*), Compactin (by *Penicillium*), Cyclopaldic acid (by *Penicillium*), and Patulin (by *Penicillium*) (El Sheikha, 2019).

Many reports have investigated regarding contamination of milk by heavy metals and toxins all over the world. According to Tables 1 and 2, which present some of the above reports, the amount of lead in Iraq, Brazil, China, Spain, and Italy was more than the maximum permissible

Table 1. Some important data about milk contamination to heavy metals (from 2014 to 2021).

Country	Contamination	Concentration	Reference
Egypt	Pb Cd	0.044–0.751 mg/L 0.008–0.179 mg/L	Meshref *et al.*, 2014
Serbia	Pb Cd	54.3–95.2 lg/kg 2.13–4.82 lg/kg	Suturović *et al.*, 2014
Iraq	Pb	32 µg/L	Alani and Al-Azzawi, 2015
Pakistan	Pb Cd	0.014 mg/Kg 0.001 mg/Kg	Ismail *et al.*, 2015
Bangladesh	Pb Cd	0.2 mg/L 0.073 mg/L	Muhib *et al.*, 2016
Iran	Pb Cd	14.0 µg/kg 1.11 µg/kg	Shahbazi *et al.*, 2016
Brazil	Pb	2.12–37.36µg/L	de Oliveira *et al.*, 2017
Mexico	Pb As	0.03 mg/Kg 0.12 mg/Kg	Castro-González *et al.*, 2018
Poland	Pb Cd	5.24 µg/L 0.15 µg/L	Halagarda *et al.*, 2018
Turkey	Pb Cd As	0.0055 mg/L 0.088 mg/L 0.002 mg/L	Seğmenoğlu and Baydan, 2021

As: Arsenic, Cd: Cadmium, Pb: Lead

Table 2. Some important data about milk contamination to mycotoxins in world from 2014 to 2021.

Country	Contamination	Concentration	Reference
Croatia	AFM_1	0.003–1.135 µg/L	Bilandžić *et al.*, 2014
China	AFM_1 OA ZEA α-ZEA	80.4 ng/kg 56.7 ng/kg 14.9 ng/kg 24.3 ng/kg	Huang *et al.*, 2014
Serbia	AFM_1	0.01–1.2 µg/kg	Kos *et al.*, 2014
Iran	AFM_1	> 0.05 µg/L	Fallah *et al.*, 2015
Macedonia	AFM_1	408.1 ng/L	Dimitrieska-Stojković *et al.*, 2016
Pakistan	AFM_1	>2610 ng/L	Aslam *et al.*, 2016
Argentina	AFM_1	293 ng/L	Michlig *et al.*, 2016
Bosnia and Herzegovina	AFM_1	60 ng/L	Bilandžić *et al.*, 2016
Italy	AFM_1	52 ng/L	De Roma *et al.*, 2017
Tanzania	AFM_1	0.627 ng/mL	Karczmarczyk *et al.*, 2017
Malaysia	AFM_1	144 ng/L	Shuib *et al.*, 2017
Kosovo	AFM_1	83 ng/L	Camaj *et al.*, 2018
El Salvador	AFM_1	Approximately 100 ng/L	Peña-Rodas *et al.*, 2018
Turkey	AFM_1	78.69 ng/L	Eker *et al.*, 2019
Ethiopia	AFM_1	207 ng/L	Zakaria *et al.*, 2019
Kenya	AFM_1	4563 ng/L	Kuboka *et al.*, 2019
Brazil	AFM_1	45.18 ng/L	Venâncio *et al.*, 2019
Ecuador	AFM_1	0.0774 µg/kg	Puga-Torres *et al.*, 2020
Spain	AFM_1	0.009–1.36 µg/kg	Rodríguez-Blanco *et al.*, 2020
India	AFM_1	1116 ng/L	Sharma *et al.*, 2020
Morocco	AFM_1	4.46 ± 14.09 ng/L	Mannani *et al.*, 2021
Malawi	AFM_1	0.551 µg/L	Njombwa *et al.*, 2021
Spain	AFM_1 AFB_1	12.6 ng/kg 0.61 µg/kg	Bervis *et al.*, 2021

AFM_1: Aflatoxin M_1, AFB_1: Aflatoxin B_1, OA: Ochratoxin A, ZEA: Zearalenone, α-ZEA: α-zearalenone.

limits. Also, AFM_1 in China and India, and cadmium in Poland and Spain, were higher than permissible limits. This information confirms the importance of decontamination in milk and dairy products.

There are different methods for the decontamination of dairy products, such as physical, chemical (reverse osmosis, ion exchange, freeze concentration, and evaporation) (Patterson and Minear, 2013), and biological methods (using different biomaterials such as bacteria and yeasts biomass, plants, and seaweeds) (Abdelmotilib *et al.*, 2018; Hashim and Chu, 2004; Hayat *et al.*, 2017; Satyapal *et al.*, 2016; Sulaymon *et al.*, 2013; Vishnoi *et al.*, 2014). Adsorption is one of the most important decontamination strategies in dairy products (Giovati *et al.*, 2015; Massoud *et al.*, 2019; Milanowski *et al.*, 2017; Porova *et al.*, 2014). There are different biosorbents, such as "algae, plants, yeasts, fungi, and bacteria," for the bioremoval of toxins and metals in fermented dairy products (e.g., kefir, kumis, yogurt, and doogh). Probiotic bacteria can also be used for this purpose. Fermented dairy products are very popular, and they have a perfect taste (El Sheikha *et al.*, 2018; Yerlikaya, 2014). Probiotics can reduce contamination (heavy metals and aflatoxins) in fermented dairy products (Zoghi *et al.*, 2014). They are widely used for bioremoval of toxins (Massoud *et al.*, 2018; Zoghi *et al.*, 2017, 2019) as well as heavy metals (arsenic, mercury, lead, and cadmium) (Hadiani *et al.*, 2018, 2019; Khosravi-Darani *et al.*, 2019), heterocyclic aromatic amines (Khosravi-Darani *et al.*, 2019; Sarlak, 2020), and even pesticides (Wochner *et al.*, 2018).

In this article, reports about the influence of adding starters and probiotics into the formulation of dairy products on the bioremoval of contaminations such as toxins and heavy metals are reviewed.

Starters and Probiotics in Dairy Products

Food fermentation by microorganisms is one of the most economic and widely practiced methods for improving

texture, flavor, and functionality, and also for enhancing the shelf life of food products (Ray *et al.*, 2014; Salque *et al.*, 2013). The fermentation process can be carried out with starter cultures to certify consistency in commercial products by using familiar microorganisms with favorable traits, such as a high amount of acidification via the manufacture of lactic acid and/or the sprinkling of secondary metabolites in the product matrix (Ryan *et al.*, 2015). Different starters have been used for producing various dairy products all around the world. Some of these products and their starters are mentioned in Table 3.

Table 3. Some fermented dairy products and related starters.

Fermented dairy products	Country/Region of origin	Starters	Reference
Acidophilus milk	—	*Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium bifidum, Lactobacillus bulgaricus, Streptococcus thermophilus*	Raftaniamiri *et al.*, 2010
Buttermilk	Egypt and Ethiopia	(cultured buttermilk) Lactic acid bacteria (e.g., *Lactococcus*, *Lactobacillus*, *Streptococcus*, and *Leuconostocs*)	El Sheikha and Montet, 2014; Kumar *et al.*, 2015
Cheese	—	(cheddar cheese) Lactic acid bacteria starter culture *(Lactococcus lactis ssp. lactis, Lactococcus lactis ssp. cremoris, and Streptococcus salivarius spp. thermophilus*)	Ferreira and Viljoen, 2003
Matzoon	Armenia	Lactic acid bacteria	Macori and Cotter, 2018
Leben	Arab World	(Leben from camel milk) *Lactococcus lactis, Lactobacillus pentosus, Lactobacillus plantarum, Lactobacillus brevis,* and *Pediococcus pentosaceus*	Fguiri *et al.*, 2013
Kishk	Arab World	Freeze-dried yogurt starter culture	Tamime *et al.*, 2000
Kumis	Central Asia Turkic countries Central Asia	*Lactococcus lactis subsp. lactis, Streptococcus thermophilus, Lactobacillus delbrueckii subsp. bulgaricus, Lactobacillus helveticus, Lactobacillus casei subsp. Pseudoplantarum, and Lactobacillus brevis* *Kluyveromyces marxianus var. lactis, Saccharomyces cerevisiae, Candida inconspicua, and Candida maris*	Simova *et al.*, 2002
Ymer	Denmark	*Streptococcus lactis, Streptococcus diacetilaclis., Streptococcus cremoris, and Leuconostoc citrovorum*	Poulsen, 1970
Kefir	Estonia, Hungary, Greece, Latvia, Romania, Slovakia, Bosnia and Herzegovina	*Lactobacilli* *Lactococcus* Acetic acid bacteria and yeast	Garrote *et al.*, 2001
Dahi	India	*Lactobacillus case* or *Lactobacillus acidophilus*	Yadav *et al.*, 2005
Mishti doi	India	*Streptococcus salivarius ssp. Thermophiles, Lactobacillus acidophilus, Lactobacillus delbrueckii ssp. bulgaricus, Lactobacillus acidophilus, Lactococcus lactis ssp. lactis, Saccharomyces cerevisiae*	Gupta *et al.*, 2000
Matsoni	Georgia	*Lactobacillus Streptococcus, Kluyveromyces marxianus, Candida famata, Saccharomyces cerevisiae, Lodderomyces elongisporus, Kluyveromyces lactis*	Bokulich *et al.*, 2015
Wara	Africa	*Lactobacillus sp., Leuconostoc sp., Pediococuus sp., Lactococcus sp., yeasts*	El Sheikha and Montet, 2014
Biruni	Sudan	*Lactic acid bacteria*	El Sheikha and Montet, 2014
Mish	Sudan and Egypt	*Lactic acid bacteria*	El Sheikha and Montet, 2014
Rob	Sudan	*Lactic acid bacteria*	El Sheikha and Montet, 2014
Doogh	Iran	*Lactobacillus acidophilus, Lactobacillus rhamnosus, Lactobacillus casei, Bifidobacterium lactis*	Sarlak *et al.*, 2017
Yogurt	Serbia	*Streptococcus thermophilus and Lactobacillus bulgaricus*	Elsanhoty *et al.*, 2014
Clabber	United States	Starters like Kefir	Dyomina *et al.*, 2017

FAO (2001) defined probiotics as, "viable microorganisms, that while ingested in sufficient amounts, exert health benefits on the host (FAO/WHO, 2001)." The main beneficial effects of probiotics on human health include mucosal immunity support, decreasing lactose intolerance, preventing respiratory infections or diarrheas, feasible hypocholesterolemia effects, prevention of intestinal pathogens, inhibition of colon cancer or inflammatory bowel disease (Sanders *et al.*, 2014; Yu *et al.*, 2015).

The application of microorganisms, especially probiotics, recently has been investigated for their potential to heavy metals and aflatoxins reduction (Zoghi *et al.*, 2014). Most species known as probiotic bacteria are *Bifidobacterium* (*B.*), *Lactobacillus* (*L.*), *Bacillus*, and yeast *Saccharomyces* (*S.*) *cerevisiae*, and some strains of *Escherichia (E.) coli*. A practical taxonomy of nonpathogenic, fermentative, and nontoxigenic probiotic bacteria is lactic acid bacteria (LAB), which are used widely in food industries (Zoghi *et al.*, 2017). LAB usually have gram-positive cell walls, and peptidoglycan is their main cell wall structural component; teichoic acid, lipoteichoic acid, some neutral polysaccharides, and a proteinous S-layer are their minor components (Zoghi *et al.*, 2014).

Toxins' Bioremoval in Milk and Dairy Products

In recent decades, several scientific studies have been done regarding decontamination in dairy products, especially the biological decontamination method. Some of these researches are mentioned in Table 4.

El Khoury *et al*. (2011) investigated the application of LAB including *L. bulgaricus* and *Streptococcus thermophiles* on the reduction of AFM_1. They showed that using LAB is a potential method to decrease AFM_1 with the higher efficiency of *L. bulgaricus* compared to *Streptococcus thermophiles*. They also mentioned that the level of AFM_1, which is bound by LAB, enhanced with increasing the time of inoculation (El Khoury *et al.*, 2011). The binding ability of yogurt cultures was different. It is suggested that the difference in the binding ability of LAB is attributed to the difference in their cell-wall structure (Sarimehmetoğlu and Küplülü, 2004).

In addition to LAB, using *S. cerevisiae* is considered as an effective way for microbial detoxification (Karazhiyan et al., 2016). A systematic review by Campagnollo *et al*. (2020) focused on parameters influencing the binding process of AFM_1 by yeast. The overall binding level of yeast was reported as 52.05%, in which the lowest binding capacity was related to the yeast extract peptone and the highest binding was associated with the ruminal fluid. Also, different factors, including temperature, yeast, pH, and the type of aflatoxin, have been mentioned as the major parameters in the process of decontamination (Campagnollo *et al.*, 2020). Moreover, the effect of different treated *S. cerevisiae*, including heat, acid, and ultrasound treated, on the binding with AFM_1 was assessed by Karazhiyan *et al*. (2016). Among all treated yeasts, acid treatment had the most positive impact on yeast cells for improving their binding ability to aflatoxins which can be attributed to the release of monomers from polysaccharides under acidic conditions and their further changes into aldehydes after breaking down of glycosides linkages. After acid treatment, heat-treated yeasts showed the highest binding ability due to protein denaturation and Maillard reaction product formation, which caused an increase in the permeability of cell walls. Comparison between viable and unviable yeasts (heat, acid, and ultrasound treated) exhibited higher efficiency of unviable cells, which indicates that such treatments increase the binding capacity of yeasts (Karazhiyan *et al.*, 2016).

In a study performed by Taheur *et al*. (2017), a novel strategy for the reduction of mycotoxins using kefir grains was examined. The results showed that kefir microorganism grains could adsorb 82 to 100% of AFB_1, zearalenone, and ochratoxin A after cultivation in milk. The main strains that were able to adsorb mycotoxins were *L. kefiri*, *Kazachstania servazzii*, and *Acetobacter syzygii*. The *L. kefiri* KFLM3 was found to be the most active strain with an adsorption rate of 80 to 100% of the mycotoxins, and *K. servazzii* KFGY7 was found to retain higher mycotoxin than others after the desorption experiments. As a result, kefir consumption can assist in diminishing gastrointestinal absorption of mycotoxins and their toxic effects (Taheur *et al.*, 2017).

Heavy Metals' Bioremoval in Milk and Dairy Products

In Table 5, investigations regarding heavy metal bioremoval in milk and dairy products are illustrated.

In two different studies by Massoud *et al.* (2019, 2020a), application of *S. cerevisiae* to reduce the concentrations of lead and cadmium in milk was examined. The optimization process was also performed considering three factors including contact time, concentrations of biomass, and initial content of heavy metals (Massoud *et al.*, 2019, 2020a). Generally, the rate of removal of heavy metals increased with an increase in the biomass, contact time, and concentration of heavy metals. They concluded that optimized conditions for lead removal were obtained after 4 days (at the end of storage time) with the content of 22×10^8 CFU/mL of yeast and 70 μg/L of lead in milk (Massoud *et al.*, 2019). Similarly, the optimized process for cadmium bioremoval was achieved after 4 days with 80 μg/L of cadmium and 30×10^8 CFU/mL of *S. cerevisiae*

Table 4. Aflatoxin decontamination in milk and dairy products.

Product	Microorganism	Removal w/w%	Contaminant	Conditions	Reference
Milk	*Lactobacillus rhamnosus* (milk whey medium)	46.0%	AFB_1	Optimal condition: 60 min in pH 3.0	Bovo *et al.*, 2014
Milk	Kefir starters 1. *L. acidophilus*, *Bifidobacterium*, & *Streptococcus thermophiles* (*thermophilic lactic* culture) 2. *Lactococcus lactis subsp. cremoris, Leuconostoc*, *Lactococcus lactis subsp. lactis biovar diacetylactis, Lactococcus lactis, subsp. lactis,* 3. *Debaryomyces hansenii, Kluyveromyces marxianus subsp. marxianus.*, yeast pool, Lactic acid bacteria pool	Full kefir starters 11.67–34.66% Yeast pool 65.33–68.89% LAB pool 65%	AFM_1	Toxin Concentration: 150, 200, and 250 ng/L Temperature: 4 °C Time: 7 days	Kamyar and Movassaghghazani, 2017
Milk	*Lactobacillus helveticus*	85%	AFM_1	Time: 60 min	Ismail *et al.*, 2017
Milk	*Saccharomyces cerevisiae*	81.3%	AFM_1	Time: 48 h	Foroughi *et al.*, 2018
Yogurt	A: *S. thermophilus* & *L. bulgaricus* B: 50% *S. thermophilus* & *L. bulgaricus* 50% *L. planetarum* C: 50% *S. thermophilus and L. bulgaricus,* 50% *L. acidophilus*	Treatment B: Highest reduction 31.5–87.8%	AFM_1	Temperature: 5°C, Storage time: 1, 3, 5, and 7 days	Elsanhoty *et al.*, 2014
Yoghurt	*Lactobacillus acidophilus*	90%	AFM_1	10^8 CFU/ mL, Initial concentration of AFM_1:0.1, 0.5, 0.75 µg/L	Adibpour *et al.*, 2016
Yoghurt	*Saccharomyces cerevisiae*	76.46%	AFM_1	Aflatoxin M_1: 100, 500, and 750 g/ M_1, in 1, 7, 14, and 21 days, yeast treatments: heat, acid, and ultrasound	Karazhiyan *et al.*, 2016
Yoghurt	*Lactobacillus* plantarum, *Bifidobacterium animalis*, *Bifidobacterium* bifidum	Yogurt starters and *B. bifidum*, *B. animalis* (60.8%), Yogurt starters and *L. plantarum*, *B. Bifidum* 55.1%)	AFM_1	Storage time: 1 or 10 days	Sevim *et al.*, 2019
Yogurt	*L. plantarum, B. animalis,* & *B. bifidum, L. plantarum*	49–60%	AFM_1	Contact time: 4 h Temperature: 42°C	Sevim *et al.*, 2019
Kefir	*Lactobacillus casei* & kefir starter	88.17%	AFM_1	Aflatoxin M_1 500 pg, Kefir starters 2, 4, 6, 8, 10%, *L. casei:* 0.1, 0.3, 0.5, 0.7, 0.9 % in 48 h	Sani *et al.*, 2014
Kefir	Kefir-grains	96.8%	AFG_1	Toxin concentration 5, 10, 15, 20, 25 ng/g, Kefir grain:5, 10, 20, 10, 25%, in 0, 2, 4, 6, 8 h, at 20, 30, 40, 50, 60°C	Ansari *et al.*, 2015

(*continues*)

Table 4. Continued

Product	Microorganism	Removal w/w%	Contaminant	Conditions	Reference
Kefir	Kefir grains: *Lactobacillus kefiri, Kazachstania servazzii, Acetobacter syzygii*	82–100%	AFB_1, ZEA, OA	1 µg/Ml mycotoxin, Kefir grains 10% w/v in 24 at 25°C	Taheur *et al.*, 2017
UHT skim milk	Lactic acid bacteria (*Lactobacillus rhamnosus, Lactobacillus delbrueckii spp. Bulgaricus, Bifidobacterium lactis*), *Saccharomyces cerevisiae*	LAB pool (30 min): 11.5 ±2.3% LAB (60 min): 11.7 ± 4.4%, Saccharomyces: (30 min), 90.3 ± 0.3%, Saccharomyces: 60 min, 92.7 ± 0.7%	AFM_1	0.5 ng AFM_1 mL^{-1}, LAB pool: 10^{10} cells mL^{-1} Yeast: 10^9 cells mL^{-1} Contact time: 30 min or 60 min	Corassin *et al.*, 2013
Fermented milk drink	*Lactobacillus casei Shirota*	AFB_1-lys reduction: 82.37%	Serum AFB_1-lysine adduct	4-week intervention phases, (A): probiotic drinks 2 twice a day (B): placebo for 6, 8, or 10 weeks	Redzwan *et al.*, 2016
Doogh	*Lactobacillus acidophilus, Lactobacillus rhamnosus, Lactobacillus casei, , Bifidobacterium lactis*	Day 28, *Lactobacillus acidophilus*: 98.8 ± 1.3%	AFM_1	0.500 ppb toxin, 1,14, or 28 days at 5 °C, *L. acidophilus* 9 log cfu/mL	Sarlak *et al.*, 2017
Ergo fermented milk	*L. plantarum Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus casei* subsp. *casei, Lactobacillus helveticus, Streptococcus faecalis, Streptococcus thermophiles, Leuconostoc mesenteroides,* subsp. *cremoris*	57.33% 54.04%	AFM_1	Time: 1–5 days Temperature: 25°C	Shigute and Washe, 2018

AFM_1: Aflatoxin M_1, AFB_1: Aflatoxin B_1, OA: Ochratoxin A, ZEA: Zearalenone, AFG_1: Aflatoxin G_1.

Table 5. Heavy metals decontamination in milk and dairy products.

Product	Microorganism	Contaminant	Removal% W/W	Conditions	Reference
Milk	*Saccharomyces cerevisiae*	Pb	70%	Opt. at 22×10^8 CFU inoculation of yeast, Lead content 70 µg/l	Massoud *et al.*, 2019
Kefir	*Lactococcus lactis, Kluyveromyces marxianus,* co-culture	Ni, Cu, Cd, Pb, Fe	81.53%, 73.45%, 79.48%, 68.53%, 58.17%	Time: 10 days	Cherni *et al.*, 2020
Milk	*Saccharomyces cerevisiae*	Cd	70%	Cadmium content in milk 80 µg/L, 30×10^8 CFU *Saccharomyces cerevisiae,* storage time the 4th day,	Masoud *et al.*, 2020
Milk	*Lactobacillus acidophilus*	Pb Cd	80% 75%	1×10^{12} CFU of *L. acidophilus*, in 4 days with the initial pollution of 100 µg/L.	Massoud *et al.*, 2020b
Milk	*Saccharomyces cerevisiae*	Hg	70%	Contact time: 30 days, initial concentration of Hg: 80 µg/L and biomass dosage 22×10^8 CFU	Massoud *et al.*, 2021

Lead: Pb, Nickel: Ni, Copper: Cu, Cadmium: Cd, Iron: Fe, Mercury: Hg.

(Massoud *et al.*, 2020a). Therefore, they have introduced applying *S. cerevisiae* as a novel and useful technology for the bioremoval of heavy metals from foodstuff (Massoud *et al.*, 2019, 2020a)

Different treatments, such as caustic, ethanol, acidic, and heat, can enhance the biosorption of heavy metals by microorganisms. In a study by Yekta Göksungur *et al.* (2005), "potential of baker's yeast in bioremoval of cadmium and lead with 3 pretreatments (caustic, heat and ethanol)" was examined. Ethanol-treated yeast strains could remove the most content of metals and it can be explained by improving the availability of yeast binding sites and maybe enhancing the metals accessibility (Göksungur *et al.*, 2005).

Mechanisms of Bioremoval and Stability of Complexes (Probiotics/Starters-heavy Metal/Toxin)

AFM_1 and other toxins are accumulated in milk and dairy products because they are able to bind to milk protein components such as casein (Dyomina *et al.*, 2017; Granados-Chinchilla, 2016; Sarlak *et al.*, 2017). Therefore, numerous investigations have been focused on the removal of toxins using microorganisms, such as LAB (Dyomina *et al.*, 2017; Sarlak *et al.*, 2017).

Although the mechanism of bioremoval of toxins and heavy metals by LAB was not well known until now, it is proposed that toxins are highly linked by cell wall components of microorganisms and are not metabolically degraded (Zoghi *et al.*, 2014). Yeast and LAB are used widely to reduce toxins and metal ions. As both viable and dead cells are capable of adsorbing toxins, it is sensible to conclude that the removal of toxins is by adhesion to the components of microorganism's cell wall relative to covalent binding, as reviewed by Shetty *et al.* (2006) (Shetty and Jespersen, 2006). It is indicated that mannan components of the *S. cerevisiae* cell wall play an important role in toxin binding (Devegowda *et al.*, 1996). Generally, the cell wall proteins of *S. cerevisiae* are bound to β-1,3-glucans by covalent linkage by β-1,6-glucan chains (Shetty and Jespersen, 2006). Apart from this, the major part of the LAB cell is made up of peptidoglycan, which contains teichoic and lipoteichoic acids. Also, a proteinous S-layer and neutral polysaccharides as components of the LAB cell wall have been recognized and reviewed by Lahtinen *et al.* (2004).

A study by Yiannikouris *et al.* (2004) indicated the interactions between zearalenone and β-D-glucans, in which β-1,3 D-glucan chains constitute a stable helical link with zearalenone and stabilized by β-1,6 D-glucan chains (Yiannikouris *et al.*, 2004). In order to investigate the mechanism of binding of aflatoxins to *L. rhamnosus* it is indicated that carbohydrates in the cell wall are predominantly responsible for binding to aflatoxins. In samples treated by urea, it is shown that hydrophobic interactions play a significant role in binding, and treatment by NaCl and $CaCl_2$ showed that electrostatic interactions played a minor role (Haskard *et al.*, 2000).

Also, it is stated that AFM_1 is bound to LAB cell wall components by weak noncovalent interactions. The difference in the binding ability among different microorganisms is attributed to the cell wall and cell envelope structures (El Khoury *et al.*, 2011). Similarly, Turbic *et al.* (2002) mentioned that the different binding ability of LAB highly depended on the strain of the microorganisms (Turbic *et al.*, 2002).

Another study associated with the mechanism of biosorption illustrated that nonviable cells, including heat and acid-treated cells, produced complexes with higher stability, which means better access of groups in treated cells rather than viable ones. This phenomenon emphasizes that the viability of cells is not an important factor for the binding ability of cells (Haskard *et al.*, 2001). Furthermore, it is shown that acids might be capable of breaking amine binding in peptides and proteins, which leads to the production of peptides and even amino acids, and consequently, more accessible aflatoxin binding sites will be available (El-Nezami *et al.*, 2002). Similarly, it is noted that hydrophobic interactions are highly expected in LAB, which is treated by acid because acid treatment leads to denaturation of proteins and enhanced hydrophobic binding sites (Haskard *et al.*, 2000).

Moreover, the mechanisms of bioremoval could be influenced by various factors including types of microorganisms or even the status of biomass (living or nonliving microorganism), chemical properties of toxic materials, and environmental factors, such as temperature as well as pH (Javanbakht *et al.*, 2014).

For more illustration, Javanbakht *et al.* (2014) investigated the mechanism of removal of heavy metals by microorganisms. They suggested that two different types of pathways are involved in biosorption, which depends on cell metabolism and is divided into metabolism-dependent and metabolism-independent groups. The first pathway only occurs in viable cells through the transformation of metals across the cell wall. The second mechanism is involved in the physicochemical interaction between metals and functional groups of cell surface such as physical adsorption and ion exchange without depending on the cell metabolisms (Javanbakht *et al.*, 2014).

To investigate the stability of complexes, Haskard *et al.* (2001) evaluated the stability of 12 complexes between

LAB and AFB_1 considering both viable and nonviable cells and concluded that 71% of AFB_1 remained bound, indicating the high stability of the complexes. Also, they showed that nonviable cells retained a higher amount of AFB_1, as mentioned above (Haskard *et al.*, 2001). Based on their results, the stability of complexes depends upon three factors including strain, treatment type, and environmental conditions. Fazeli *et al.* (2009) conducted a study to investigate the effect of strains, including *L. casei*, *L. plantarum*, and *L. fermentum*, on the reduction of AFB_1 and concluded that all the strains were able to remove AFB_1, although *L. casei* was found to be a stronger binder of AFB_1 rather than other bacteria (Fazeli *et al.*, 2009).

A Study by Zoghi *et al.* (2020) showed that adsorption of patulin by LAB can be reversible in simulated gastrointestinal conditions. The reversibility of binding between LAB and patulin can be explained by the sense of noncovalent electrostatic bonds (Van der Waals and hydrogen bonds) (Zoghi *et al.*, 2020). Similarly, in another study, the adsorption of AFB_1, zearalenone, and ochratoxin A by kefir grains in simulated gastrointestinal pH was reversible. In pH 3, further amounts of toxins were released (Taheur *et al.*, 2017). Moreover, reduction of AFB_1 from a gastrointestinal model by several cells, including *L. rhamnosus, L. plantarum,* and *L. acidophilus*, were examined by Motameny *et al.* (2012), and they concluded that *L. plantarum* was the most active cell (Motameny *et al.*, 2012).

Conclusions

Aflatoxins and heavy metals frequently contaminate milk and dairy products at different levels. In the food industry, controlling aflatoxin and heavy metal levels in dairy products is a challenge for researchers. According to the recent studies summarized in this review, it is revealed that using different microorganisms (such as probiotics) in different dairy products could result in the removal of toxins and heavy metals by creating bonds between contaminants and these microorganisms. Using the starters in fermented dairy products can be helpful in the decontamination of toxins and heavy metals. According to this review, *L. bulgaricus*, Kefir grains, *L. acidophilus*, and *L. rhamnosus* could be useful for decreasing AFM_1 and other toxins in milk and dairy products. Also, for decontamination of heavy metals, kefir grains had the best ability for the bioremoval of different metals.

Future directions

More investigations are needed regarding the stability of binding between probiotics and toxins/heavy metals in *in vivo* and *in vitro* conditions. Also, more experiments should be done for finding optimum conditions for special starters in special dairy products for better decontamination.

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RM was involved in writing and original draft preparation; AZ was responsible for writing, review, and editing; KKD was concerned with conceptualization and supervision; FM was involved in writing and editing; and SJ, RM, and YR were responsible for review and editing.

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The prevalence and risk assessment of aflatoxin in sesame-based products

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PAPER

Abstract

The contamination of aflatoxins (AFs) in 120 samples of sesame seeds, tahini, and tahini halva collected from Iran's market were evaluated. The exposed risk due to ingestion of aflatoxin B_1 (AFB_1) via their consumption was estimated with the aid of the Monte Carlo simulation (MCS). The highest prevalence of AF (55%) was associated with sesame seed samples, followed by tahini (45%) and tahini halva (32.5%). The AFB_1 concentration in sesame seeds, tahini, and tahini halva was in the ranges of 0.21–12.35, 0.23–5.81, and 0.27–3.56 µg/kg, respectively. The concentration of the total aflatoxin (TAF) in 7 (17.5%), 8 (20%), and 2 (5%) samples of sesame seeds, tahini, and tahini halva, respectively, was below the limit of European regulations (4 µg/kg), while the levels of AFB_1 in 10 (25%), 7 (17.5%), and 6 (15%) samples of sesame seeds, tahini, and tahini halva, respectively, were higher than the European regulations (2 µg/kg). As the percentile 50 and 95 of margin of exposure (MOE) with AFB_1 for sesame seed, tahini, and tahini halva was more than 10,000, it could conclude the intake of aflatoxin through the consumption of mentioned products did pose a not remarkable cancer risk for adults.

Keywords: mycotoxin; contamination; risk assessment; traditional products; sesame based

Introduction

Today, humans pay considerable attention to food safety and contaminants (Milicevic *et al.*, 2021; Rapa *et al.*, 2021; Xinyu *et al.*, 2020). Mycotoxins produced by fungi are among the most important food contaminants and have a negative impact on public health, food safety, and the national economy of many countries, especially developing countries (Batrinou *et al.*, 2020; Grumi *et al.*, 2020). The most critical factors in food contamination with mycotoxins are moisture, intrinsic properties and nutrients, long shelf life and pH, and high-water activity (Wang *et al.*, 2018). Aflatoxins (AFs) are produced by *Aspergillus* fungi, especially *A. flavus* and *A. parasitics* and rarely by *A. nomius* (De Souza *et al.*, 2021; Heshmati *et al.*, 2019). They pose carcinogenic, mutagenic, immunosuppressive, and teratogenic consequences. The most common AFs are aflatoxin B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), and G_2 (AFG_2) (Heshmati *et al.*, 2017). AFB_1 is the most carcinogenic type for humans and animals (Mokhtarian *et al.*, 2020). The International Agency for Research on Cancer (IARC) classified AFB_1 as a group 1 carcinogen (Elzupir *et al.*, 2010).

Aflatoxins production may occur during harvesting, transportation, storage, or on the farm. AFs are very stable chemical compounds resistant to heat and food processes (Cui *et al.*, 2020). They can contaminate various foods, including cereal, dairy products, oilseeds,

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spices, and nuts (Javanmardi *et al.*, 2020; Khaneghah *et al.*, 2018; Mozaffari Nejad *et al.*, 2020).

Due to the high toxicity of AFs, exposure to these contaminations could threaten human health. The Committee on Food Additives of Joint FAO/WHO (JECFA) recommends that the presence of mycotoxins in meals be minimized to reduce the potential risk (Di Sanzo *et al.*, 2018). Therefore, in order to control AFs in food products, some regulations were established in many countries; the measurement and monitoring of AFs in food products are crucial (Sebaei *et al.*, 2020).

With the scientific name of *Sesamum indicum* L., sesame seed belongs to the Pedaliaceae family and is one of the oldest and most momentous oilseeds globally (Lee *et al.*, 2020). It contains 58%–44% oil, 25%–25% protein, 13.5% carbohydrates, and 5% ash (Kollia *et al.*, 2016). It is also a significant source of dietary fiber and micronutrients such as minerals, including calcium, phosphate, iron, potassium, vitamins such as E, thiamine, and niacin, lignans tocopherols, and phytosterols (Elleuch *et al.*, 2011; Yao *et al.*, 2021). Sesame seeds have antioxidant, anti-inflammatory, anti-fungal, anti-viral, and natural antibacterial effects (Dravie *et al.*, 2020). Sesame seeds are consumed in different forms in the world (Namiki, 2007). It is widely used in the Iranian food industry as an ingredient in confectionery products, bread, and pastries. Therefore, ensuring the mycotoxicological quality of sesame is very important (Asadi *et al.*, 2011; Eghbaljoo-Gharehgheshlaghi *et al.*, 2020; Elleuch *et al.*, 2011; Kollia *et al.*, 2016).

Tahini is made by grinding and roasting sesame seeds known in the Middle East (Gholami *et al.*, 2020; Sebaei *et al.*, 2020). Tahini halva, also known as halva, helva, halawi, halawh, is produced by mixing tahini with sugar, citric or tartaric acid, and *Saponaria officinalis* root extract (Öğütcü *et al.*, 2017; Osaili *et al.*, 2018a; Var *et al.*, 2007). Consumption of tahini halva has increased due to its excellent nutritional value and health properties in other countries, including the United States and European countries, Iran, Turkey, Saudi Arabia, Iraq, Greece, Jordan, Bulgaria, and Bosnia and Herzegovina (Anilakumar *et al.*, 2010). Tahini halva consists of about 45% tahini, 45%–55% sugar, 2% ash, and 2.5% moisture and is consumed with bread in breakfast and dinner (Osaili *et al.*, 2018b).

When sesame seeds were stored improperly, they can be contaminated with mycotoxins, especially AFs (Anthony *et al.*, 2014). Therefore, the EU sets limits for AFB_1 and TAF in sesame and its products as 2 and 4 μg/kg, respectively. In contrast, the limit of these mycotoxins, according to the Iranian Institute of Standards and Industrial Research (ISIRI), was 5 and 15 μg /kg (INSO, 2020). The prevalence of AFs in sesame seeds and their products was demonstrated by previous studies (Anthony *et al.*, 2014; Apeh *et al.*, 2016; Asadi *et al.*, 2011; Esan *et al.*, 2020; Fapohunda *et al.*, 2018; Hosseininia *et al.*, 2014; Kollia *et al.*, 2016; Li *et al.*, 2009; Reddy *et al.*, 2011; Sabry *et al.*, 2016; Sebaei *et al.*, 2020; Sirhan *et al.*, 2014; Tabata, 2007; Torlak and Akan, 2013; Var *et al.*, 2007).

No studies have been performed to evaluate mycotoxin contamination in tahini and tahini halva samples available in Iran's market. Therefore, the current study aimed to determine the level of AFs in sesame seed, tahini, and tahini halva, and halva consumed in western Iran. Furthermore, the exposed risk due to ingestion of AFB_1 via their consumption was estimated with the aid of the Monte Carlo simulation (MCS).

Material and Methods

Materials

Acetonitrile, HNO_3 (65%), phosphate buffer solution (PBS), methanol, Potassium bromide (KBr), were purchased from Merck (Darmstadt, Germany). The AF standards were purchased from Sigma-Aldrich (St. Louis, MO, USA)

Sample collection

Samples (n = 120; 40 fresh sesame seed, 40 tahini, and 40 tahini halva) were collected from the local market in Hamadan province, Iran, from April 2020 until August 2020. Samples were stored in a refrigerator (4°C) until analysis.

Chemical properties

Moisture content (%) of sesame seed, tahini, and tahini halva was determined by drying in an oven at 100°C. The protein and fat contents of samples were determined by the Kjeldahl method and Soxhlet extraction, respectively (Zebib *et al.*, 2015). Extracted fat acidity was measured by titration by sodium hydroxide (0.01°N) in the presence of phenolphthalein as an indicator.

Extract and cleanup of AF

The method applied for the extraction and cleanup of AF was similar to the previous one with slight modifications (Torlak and Akan, 2013). Samples were wholly powdered and mixed. Fifty grams of ground sample was weighed and transferred into a 250 mL flask. Then, 150 mL of a mixture of water and methanol (30:70; v/v) was added and placed

on a shaker and stirred for 10 min. Then, it was filtered through filter paper of Whatman No. 2. Twenty milliliters of filtered solutions were collected and transferred into a 100 mL flask, and 40 mL of distilled water was added and mixed for 5 min and filtered. For the cleanup of AFs, 15 mL of filtrate was transferred through the immunoaffinity column at a flow rate of 2–3 drops/s. Further, the column was washed three times with 10 mL distilled water at the same flow rate. The elution of AFs was performed by acetonitrile (1 mL). The eluate was gathered in a vial, and its volume was reached 2 mL with acetonitrile. Then, 100 µL of eluted AFs was injected into a high-performance liquid chromatography (HPLC) instrument.

Analysis of AFs

The quantification of AFs in sesame products was carried out by HPLC (Knauer-Germany), equipped with a Smartline Pump, fluorescence detector, reverse phased C18 (150 mm × 4.6 mm i.d and 5 µm particle size). In the fluorescence detector, an excitation wavelength of 333 nm and emission wavelength of 430 nm was applied for AFs determination. The mobile phase was water/methanol/acetonitrile (6:102:94, v/v/v) and contained 100 mg KBr and 100 µL HNO_3. Then, it is diverted into HPLC in the isocratic method with a flow rate of 0.8 mL/min. The column temperature of HPLC was maintained at 40°C.

The validation of AFs analysis method

The linearity, accuracy, repeatability, limit of detection (LOD), and limit of quantification (LOQ) were determined (Heshmati *et al.*, 2017).

Risk assessment

For risk assessment of AFB_1 intake through sesames seed, tahini, and tahini halva, the probabilistic approach was considered and estimated daily intake (EDI). The following equations are used to calculate the margin of exposure (MOE):

EDI (ng/kg bw/day) = the concentration of AFB_1 × average daily consumption (kg)/average body weight (kg)

Per capita consumption of sesame in Iran is 3 g/day (Eghbaljoo-Gharehgheshlaghi *et al.*, 2020). In this study, 70 kg is considered as the mean body weight for an adult in Iran.

MOE = benchmark dose lower confidence limit 10% ($BMDL_{10}$)/EDI

where, $BMDL_{10}$ is the lowest dose that is 95% certain to cause no more than 10% cancer prevalence. The EFSA Panel on Contaminants in the Food Chain (CONTAM) suggested 400 ng/kg BW/day for $BMDL_{10}$ reference value (Chain *et al.*, 2020). A MOE of 10,000 or larger has little concern for public health, while a MOE of less than 10,000 shows a potential danger for consumers (Heshmati *et al.*, 2017). EDI and MOE were estimated by MCS using Crystal ball software (version 11.1.2.3 Oracle).

Statistical analysis

For statistical analysis, the SPSS software version 20 (IBM, PASW Statistics, USA) was applied. The mean and standard deviation of AFs levels in different samples were determined. One sample T-test was used to compare the mean of AFB_1 and TAF with the allowable limit. The significant level was considered $P < 0.05$. One-way ANOVA and Tukey's test were applied to determine the significant difference of moisture, fat, protein content, and extracted fat acidity levels of these samples.

Result and Discussion

The chemical properties of samples

The moisture, fat, protein, and extracted fat acidity amount of sesame seed, tahini, and tahini halva samples are shown in Table 1. Sesame seed had a higher moisture, fat, and protein value than tahini and tahini halva samples.

Method validation

The LOD of AFs for sesame seeds, tahini, and tahini Halva samples ranged from 0.04 to 0.08, 0.05 to 0.09, and 0.07 to 0.08 µg/kg, respectively while LOQ for them ranged from 0.13 to 0.25, 0.18 to 0.31, and 0.21 to 0.27 µg/kg, respectively (Table 2). Moreover, the recovery range for AFs of sesame seeds, tahini, and tahini halva samples was 82.15–96.23, 77.36–95.63, and 83.65–98.65, respectively (Table 3). The determination coefficients ($R^2 > 0.992$) of the

Table 1. Chemical proprieties of sesame and related products.

Parameters	Sesame seed	Tahini	Tahini halva
Moisture (%)	6.87 ± 0.38[a]	1.15 ± 0.07[c]	2.5 ± 0.06[b]
Fat (%)	52.31 ± 2.89[a]	49.13 ± 2.12[b]	28.45 ± 2.32[c]
Protein (%)	22.34 ± 1.87[a]	20.67 ± 1.76[b]	9.87 ± 0.75[c]
Extracted fat acidity (%, in oleic acid)	0.82 ± 0.09[a]	0.90 ± 0.10[a]	0.76 ± 0.07[a]

The different superscript letters within each row indicated significant differences ($P < 0.05$).

regression equations showed acceptable linearity, and good recovery was obtained for spike samples and was similar to values reported in previous studies (Heshmati *et al.*, 2017). The findings obtained during method validation were conformed with accepted criteria (AOAC International, 2002).

The prevalence of AFs in sesame seeds

In this study, 40 samples of sesame seeds were analyzed for AFs. Sesame seeds had the highest prevalence (55%) of total AFs among the three analyzed samples. The detection rates of AFs were higher in sesame seed samples than in the tahini and tahini halva. AFB_1, AFB_2, AFG_1, and AFG_2 were detected in 19 (47.5%), 5 (12.5%), 6 (15%), and 5 (12.5%) of 40 sesame seeds samples, respectively (Table 4). AFB_1 was the most abundant AF, and its level varied from 0.21 to 12.35 µg/kg. In addition, 10 and 6 samples contained AFB_1 more than the accepted limit according to European (2 µg/kg) and Iranian standard (5 µg/kg), respectively, and the total AFs content of samples was lower than the permitted level in Iran (15 mg/kg).

There are several reports of AF contamination in sesame seeds (Table 5). The different levels of total AFs and AFB_1 have been reported in previous similar studies. For example, Anthony *et al.* (2014) reported that 8 (26.67%) out of 30 sesame samples studied in Nigeria were contaminated with AFB_1 at levels above the limit of European regulations (Anthony *et al.*, 2014). Esan *et al.* (2020) surveyed the contamination of total AFs in sesame seeds of Nigeria. They demonstrated that the positive samples contaminated with total AFs ranged from 0.29 to 88.5 µg/kg (Esan *et al.*, 2020). In another study, Kollia *et al.* (2016) investigated 30 samples of sesame seeds from the Greek market. They observed that the amount of AFB_1 in eight samples exceeded the limit of European regulations (Kollia *et al.*, 2016). In an investigation, among 28 samples of sesame products from Egypt by Sabry *et al.* (2016), a higher prevalence of AFB_1 and AFG_1 than other mycotoxins in the range of 60%–100% and 33.33%–100%, respectively, were reported. The mean range of AFB_1 and AFG_1 in different provinces was 18.63–66.79 and 14.88–51.47 g/kg, respectively (Sabry *et al.*, 2016).

Table 2. Validated parameters for aflatoxin analysis in sesame and related products.

Aflatoxin type	Equation of calibration curve	Range of linearity (ng/mL)	R^2	Sesame seed		Tahini		Tahini halva	
				LOD	LOQ	LOD	LOQ	LOD	LOQ
AFB_1	Y = 13562.23X + 456.03	0.15–25	0.998	0.06	0.21	0.07	0.23	0.08	0.27
AFB_2	Y =10623.15X – 4856.45	0.25–20	0.997	0.08	0.25	0.09	0.31	0.07	0.22
AFG_1	Y = 20354.67X + 120.09	0.16–20	0.996	0.06	0.22	0.05	0.18	0.08	0.26
AFG_2	Y = 14600.18X – 809.53	0.12–25	0.992	0.04	0.13	0.06	0.19	0.07	0.21

LOD and LOQ in µg/kg.
AF: aflatoxin; LOD: limit of detection; LOQ: limit of quantification.

Table 3. Recovery of aflatoxin from sesame and related products.

Aflatoxin type	Spiked level (µg/kg)	Recovery ± RSD (%)		
		Sesame seed	Tahini	Tahini halva
AFB_1	0.5	82.15 ± 4.51	84.56 ± 10.36	87.65 ± 12.74
	2	87.63 ± 8.56	88.32 ± 13.08	90.23 ± 15.32
	5	85.25 ± 3.65	87.32 ± 8.98	85.32 ± 14.56
AFB_2	0.5	90.23 ± 10.23	79.65 ± 4.56	94.36 ± 12.02
	1.5	92.35 ± 12.32	82.36 ± 7.25	98.65 ± 4.08
	3	95.63 ± 15.36	77.36 ± 14.97	94.82 ± 6.23
AFG_1	0.5	89.69 ± 12.31	83.65 ± 4.56	88.63 ± 4.51
	1.5	92.34 ± 8.96	86.53 ± 45.23	87.56 ± 14.23
	3	94.23 ± 14.85	82.03 ± 12.78	83.65 ± 14.32
AFG_2	0.5	90.23 ± 11.57	90.23 ± 15.63	89.32 ± 4.36
	1.5	96.23 ± 8.69	95.63 ± 10.56	90.56 ± 8.69
	3	94.23 ± 10.23	92.53 ± 8.02	95.36 ± 10.26

AF: aflatoxin; RSD: Relative standard deviation

Table 4. The contamination status of aflatoxin of sesame and related products.

Aflatoxin type		Contamination status	Sesame seed	Tahini	Tahini halva
AFB_1	Contamination level (µg/kg)	No. of contaminated samples	19 (47.5)	14 (35)	11 (27.5)
		Mean ± SD (µg/kg)	1.67 ± 0.45	0.85 ± 0.24	0.55 ± 0.17
		0.15–2	9 (22.5)	7 (17.5)	5 (12.5)
		2–5	4 (10)	4 (10)	6 (15)
		>5	6 (15)	3 (7.5)	0
		Range (µg/kg)	0.21–12.35	0.23–5.81	0.27–3.56
AFB_2		No. of contaminated samples	5 (12.5)	3 (7.5)	4 (7.5)
		Mean ± SD (µg/kg)	0.13 ± 0.06	0.1 ± 0.06	0.07 ± 0.03
		Range (µg/kg)	0.25–1.62	0.31–1.75	0.22–0.92
AFG_1		No. of contaminated samples	6 (15)	4 (10)	2 (5)
		Mean ± SD (µg/kg)	0.10 ± 0.05	0.07 ± 0.04	0.04 ± 0.03
		Range (µg/kg)	0.22–1.41	0.18–1.32	0.26–1.02
AFG_2		No. of contaminated samples	5 (12.5)	5 (12.5)	4 (10)
		Mean ± SD (µg/kg)	0.05 ± 0.02	0.08 ± 0.04	0.06 ± 0.03
		Range (µg/kg)	0.13–0.85	0.19–1.02	0.21–0.74
TAF	Contamination level (µg/kg)	No. of contaminated samples	22 (55)	18 (45)	13 (32.5)
		Mean ± SD (µg/kg)	1.95 ± 0.48	1.10 ± 0.30	0.72 ± 0.22
		>4	15 (37.5)	14 (35)	11 (27.5)
		4–15	7 (17.5)	8 (20)	2 (5)
		>15	0	0	0

AF: aflatoxin.

The prevalence of AFs in tahini

A lower rate of AF contamination was observed in tahini than that in sesame seed. Eighteen (45%) of Tahini samples contained total AFs. The highest prevalence of AFs in tahini halva was AFB_1 (35%), followed by AFG_2 (12.5%), AFG_1 (10%), and AFB_2 (7.5%). It was observed that the AFB_1 value of seven samples was higher than the limit of European regulations (2 µg /kg) while only three samples had AFB_1 contamination higher than the recommended standard of Iran (5 µg/kg). Also, 18 samples contained total AF with an average value of 1.10 ± 0.30 µg/kg, among which the concentration of eight samples was higher than the limit of European regulations (4 µg/kg). It seems that washing and peeling of sesame seeds before tahini preparation could reduce the amount of AFs. Before tahini preparation, sesame seeds were roasted. The previous studies indicated that roasting operation could cause AFs degradation (Emadi *et al.*, 2021; Yazdanpanah *et al.*, 2005).

The prevalence of AFs in tahini was reported in other studies. For example, in a study performed by Sebaei *et al.* (2020) in Egypt, mean AFB_1 in 16 samples of branded tahini and 101 samples of local tahini reported 0.10 ± 0.24 µg/kg and 13 ± 19.3 µg/kg, respectively (Sebaei *et al.*, 2020). Li *et al.* (2009) reported that 19% and 32% of 100 of the sesame paste (Tahini) samples studied in China contained AFB_1 at levels higher than the Chinese regulation (5 µg/kg) and European regulations (Li *et al.*, 2009). In addition, Torlak and Akan (2013) studied AFs contamination in 104 samples of tahini from the Anatolia region of Turkey. The mean of AFB_1 and total AFs of samples was 0.93 ± 0.62 µg/kg and 1.17 ± 0.55 µg, respectively, which both were lower than the Turkish standard (AFB_1: 5 µg/kg and TAF: 10 µg/kg) and our study. These authors indicated that the roasting process applied in tahini production does not eliminate AFs (Torlak and Akan, 2013).

For the traditional production of tahini, sesame is roasted for 2 h at a temperature of 100°C to 150°C (Torlak and Akan, 2013). AFs are resistant to heat and are difficult to eliminate at insufficient temperatures. In general, AFs are eliminated at 237°C to 306°C. In foods heated, important parameters determining the reduction of AF include

Table 5. The contamination status of aflatoxin of sesame and related products.

Country	Sample type	No of samples	Positive *n* (%)	Method	Mycotoxin	Range (µg/kg)	Mean ± SD (µg/kg)	Reference
Iran	Sesame seeds	269	50%	HPLC	TAF		1.43 ± 4.38	Hosseininia *et al*. 2014
Iran	Sesame seeds	269	50%	HPLC	AFB_1		1.25 ± 3.7	Hosseininia *et al*. 2014
Egyptian	Tahini (Brand)	16		HPLC	AFB_1		0.10 ± 0.2	Sebaei *et al*. 2020
Egyptian	Tahini (Local)	101		HPLC	AFB_1		13 ± 19.3	Sebaei *et al*. 2020
China	Sesame paste	100	37 (37%)	LC	TAF	0.54–56.89	6.75	Li *et al*. 2009
China	Sesame paste	100	37 (37%)	LC	AFB_1	0.39–20.45	4.31	Li *et al*. 2009
Iran	Sesame seeds	182	33 (18.1%)	LC	AFB_1		1.62 ± 1.32	Asadi *et al*. 2011
Malaysian	Sesame seeds	8	7 (87.5%)	ELISA	AFB_1	0.5–1.82	0.9	Reddy *et al*. 2011
Nigeria	Sesame seeds	60	12	LCMS/MS	TAF	0.29–88.5	16.9	Esan *et al*. 2020
Nigeria	Sesame seeds	60	12	LCMS/MS	AFB_1	0.29–79.3	14.8	Esan *et al*. 2020
Nigeria	Sesame seeds	30	26.66	HPLC	AFB_1	14.71–140.90	69.72 **±** 41.68	Anthony *et al*. 2014
Turkey	Tahini	104	14.42	HPLC	AFB_1		0.93 ± 0.62	Torlak and Akan 2013
Turkey	Tahini	104	15.38	HPLC	TAF		1.17 ± 0.55	Torlak and Akan 2013
Turkey	Tahini Helva	34	0	TLC	AFB_1	<1	<1	Var *et al*. 2007
Egyptian	Sesame seeds	28	88.89	HPLC	AFB_1		33.66 ± 1.35	Sabry *et al*. 2016
FCT, Abuja, Nigeria	Sesame seeds	24	13	LCMS/MS	AFB_1		3.6	Fapohunda *et al*. 2018
Nigeria	Sesame seeds	46	23 (50%)	TLC	AFB_1	0.79–37.25		Apeh *et al*. 2016
Japan	Sesame seeds	47	5	HPTLC	AFB_1	0.6–2.4		Tabata 2007
Jordan	Sesame seeds	46	2	HPLC	TAF	100–1280		Sirhan *et al*. 2014
Greek	Sesame seeds	30	77.60%	HPLC	AFB_1		2.0 ng AFB1 g-1	Kollia *et al*. 2016

moisture content, heating temperature, and nutrient media. It has been reported that roasting dried wheat at 150°C for 30 min, Green coffee beans at 150°C to 180°C for 10 to 15 min, and Pistachio nuts at 150°C for 30 min will reduce AF levels to 50%, 42.2%–55/9%, and 63%, respectively (Pankaj *et al.*, 2018).

The prevalence of AFs in tahini halva

The results showed that tahini halva samples contained four types of AF, i.e., AFB_1, AFB_2, AFG_2, and AFG_2, with mean values of 0.55 ± 0.17, 0.07 ± 0.03, 0.04 ± 0.03, and 0.06 ± 0.03 µg/kg, respectively (Table 4). Except for AFB_2, the detection rate of AFB_1, AFG_2, and AFG_2 was lower in tahini halva samples than in the sesame seeds and tahini samples. The average total AFs of tahini halva samples was 0.72 ± 0.22 µg/kg. According to Iranian standards, the concentration of total AFs and AFB_1 was not higher than the allowable limit. In contrast, the total AFs level of two samples was higher than the limit of European regulations (4 µg/kg). Also, in six samples, the concentration of AFB_1 was higher than the limit of European regulations (2 µg/kg). The lower level of AFs in tahini halva could be related to the dilution effect of other products, including sugar, emulsifier, in the formulation. Few studies have been performed to evaluate AFs in tahini halva. Var *et al*. (2007) investigated AFB_1 contamination in 34 samples of helva in Turkey. No AFB_1 was found in helva samples. It seemed the discrepancies in AFs contamination prevalence in different studies could be due to differences in sampling geographical areas and storage conditions and the AFs measuring method.

Risk assessment of AFB1 intake

Findings of percentile 50 (as the median of the population) of EDI of AFB_1 calculated using the MCS approach showed the lowest (0.02 ng/kg bw/day) and highest (0.05 ng/kg bw/day) of EDI in sesame seed and tahini halva, respectively (Figure 1). The percentile 95 of EDI of AFB_1 through sesame seed, tahini, and tahini halva was 0.09, 0.05, and 0.03 ng/kg bw/day, respectively.

As shown in Figure 2, the percentile 95 of MOE with AFB_1 through sesame seed, tahini, and tahini halva was calculated as 25,485, 50,092, and 77,114, respectively. Because data

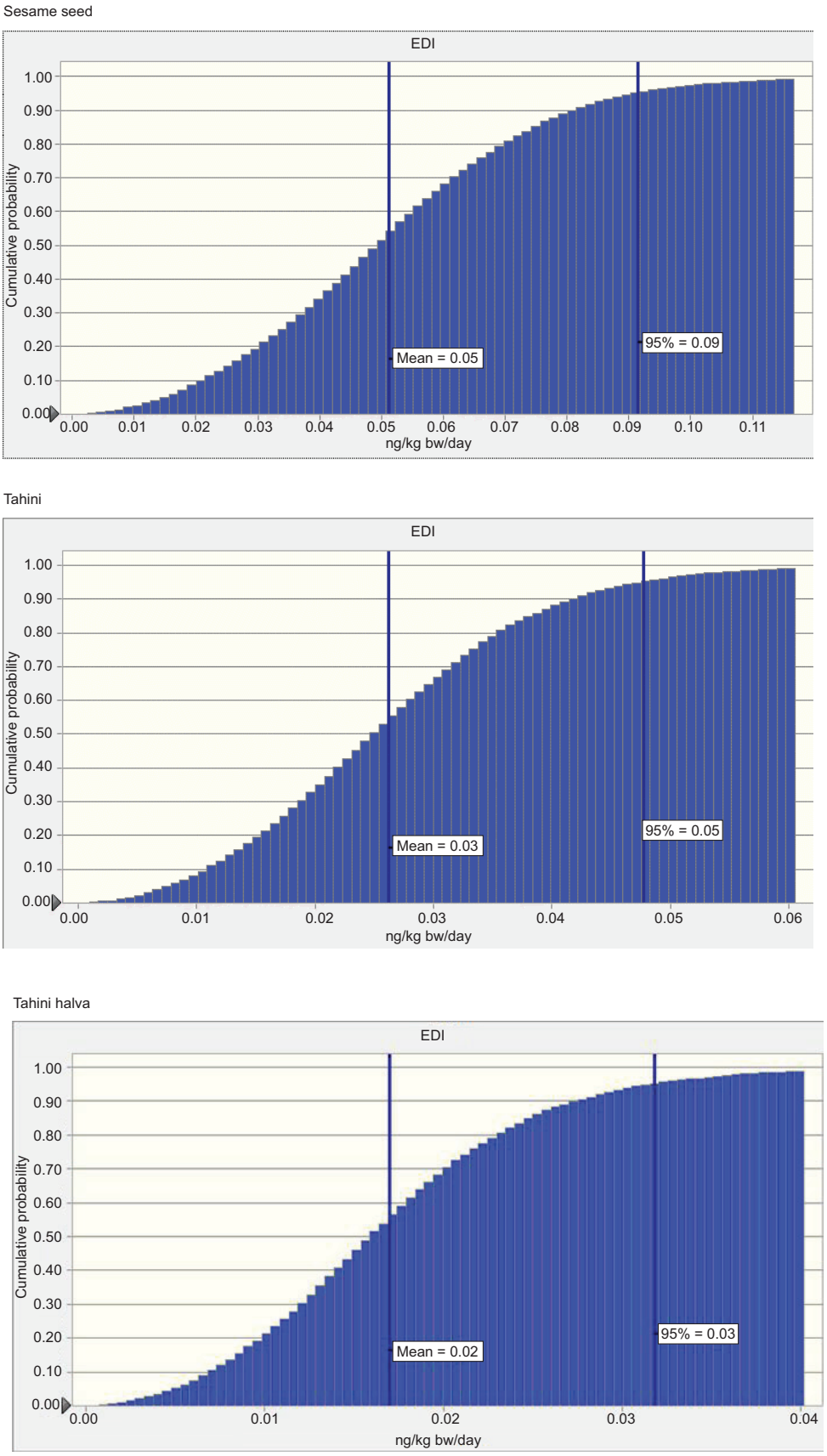

Figure 1. Cumulative probability plot of EDI of AFB1 through sesame seed, tahini, and tahini halva consumption.

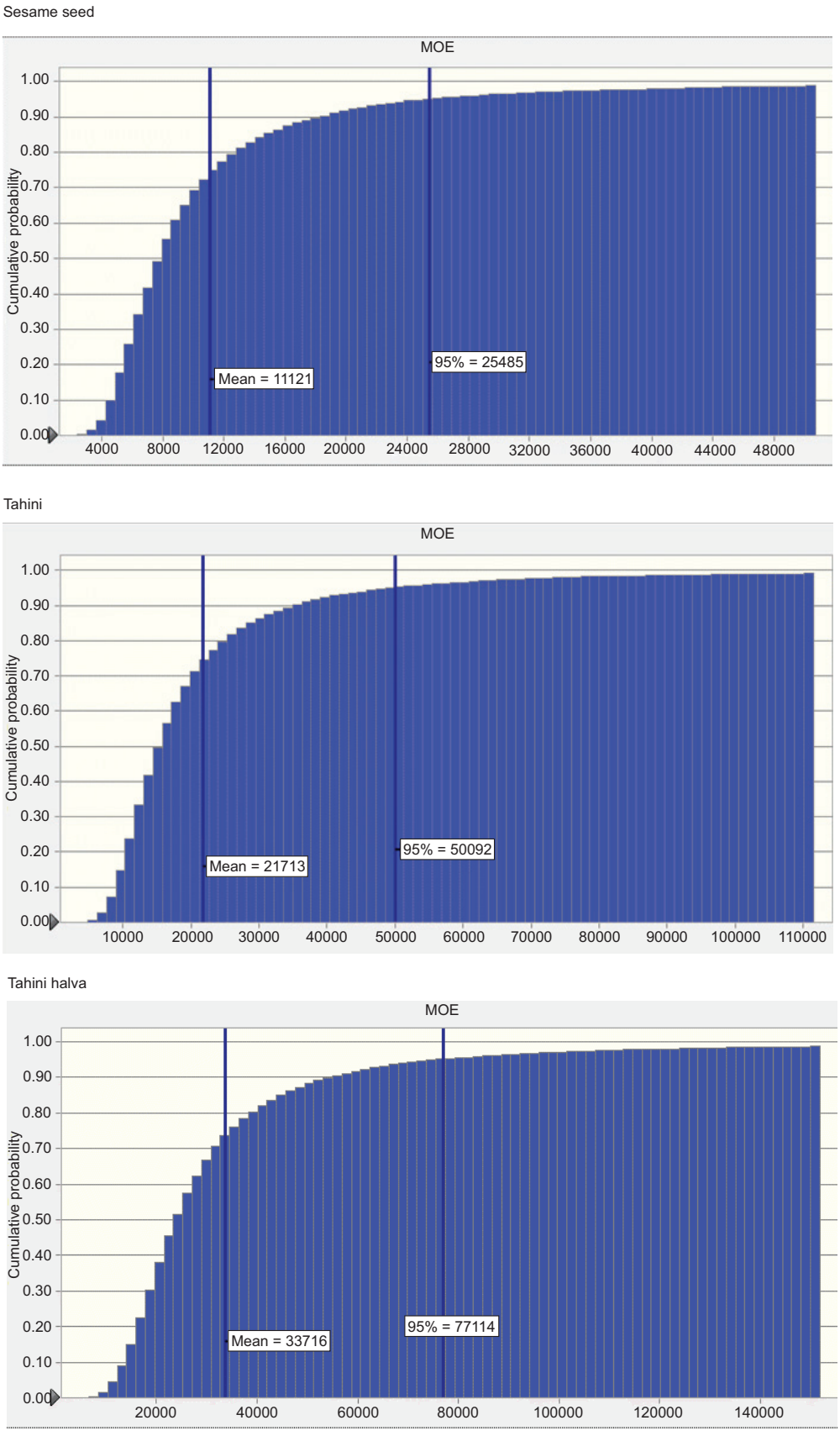

Figure 2. Cumulative probability plot of MOE of AFB_1 through sesame seed, tahini, and tahini halva consumption.

regarding the percentile 50 and 95 of MOE was more than 10,000 (Heshmati *et al.*, 2017), it could be concluded that AFB_1 intake through sesame seed, tahini, and tahini halva consumption has no remarkable cancer risk for adults.

Conclusions

The results of this study indicated the high prevalence of AFs in sesame seed (55%), tahini (45%), and tahini halva (32.5%) samples. In addition, the levels of TAF in 7 (17.5%), 8 (20%), and 2 (5%) samples of sesame seeds, tahini, and tahini halva exceeded the limit of European regulations (4 µg/kg), respectively. Furthermore, 10 (25%), 7 (17.5%), and 6 (15%) samples of sesame seeds, tahini, and tahini halva contained AFB_1 more than the limit of European regulations (2 µg/kg), respectively. However, risk assessment indicated that the intake of AF through the consumption of mentioned products had no remarkable cancer risk for adults.

Acknowledgments

The ethical and scientific committee of Hamadan University of Medical Science approved this study (ethical code: IR.UMSHA.REC.1400.177).

Conflict of interest

The authors declare that they have no conflict of interest.

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Aflatoxin M1 in traditional and industrial pasteurized milk samples from Tiran County, Isfahan Province: A probabilistic health risk assessment

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Abstract

In this study, the aflatoxin M1 (AFM1) concentration in traditional and industrial milk and risk assessment due to AFM1 exposure using the Monte Carlo simulations technique was investigated. The mean concentration of AFM1 in traditional and industrial milk samples was 53.00 ± 11.49 and 54.33 ± 12.22 ng/L, respectively, which was higher than European Union and Codex standards. Percentile 95% of hazard quotient (HQ) adults and children due to industrial ingestion milk was 1.056 and 4.956, and traditional milk was 1.031 and 5.116, respectively. Hazard quotient in all age consumers was higher than 1. Therefore, consumers are at a considerable health risk.

Keywords: aflatoxin M1; industrial and traditional; milk; risk assessment; seasonal variation

Introduction

The exposure of humans to various types of toxic chemical compounds (natural or artificial) may cause a wide range of human health problems, including genital diseases, mental disorders, dysfunction, especially in kidneys and liver, suppression and weakening the immune system, and a variety of cancers (Bahrami *et al.*, 2016; Khaneghah *et al.*, 2019; Khodaei *et al.*, 2020; Mir *et al.*, 2021; Mousavi Khaneghah *et al.*, 2021; Rahmani *et al.*, 2018; Rastegar *et al.*, 2017; Shahbazi *et al.*, 2015; Sipos *et al.*, 2021). Among them, the mycotoxins are the secondary metabolites produced by fungi, mainly the *Aspergillus*, *Fusarium*, *Penicillium*, and *Alternaria* genera, causing substantial concerns all over the world over the last few decades (Coppa *et al.*, 2021; de Souza *et al.*, 2021; Ertas *et al.*, 2011; Fakhri *et al.*, 2019; Mahmood Fashandi *et al.*, 2018; Roohi *et al.*, 2020). Mycotoxins belong to the *aflatoxins* group (around 300 different mycotoxins) and are produced by three filamentous fungi species called *Aspergillus parasiticus*, *Aspergillus flavus* rarely by *Aspergillus nominus* (Campagnollo *et al.*, 2016; Fakhri *et al.*, 2018; Heshmati *et al.*, 2019).

Aflatoxin B1 (AFB1) is the typical type and most toxic one among 18 aflatoxin types. Aflatoxin M1 (AFM1) is an AFB1 hydroxylated metabolite deformed by the enzymes of cytochrome P450 in the liver. This is secreted through the milk glands in lactating cows by AFB1, which is consumed through food (Marhamatizadeh and Goosheh, 2016; Škrbić *et al*., 2014); and about 0.5% to 6% of AFB1 is converted into AFM1. When AFB1 contaminated animal feeds with lactating animals, after 12 to 24 h, AFM1 could be detected and reached a maximum after 72 h when the feeding with the contaminated animal feeds is reached (Asi *et al*., 2012; Iqbal *et al*., 2014; Mousavi Khaneghah *et al*., 2018). After AFB1 intake, about 72 h, the AFM1 concentration was decreased to an undetectable level (Khaneghah *et al*., 2018). The ratio of swallowed AFB1 to the excreted AFM1 has been reported with various percentages in different sources; however, typically, it has been in the range of AFB1 averages 1%–2% that varies from day to day, type of animal, and type of milk (Bervis *et al*., 2021; Gonçalves *et al*., 2017; Heshmati *et al*., 2019).

Milk is a very nutritious food rich in micro and macronutrients, vital to human health evolution and human body growth. Therefore, the safety and hygiene of milk play a critical role in human health (Bahrami *et al*., 2016; Iqbal *et al*., 2015). Aflatoxin M1 in dairy products and milk is highly resistant to conventional milk processing methods, including pasteurization, ultra-high temperature (UHT), and other processing methods (De Roma *et al*., 2017; Khaneghah *et al*., 2021; Rahmani *et al*., 2018; Turkoglu and Keyvan, 2019). The pasteurization method can reduce the concentration of AFM1; however, it cannot eliminate this contaminant (Mahmood Fashandi *et al*., 2018; Naeimipour *et al*., 2018). Some adverse health effects such as carcinogenicity, teratogenicity, preventing RNA encoding and protein synthesis, brain damage, colon damage, lung, liver, kidney, mutation, immune suppression, and digestive disorders were associated with the consumption of contaminated milk by AFM1 (Fallah *et al*., 2016; Kaur *et al*., 2021; Pokharel *et al*., 2021).

In order to assess the overall prevalence of AFM1 in industrial and traditional milk, several studies have been carrying out. The study by de Souza *et al.* (2021) stated that the AFM1 concentration had been reduced in the contaminated milk, which was mainly carried out in traditional products. However, preventative approaches are recommended, and it is necessary to reduce the concentration of aflatoxin. Tajik *et al*. (2016) investigated the AFM1 concentration in the West-Azerbaijan in the pasteurized milk. Their study showed that 77.7% of the samples were contaminated with AFM1; and in the pasteurized milk, about 70% of them exceeded the standard value given by European Commission (50 ng/L). Makhdoumi *et al*. (2021) showed that the prevalence of AFM1 in conventional and industrial dairy products was due to the cold season (autumn and winter). They reported that AFM1 could be found in dairy products with an overall prevalence percentage of 63.53 and 54.05 based on the type of sample and production process, respectively. A study by Ansari *et al*. (2019) reported that the autumn and winter seasons had shown the highest concentration and contamination (above 50 ng/L). To control and prevent exposure to AFM1, more than 60 countries in the world have set the maximum permissible limit for AFM1 in dairy products. The Codex Alimentarius and European Union (EU) have determined the 50 ng/kg of AFM1 concentration as the maximum amount of AFM1 remaining in raw and warmed milk (de Souza *et al*., 2021; Khaneghah *et al*., 2021; Mannani *et al*., 2021). The FAO (Food and Agriculture Organization, the United Nations) has set the maximum allowable concentration of AFM1 to 500 ng/L (Cai *et al*., 2012; Cavallarin *et al*., 2014). Also, the Iranian Institute of Standards has declared the concentration of AFM1 to 100 ng/L in raw milk samples (Hashemi, 2016; INSO 5925; Khaneghahi Abyaneh *et al*., 2019).

The health risk assessment of AFM1 exposure due to dairy products and milk consumption is a valuable way to risk and evaluate the liver's developing cancer (Serraino *et al*., 2019; Tsakiris *et al*., 2013). The AFM1 risk assessment is defined as assessing the daily oral intake and hazard quotient (HQ). These data and studies can be used for assessing the cancer risk in toxicological studies to estimate the severity and probability of toxin contamination (Heshmati *et al*., 2017, 2019; Nabizadeh *et al*., 2018; Oteiza *et al*., 2017; Škrbić *et al*., 2015).

Some studies in Iran have been done for characterizing the concentration of AFM1 in various cities; however, the available evidence on the risk assessment of AFM1 exposure is limited in developing countries (e.g., Iran) (Bahrami *et al*., 2016; Mohajeri *et al*., 2013). In developing countries, milk and its products are produced with traditional and industrial methods, increasing the risk of exposure to AFM1. Moreover, traditional livestock, especially in rural areas, can exacerbate this risk (Bahrami *et al*., 2016). Traditional Iranian dairy products made from milk are native to some areas of Iran. Also, in consuming this type of milk, the usual industrial processes of milk preparation do not occur, and boiling milk for consumption is done by individuals in their home. In the present study, samples of traditional milk were cow's milk, which were purchased from local distributors in the same area (rural regions) that are manufactured in rural households under unacceptable hygiene conditions (Bahrami *et al*., 2016; de Souza *et al*., 2021; Kaur *et al*., 2021).

In this regard, the current investigation was undertaken to determine the AFM1 level in traditional and different industrial brands available in the markets of Tiran

County besides assessing the effects of seasonal changes on the AFM1 level in milk. Also, to conduct a probabilistic risk assessment of AFM1 exposure due to consumption of milk.

Materials and Methods

Sampling area

The present study was conducted in Tiran city, Isfahan province, Iran. The geographical location of the Tiran County is at 32°42′12.96″ east and 51°9′ 6.84″ north and 1640 m above the sea level (Figure 1). Tiran has arid climates, and the annual average rainfall is 116.9 mm (Jafari *et al.*, 2018). Based on the last census (Iran Statistical Center, 2016), the county population was 71,583, and per capita milk consumption in this province is 90 kg/person/year. Moreover, Isfahan province, with 1,250,000 tons of milk in 2018, has been the first raw milk producer in Iran.

Sampling and preparation

In this study, 156 traditional and industrial milk samples (60 traditional milk (*a*) and 96 industrial milk samples from 15 brands of *b-p*) were collected from 15 best-selling milk brands during autumn 2017 and winter 2018 (26 samples were taken in each month). Sampling was performed randomly from all locations of the milk supplier in the study area. Industrial pasteurized milk was randomly obtained from large and busy stores in the city, and traditional milk samples (unheated raw milk) were also obtained from traditional dairy stores in Tiran. The samples were transferred to the laboratory in pre-sterilized stainless steel containers (for traditional) and under dark environment conditions, away from smell and light at 1 to 5°C. Sterilized milk samples (industrial samples) and traditional samples were stored at 4°C. Samples preparation was conducted to determine AFM1 in samples of milk by using enzyme-linked immunosorbent assay (ELISA) method according to the instructions given in the manufacturer kit (R-Biopharm, Darmstadt, Germany).

Traditional and industrial milk (pasteurized)

Traditional milk means milk that is produced in villages and does not go through the industrial process. In the present study, the meaning of traditional milk is milk produced and marketed in traditional livestock farms in villages around Tiran County without any industrial process for its preparation. Pasteurized milk is the samples prepared in the dairy industry with mechanized equipment and methods.

Analysis of aflatoxin M1 by ELISA method

Based on the information recommended by the kit catalog, the limit of detection (LOD) was 5 ng/kg, and the analysis area was 50%–150%. The AFM1 cross-reactivity in these kits is 100%, and the probability of crossover reaction with aflatoxin G1, G2, B1, and B2 is zero. Validation of ELISA was carried out by determining recoveries, and the mean variation coefficient (CV) for milk samples spiked with different concentrations of AFM1. The mean recovery score in spiked milk samples (5, 10, 20, 40, and 80) was 99.96%,

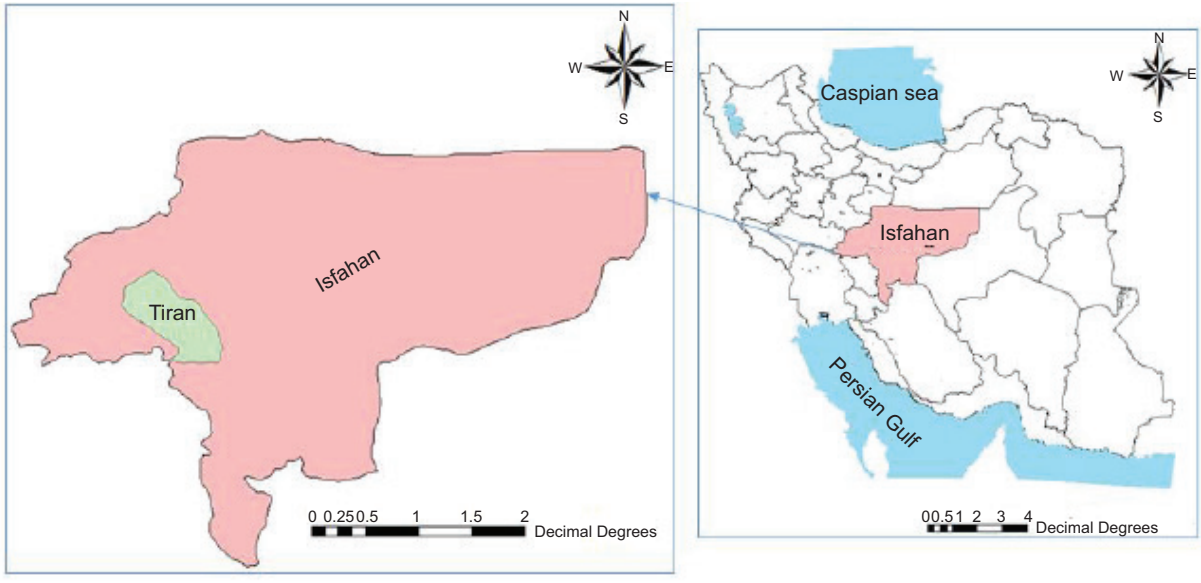

Figure 1. Location of Tiran County in Isfahan province and Iran (Jafari *et al.*, 2018).

with a CV of 1.2%. According to manufacturer's guideline, the recovery rate in spiked milk was 95%, with a CV of 15%. Also, the detection range of these kits is 50–80 ng/kg. AFM1 stock solution (50 mg/L) was obtained by solubilizing the AFM1 standard powder (Sigma Aldrich, Germany) in a chloroform/methanol solution with a volume ratio (v/v) of 19:81 and kept at a temperature of –20°C. Before analysis, by chloroform:methanol at a 1:1 v/v ratio, the stock solution to obtain different standard solution concentrations was diluted (Fallah, 2010; Maggira *et al*., 2021; Tajik *et al*., 2016). AFM1 analysis was carried out according to the instructions given in the kit. Notably, samples remained fully protected from light (prevention from AFM1 inactivation).

Health risk assessment

The health risk of AFM1 exposure in the milk samples was calculated according to the United States Environmental Protection Agency (USEPA, 2011; Hooshfar *et al*., 2020; Kaur *et al*., 2021). The daily exposure to AFM1 by milk was calculated as following:

$$EDI_{ing} = \frac{C_m \times IR_m \times EF \times ED}{BW \times AT} \quad (1)$$

where, *EDI* is the estimated daily intake per day by AFM1 consumed in milk (ng/kg/day). C_m denotes AFM1 concentration in milk (ng/L), and IR_m is the milk ingestion rate according to liter/day. Based on the approximate information available, the amount of milk consumption for people with high consumption was 222 g/day, the average consumption was 174 mL/day, and the minimum consumption was 74 mL/day. However, according to the previous study in Iran (March 2011–March 2012), Iranians approximately consumed 190 mL or less than one glass of milk/dairy products per day.) (Abyaneh *et al*., 2019; Home Society Economy Politics Sports Culture International Multimedia Tourism, 2019; Nejad *et al*., 2019; Pérez-Gregorio *et al*., 2011), *EF* is exposure frequency (day/year), *ED* is exposure duration (according to years), *BW* is body weight (kg), and *AT*, the averaging time (based on days).

The HQ was investigated to determine the AFM1 non-carcinogenic risk induced by milk intake. If the HQ is higher than 1, the risk of liver cancer for the consumer is more (Fakhri *et al*., 2018; Kuiper-Goodman, 1990; Nabizadeh *et al*., 2018). While HQ values less than 1 indicate that milk intake does not cause harmful effects for the consumers (Bahrami *et al*., 2016). The risk index was calculated according to the following equation:

$$HQ = \frac{EDI}{RfD} \quad (2)$$

where *RfD* is the reference dose of aflatoxin.

IR was 0.19 L/day, *EF* was 350 days, *ED* was 30 and 6 years for adults and children, respectively, and *BW* was 70 and 15 kg for adults and children, respectively. *AT* was equal to 10,950 and 2190 days for adults and children, respectively. The *RfD* was also 0.2 ng/kg per day (Xiong *et al*., 2021).

Monte Carlo simulation (MCS) method

The simulation of Monte Carlo was used to minimize uncertainties in the results. When the point values of a variable were used to assess the exposure risk of pollutants with a population, the probability of interference and error, and finally, the uncertainty in the result is obtained (Fakhri *et al*., 2018; Huang *et al*., 2017; Keramati *et al*., 2018, 2019). Crystal Ball software (version 11.1.1.1 Oracle, Inc. United States of America) was used to determine uncertainties with 5000 trails in the MCS. The percentile 95% of HQ was selected as a worse scenario for the health risk of consumers.

Statistical analysis

The Shapiro–Wilk normalization test was performed to verify the data normality. The difference between the concentration of AFM1 in the two groups of samples (traditional vs. industrial samples) and seasons was assessed using the Mann–Whitney test. Furthermore, the association between AFM1 concentrations and different brands was investigated by the Post-Hoc test. A P-value of <0.05 was considered as a significance level for all tests. All statistical analyses were carried out using $SPSS_{21}$ software.

Results and Discussion

AFM1 concentration

The AFM1 concentration in industrial and traditional samples in autumn and winter is shown in Figure 2. Also, the results of AFM1 concentration based on the different seasons and different types of milk are shown in Tables 1 and 2. While the AFM1 mean concentration in samples of traditional milk produced in autumn and winter is 54.33 ± 11.65 and 53.00 ± 12.22 (ng/L), the overall AFM1 mean concentration in traditional and industrial milk samples is 53.00 ± 11.49 and 54.33 ± 11.22 ng/L, respectively (see the Table 1). In the industrial milk brands, b and h with a mean concentration of 60.00 (12.64) ng/L had the highest concentration of AFM1, and l and o brands with a mean concentration of 48.33 (13.29) ng/L had the lowest AFM1 concentration. The total mean of AFM1 concentration in samples of milk was 53.33 (11.87) ng/L, which was more than the recommended standard

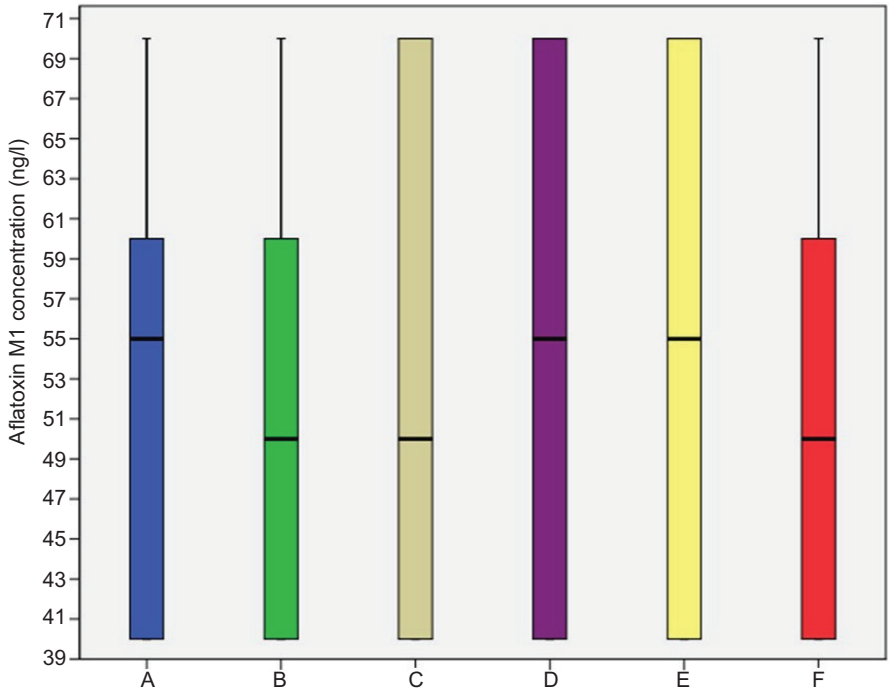

Figure 2. AFM1 concentrations in traditional and industrial milk samples based on the season (A: AFM1 in traditional milk, autumn; B: AFM1 in traditional milk, winter; C: Industrial milk, autumn; D: Industrial milk, winter; E: total AFM1 concentration in industrial samples; F: total AFM1 concentration in traditional samples).

Table 1. AFM1 concentration in milk samples based on the brand.

Brand of milk	Number	AFM1 concentration according to number of samples			Mean concentration ± SD
		Lower than 50 ng/L	Standard or 50 ng/L	Higher than 50 ng/L	
Traditional (a)	60	19	13	18	53.66 ± 11.49
b	6	1	1	4	60 ± 12.64
c	12	6	2	4	50 ± 12.06
d	6	3	0	3	51.66 ± 13.29
e	6	3	0	3	55 ± 16.43
f	6	1	3	2	56.66 ± 10.32
g	6	2	2	2	51.66 ± 11.69
h	6	1	1	4	60 ± 12.64
i	6	3	0	3	53.33 ± 15.05
j	6	2	2	2	51.66 ± 11.69
k	6	1	3	2	55 ± 12.24
l	6	4	0	2	48.33 ± 13.29
m	6	2	2	2	51.66 ± 11.69
n	6	2	2	2	50.00 ± 8.94
o	6	4	0	2	48.33 ± 13.29
p	6	1	2	3	57.14 ± 11.12
Total	156	55	31	70	53.66 ± 11.49

Table 2. AFM1 concentration range in the traditional and industrial pasteurized milk samples based on the season.

Season/month	Under 50 ng/L (%)	50 ng/L (%)	Higher than 50 ng/L (%)	Total samples
Autumn	7 (26.92)	5 (19.23)	14 (65.38)	26
Autumn	10 (38.46)	7 (26.92)	9 (34.61)	26
Autumn	10 (38.46)	2 (7.69)	14 (65.38)	26
Winter	10 (38.46)	4 (15.38)	12 (46.15)	26
Winter	10 (38.46)	5 (19.23)	11 (42.30)	26
Winter	8 (30.76)	8 (30.76)	10 (38.46)	26
Total	55 (35.25)	31 (19.87)	70 (44.87)	156

of AFM1 in the EU and the Codex exceeding the permissible limit (50 ng/L) (Bahrami *et al*., 2016). Fifty-five cases from 156 samples had contamination lower than 50 ng/L, 31 samples were 50 ng/L, which complied with the European Union's recommended standards of 50 ng/L (Fallah, 2010; Fallah *et al*., 2016), 70 cases had a high contamination rate of AFM1 that were higher than the recommended standard of 50 ng/L. Overall, the lowest AFM1 concentration was 40 and the highest was 70 ng/L. However, this concentration of AFM1 was lower than the Iranian standard limit (100 ng/L) (Khaneghahi Abyaneh *et al*., 2019). In another study, the mean concentration of AFM1 in the pasteurized milk was 76.2 ng/L (Tajik *et al*., 2016), which is higher than the results of our study. One of the reasons for the higher level of AFM1 in West-Azerbaijan may be due to geographical conditions, more contamination of animal feed with AFB1. So, control and monitoring of milk and local health conditions are crucial. AFM1 concentration in traditional milk, surveyed by Omeiza Gabriel Kehinde *et al*. (2021), that AFM1 concentration in the traditional products was the highest concentration compared to industrial products. There was a significant difference between the type of feed, type of dairy herds, and holding capacity of the dairy herds (Kehinde *et al*., 2021). Xiong *et al*. (2021) showed the health risk for child group 2–4 years old that consume contaminated milk with AFM1. About 5.3% of the samples were above the 50 ng/L limits, and the AFM1 level in UHT milk was lower than pasteurized milk.

Comparing the AFM1 concentration in traditional milk and industrial samples were carried out (it is notable, the traditional milk was purchased from rural areas around Tiran county and was prepared as well as sold by individuals on private farms. Also, boiling and milk preparation was carried out at the buyer's house by themselves.). Moreover, comparing the AFM1 concentration in the traditional and industrial samples with the EU, the Codex limit, and European Union standards (50 ng/L) indicated that 44.9% of samples had a concentration higher than the maximum allowable level. In a study conducted by Xiong *et al*. (2018), the mean concentration of AFM1 100 ng/L was assigned as the AFM1 concentration in UHT and pasteurized samples of milk collected from China's market by using ELISA while about 1.8% of UHT milk samples and 59.5% of pasteurized milk were above the EU and Codex standards (Xiong *et al*., 2018). According to the results shown in Table 2, the AFM1 mean concentration in traditional and industrial milk samples in autumn was higher than in the winter season, which may be due to storage conditions and increased moisture in autumn, but P-value was not significant (Aydemir Atasever *et al*., 2021; Makhdoumi *et al*., 2021; Rahmani *et al*., 2018). In another investigation, the concentration of AFM1 in UHT milk was significantly lower in spring and winter than in summer and autumn (Heshmati and Milani, 2010). Similar results regarding the AFM1 levels in milk and UHT from Brazil and Serbia were reported as higher AFM1 concentrations in summer and autumn than spring and winter (de Oliveira *et al*., 2013; Tomašević *et al*., 2015). In another study, AFM1 concentration in raw milk of cattle, sheep, and goat samples collected among autumn and spring were investigated, while no significant correlation in AFM1 among seasons was noted (Bilandžić *et al*., 2017). In another study, 119 samples out of 125 powder milk UHT and pasteurized milk collected from Brazil were contaminated with AFM1 in the range of 10 to 200 ng/L and the mean concentration of 31 ng/L (Shundo *et al*., 2009).

No significant association regarding AFM1 concentration among different seasons was noted (P-value was 0.704 according to Mann–Whitney test). According to De Roma *et al*. (2017), seasonal changes were considered as an influential factor in the concentration of AFM1. Also, Bahrami *et al*. (2016) demonstrated that AFM1 levels were significantly higher in winter than their corresponding values in summer. Therefore, seasonal variation was considered as an effective parameter on the concentration of AFM1 (Akbar *et al*., 2019; Ansari *et al*., 2019).

Moreover, no significant difference in the AFM1 level was observed among the traditional and industrial samples (P-value was 0.563 according to the Mann–Whitney test). However, based on the findings of some similar studies conducted in Iran, the level of contamination in the traditional milk samples was notably higher than the collected samples from industrial ounces, which

were associated with the non-hygienic condition of livestock storage among a variety of livestock, including cattle, goats, and sheep (Bahrami *et al*., 2016; Fallah, 2010; Fallah *et al*., 2016; Mohammadi, 2011). Moreover, the variety in the type of measurement method, geographical location, weather, seasonal, feeding systems, food storage, and farmland and pasture techniques are among other possible reasons (Bahrami *et al*., 2016; Iqbal *et al*., 2015). In the present study, almost all samples were prepared from the exact geographical location and climate. Therefore, the high AFM1 in some samples could be correlated with the type of feeding, poor health conditions of forage, and livestock feed (Bilandžić *et al*., 2017; De Roma *et al*., 2017; Khaneghah *et al*., 2021; Visciano *et al*., 2015). However, according to the sampling season, the level of AFM1 was higher in winter than in autumn (not significant).

Health risk assessment

Percentile 95% of HQ in adults and children due to industrial ingestion milk was 1.056 and 4.956 and due to traditional milk was 1.031 and 5.116, respectively. The HQ value in both adult and children consumers was higher than 1. Therefore, consumers are at a considerable health risk (Figures 3 and 4). In a study by Bahrami *et al*. (2015), the health risk assessment of AFM1 was assessed by consuming traditional dairy products in the western part of Iran. In this study, the average body weight for each adult Iranian was considered to be 60 kg. The results showed that the HQ was higher than 1 for dairy products in winter (1.24 ng/kg body weight in the ELISA method). In summer, the HQ was less than 1 for both methods. In this study, it is noteworthy to assess health risk, and the amount of milk and dairy consumption per Iranian in 2013 was calculated to be equal to 70 kg (annual statistics of Iranian agriculture) (Bahrami *et al*., 2016).

In another study in Iran, the average body weight of Iranian adults was considered to be 70 kg, which can effectively calculate the results. In this study, the mean concentration of AFM1 in the traditional cheeses was 139.4 ± 2.4 ng/kg. The AFM1 concentration was not higher than 500 ng/kg as the maximum permissible limit. After performing a health risk assessment, the HQ was less than 1 (Shahbazi *et al*., 2017; Tomašević *et al*., 2015).

Assessment of the health risk of AFM1 in infant formula milk for infants with 0 to 6 months old, an average weight of 5.7 kg, and the consumption rate of infant formula milk equal 19.4 g/day showed that HQ was less than 1 (Hooshfar *et al*., 2020). The difference in results with our study was the difference in the amount of AFM1 concentration in milk samples, age, body weight, and milk consumption rate (Bahrami *et al*., 2016; Duarte *et al*., 2013; Kos *et al*., 2014; Milićević *et al*., 2017b; Serraino *et al*., 2019; Tsakiris *et al*., 2013).

The risk assessment of AFM1 in milk and dairy products in Lebanon measured AFM1 concentration in the raw milk, UHT, and pasteurized milk was 0.011–0.440, 0.015–7.350, and 0.013–0.219 μg/L, respectively. The health risk assessment for adults with an average body weight of 75.49 kg was at a safe level (HQ < 1). According to a study by Daou *et al*. (2020), the milk consumption rate for Lebanon was 113.7 g/person/day that was lower than the milk consumption rate in our study (0.19 L/day). This difference in the body weight and daily milk consumption rate can be one of the reasons for the low HQ value compared to our study.

The AFM1 concentration in all brands was safe regarding non-carcinogenic risk due to milk consumption in Iranian consumers, which agrees with one of the previously published reports (Hooshfar *et al*., 2020). However, the calculated HQ in summer and winter after measuring the AFM1 by ELISA and HPLC techniques were reported as 0.54 and 1.245, 0.88, and 1.45, respectively (Bahrami *et al*., 2016). In another similar investigation performed after AFM1 level assessment by ELISA method, HQ was higher than 1 (Milićević *et al*., 2017a), while the findings of both investigations, as mentioned earlier, demonstrated a potential risk of liver cancer for consumers. AFM1 was measured in dairy products from Turkey; and in cheese samples, the HQ of receiving AFM1 was more than 1 (Sakin *et al*., 2018). In a study by Rahmani *et al*. (2018), probabilistic health risk assessment of AFM1 in milk samples of the east region was carried out. In many Middle East countries, the HQ was higher than 1 for children at *a* considerable risk of cancer, unlike adults (Rahmani *et al*., 2018).

The risk of cancer and non-carcinogenic diseases in Brazilian children aged 0–5 years was assessed by exposure to AFM1 in samples of UHT milk, powder milk, and infant formula. The results of the study showed that the range of AFM1 was from 150 to 1020 ng/kg and all positive samples exceeded the limit set by the European Union, and the number of hepatocellular carcinoma cases associated with AFM1 exposure was higher than 0.001 cases per 100,000 people (Conteçotto *et al*., 2021). As in the present study, there was a risk of cancer for children. The study of Sharma *et al*. (2020) showed the presence of AFM1 in raw and pasteurized milk samples of India and it is necessary to pay attention to the amount of this mycotoxin. The study that evaluated cancer and non-carcinogenic risk in the infant with an age of below 6 months in Iran showed only one of the infant formula milk samples were contaminated with AFM1 and did not concern the health risk to consumers (Hooshfar *et al*., 2020). Therefore, attention to different methods for detoxification of dairy products, attention to the manner

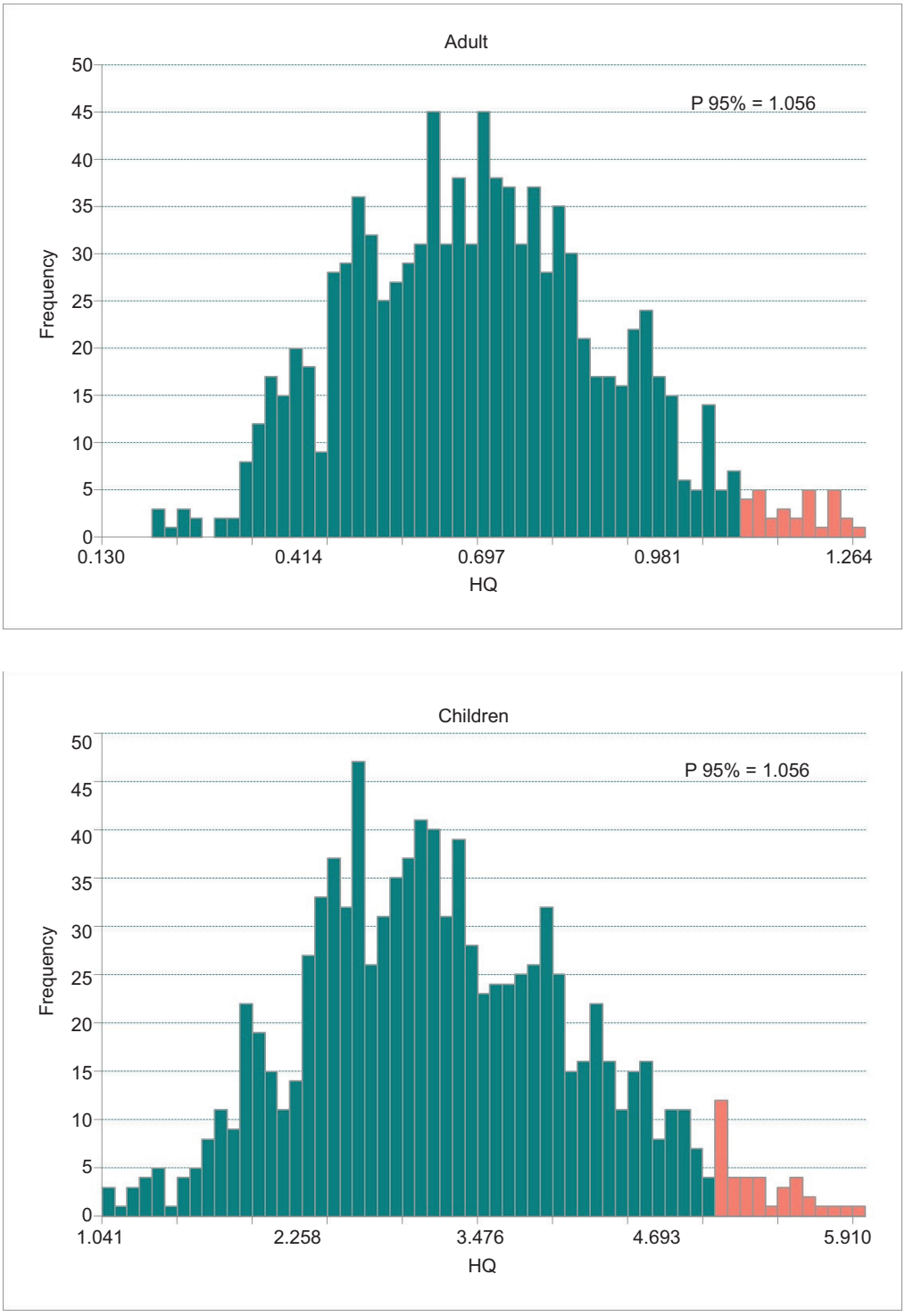

Figure 3. Percentile 95 of HQ in adults and children due to ingestion industrial pasteurized milk content of AFM1.

of storage, the lifetime of food intended for feeding livestock and poultry, as well as the need for stricter legal requirements and monitoring legal recommendations are recommended (Min *et al*., 2020).

A study that evaluated the prevalence of milk contamination with AFM1 worldwide found that most countries had moderate levels of AFM1 in milk in recent years, which were lower than EU levels. However, several countries, including Pakistan, India, and several countries around sub-Saharan Africa, have high levels of AFM1 that exceeded EU and US standards. Therefore, it has been speculated that high levels of AFM1 in milk can indicate high levels of AFB1 in animal feed, products

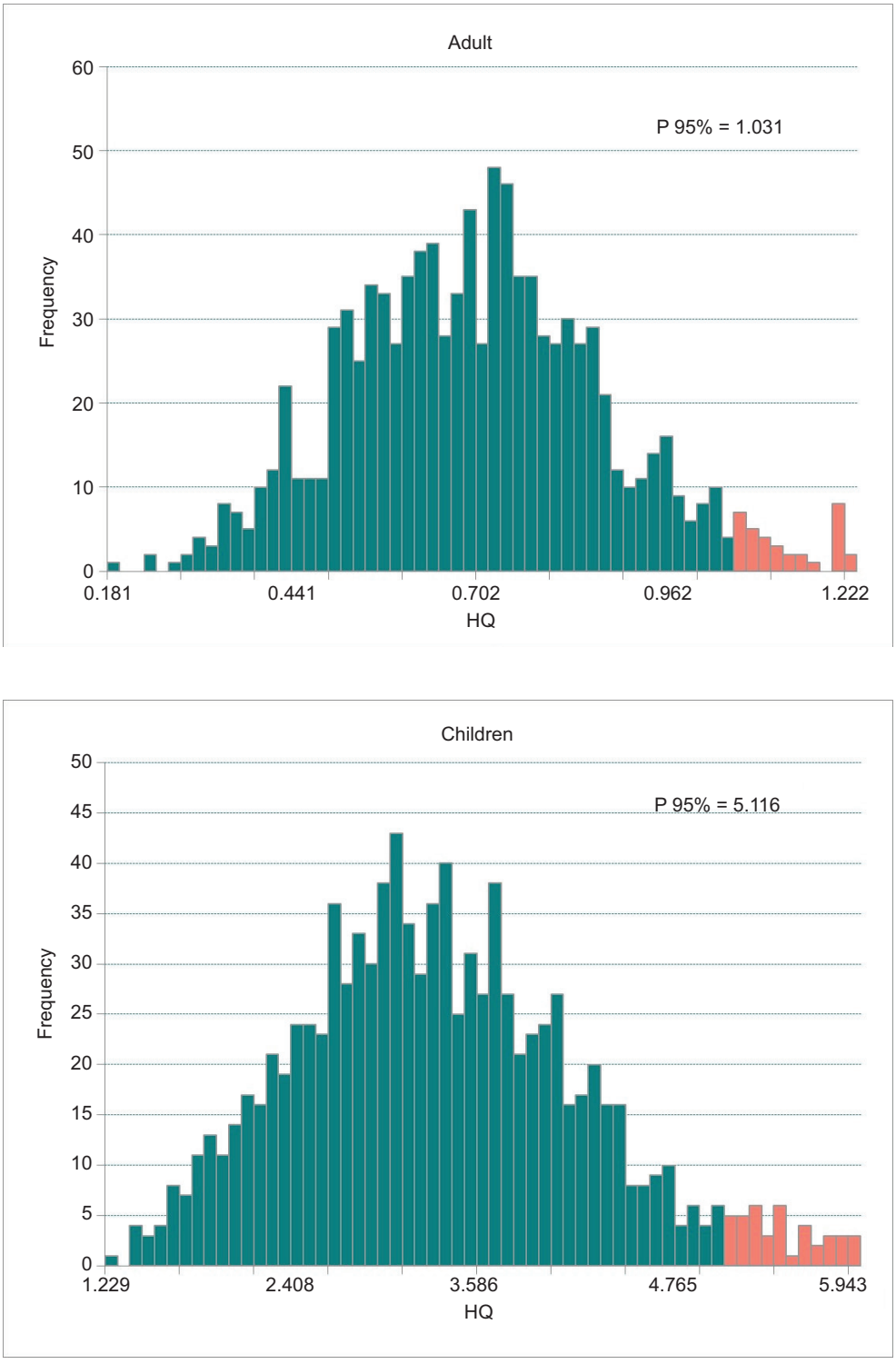

Figure 4. Percentile 95 of HQ in adults and children due to ingestion traditional milk content of AFM1.

such as corn used to make animals feed may have high levels of AFB1, which after consumption can lead to animal and humans health adverse effect (Turna and Wu, 2021). Contamination of goat's milk with AFM1 in Brazil showed that in all milk samples, the recommended levels of AFM1 in milk were lower than the permissible limit. However, the potential risk of liver cancer and EDI levels for AFM1 through goat's milk for 1-year-old children were higher than the tolerable daily intake and estimated values (de Matos *et al.*, 2021). The results of the study about the amount and risk of AFM1 in pasteurized milk products, ESL and UHT milk from China in summer and winter

showed that 5.3% of milk samples had a level of AFM1 more than the standard limit of 50 ng/kg, which is less contamination compared in these studies. Similar to the present study, AFM1 concentrations were higher in winter. Children aged 2–4 years had the highest risk of exposure to AFM1 in milk, which is due to the type of storage and observance of hygienic conditions, especially in winter is necessary (Xiong *et al*., 2021). AFM1 exposure through yogurt consumption and the risk of liver cancer were studied in Hamedan, Iran. Although it was found that a high percentage of yogurt samples in Iran were contaminated with AFM1 content, there was no particular concern about the risk to public health according to European standards (EC) and the Iranian Institute for Standards and Industrial Research (ISIRI) (Heshmati *et al*., 2020)

Conclusions

In this study, AFM1 concentration in milk samples [traditional (60 samples) and industrial milk (from 15 brands, *b–p*) was measured. Also, the AFM1 concentration in two seasons of the year (autumn and winter) was measured. According to the results, approximately 45% of traditional and industrial milk samples were contaminated with AFM1 more than the standard level (EU and Codex). Aflatoxin levels in autumn were higher than in winter, but P-value was not significant. Moreover, the amount of AFM1 in samples of traditional milk was higher than that of industrial. The non-carcinogenic risk of exposure to AFM1 showed that HQ was higher than 1 for adults and children, indicated that milk consumers in Tiran County are at considerable risk of developing liver cancer-related AFM1.

Recommendation

It is also important to enforce strict rules and more supervision in livestock farms and production centers. The most important idea to reduce the exposure of AFM1 is to prevent the production of AFB1 in the fields, and the best solution is to secure farms, industrial and traditional livestock farms, educate people about the dangers and appropriate ways to store livestock feeds, the type of food that is used, and improving their storage environment to reduce AFB1. A broader and more detailed examination of dairy products produced in various regions of Iran during different periods and four seasons is needed to achieve more accurate results.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Effect of natural antioxidants and vegetable fiber on quality properties of fish sausage produced from Silver carp (*Hypophthalmichthys molitrix*)

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Abstract

In this study, production of low-fat, nitrite-free sausage based on Silver carp surimi was performed. In order to replace oils, inulin fiber (IF) was used; natural preservatives such as grape pomace extract (GE) and nisin (NI) were used as nitrite replacements with modified atmosphere packaging (MAP: 70% CO_2 + 30% N_2). For this purpose; five treatments including, T1: control, T2: control + MAP, T3: IF 5% + MAP, T4: IF5% + GE0.5% + MAP, and T5: IF5% + GE0.5% + NI 0.5% + MAP, were prepared. The physicochemical and texture properties of sausage at the beginning of storage and chemicals (Peroxide value, pH, and color index) and microbial index (Total count and psychrotrophic bacteria) during 42 days' storage in the refrigerator (4 ± 1°C) were evaluated. The results showed that the use of natural preservatives had no effect on the physicochemical properties of the sausage ($P > 0.05$), but the FI had a positive effect on the texture characteristics (the firmness increased and elasticity decreased), increased moisture and ash, and reduced the fat content of sausages ($P < 0.05$). All in all, the best results among the treatment containing natural preservative was T5, in all microbial and chemical tests there was no significant difference with T1 ($P > 0.05$). All in all, a functional product with properties such as fiber and natural antioxidants, low fat and nitrite-free can be produced.

Keywords: fat replacement; inulin fiber; nitrite; plant extracts; nisin; fish sausage

Introduction

Seafood is a valuable source of protein for humans and plays an important role in healthy nutrition. Fish have a very high nutritional value and provide most of the essential nutrients for humans, but what makes fish a specifically important food item is the presence of fatty acids. The human body is not capable of producing some unsaturated fatty acids such as omega-3 (Kwasek *et al*., 2020).

Hypophthalmichthys molitrix is one of the warm water fish species that is widely cultivated in many countries of the world for its rapid growth, high feed conversion ratio, and good nutritional value (Fan *et al*., 2008; Stepien *et al*., 2019). Despite intermuscular bones and unpleasant odor (mud), this fish, like other fish, contain high-quality protein and unsaturated fatty acids. To solve the abovementioned problems, as well as produce high value-added products from Silver carp, it needs to be processed. On the other hand, with a glance at people's livelihoods, mechanical life, and lack of time to prepare food, the idea of producing or supplying ready-to-eat or semi-prepared food, such as fish paste, fish ball, fish cake, fish sausage, and burger from seafood, seems to be a good solution (Fan *et al*., 2008; Ozpolat and Patir, 2015).

Nitrite is used to prevent spoilage (undesirable physicochemical and biological changes) in sausage and ham (from production to consumption). Nitrate and nitrite

are preservatives that give a good pink color (Hernández-Hernández *et al*., 2009). Due to the potential dangers of using synthetic preservatives such as nitrite and consumers' interest in using healthy and natural products, food industry researchers favor the use of natural antioxidants in food products. The use of natural preservatives, such as extracts, powders, and essential oils of plants, to increase the shelf-life of meat products is a promising new technology, were . The active ingredients found in plants have antimicrobial and antioxidant properties (Hernández-Hernández *et al*., 2009; Odunayo Olatunde and Benjakul, 2018). The following are some of the compounds that have antioxidant and antimicrobial activity as nitrite substitutes.

Grape pomace is one of the waste products (5000 tons a year) produced annually in juice factories, a rich source of several valuable compounds such as citric acid, tartrate, dietary fiber, and phenolic compounds. Anthocyanin (malvidin and penonidine), flavonol (quercetin and meristine), acetylbene, and phenolic acids are the major phenolic compounds, and flavan-3-L, catechin and epi-catechin, and gallic acid are the predominant phenolic compounds in the pulp (Drosou *et al*., 2015). Also, due to its red color, it seems to have a pink effect on the sausage.

Nisin (NI) is a biphasic bacteriocin polypeptide containing 34 amino acids, produced by specific strains of *Lactococcus lactis* subsp. *Lactis*. Due to its antimicrobial properties and low toxicity to humans, it has long been regarded as a GRAS[1] food preservative in the food industry. NI is known in two forms, A and Z, that are similar in structure and antimicrobial activity and differ in their amino acid in position 27; in type A, histidine and the Z variant is asparagine (Biswaro *et al*., 2018).

Modified atmosphere packaging (MAP) is used in the storage of fresh (nonfrozen) foods or at least the process. The use of vacuum or modified atmosphere or high CO_2 packaging is easily feasible for processed meats, but high levels of CO_2 will have negative effects on product quality, especially texture changes and increased blood loss (Hematian *et al*., 2012).

Inulin is a nondigestible carbohydrate containing natural Fructooligosaccharides (FOS) and has dietary fiber properties that are of great interest for its consumption due to its specific health and technological properties. Inulin in the mouth creates a fatty mouth feel. The characteristic of inulin as an imitative lipid relates to its ability to bind to water molecules and form a gel-like network (Menegas *et al*., 2013).

[1]Generally Recognized as Safe.

So far, no study has been done on sausages with these preservations along with the MAP. Overall, according to the cases stated in this study, we evaluated the effect of natural antioxidants as a nitrite replacement and the use of fat substitutes to reduce the use of oil in sausage formulation produced by Silver carp surimi. MAP was also used for packaging of samples in different treatments.

Material and Methods

Raw materials

At first, 30 kg of silver carp were caught from the farms and transported to the laboratory in ice boxes, followed by washing and removing the *skin*. After this step, the underlying meat was removed without contact with the viscera and then it was ground first through a 10 mm plate and then through a 5 mm plate in a meat grinder (MKG1300P, Panasonic, Japan). All chemicals used were purchased by Merck Germany and were of an analytical grade.

Preparation of treatments

To prepare the surimi, minced meat was washed with drinking water at a temperature below 10°C for 10 min in three stages. Rinsing was performed in 0.2% brine in the third step. Surimi obtained during experiments was stored at refrigerator temperature (Shabanpour *et al*., 2017). The sausages contained crushed ice 10%, liquid sunflower oil 10%, extenders and binders 7%, NaCl 1.5%, spice mixture 1%, monosodium glutamate 0.1%, polyphosphate 0.3%, and 70% mince. Sausage was prepared by mixing the ingredients in sequence in a food processor. The mix was stuffed into five-layer synthetic casings and cooked. Diameter and length of sausages were 25 mm and 20 cm, respectively. All fish sausages were packed separately in nine labeled polyethylene zip-bags and stored at 4°C prior to the measurements.

Inulin fiber (IF) was added to the primary sausage formulation as fat substitutes. In addition, a ranking test previously performed comparing sausage samples with IF at different concentrations showed significantly lower acceptability of the samples incorporating 6% or 7% of them when compared to the rest (5% or lower) (data not shown). After these, IF at 5% was chosen as optimal for the following study. Fat decreased by 5%. Also, extracts of orange peel, grape pomace, and NI in concentration of 0.5% (concentration approved by sensory evaluators unchanged in taste of fish sausage) were added separately and combined as a substitute for nitrite and then, the same steps were taken similar to control treatment and finally the treatments were packed into three multilayer flexible

pouches (three and four layers) under modified atmosphere (MAP: 70% CO_2 + 30% N_2) (Rahmanifarah *et al*., 2013). The treatments were kept at refrigerator temperature (4 ± 1°C) for 42 days. On 0, 7, 14, 28, 35, and 42 days of storage, three sausages from each section were randomly selected and tested to determine qualitative parameters (physicochemical and microbiological). Proximate factors and texture analysis were examined at zero time. All experiments were performed with three replications.

In total, five treatments were studied.

Treatment 1: Control treatment (no additives)
Treatment 2: Control treatment + MAP
Treatment 3: 5% oil, 5% IF + MAP
Treatment 4: 5% oil, 5% IF + 0.5% grape pomace extract (GE) + MAP
Treatment 5: 5% oil, 5% IF + 0.5% GE+ 0.5% NI + MAP

Proximate composition

Moisture of samples using oven at 105°C, amount of protein by the kjeldahl method, amount of fat using petroleum ether solvent (boiling point of 40–60°C) by soxhlet extraction, and ash of samples using electric furnace at 550°C were measured (AOAC, 2005).

Cooking loss test

Thirty grams of samples were stuffed into screw top test tubes and were heated in a steam bath at 70°C for 30 min. The cooked samples were quickly immersed in cool water for 10 min. Cooking loss was determined by weighing individual sample before and after cooking, and the difference was expressed as a percentage of the original weight (Choi *et al*., 2009).

Texture analysis

To measure the texture of the sausage, the cubic pieces were cut in 1 × 1 × 1 cm^3 dimensions and subjected to compression test by a texture analyzer with a flat probe profile of 40 × 40 mm and a load of 10 kg. The force required to compress the samples to 70% of their initial height was measured at a constant rate of 200 mm/min (Vural, 2003).

Chemical analyses

Peroxide value

Peroxide value of the samples was determined according to the Pearson method (Bagheri *et al*., 2016). Results were expressed in meq oxygen kg^{-1} lipids.

pH value

Five grams of each sample were added to 45 mL of distilled water and placed in a mixer for 30 s. Then, the pH of the samples was measured with a digital pH meter calibrated to pH four and seven standards (Valipour *et al*., 2017).

Color test

The color of the sausage was measured using the Hunterlab colorflex colorimeter (Hunter Lab Inc). The color test results include three Hunter indices a*, b*, L*, where L* is a light symbol which is black (0) and white (100), a* is a green-to-red symbol, –a is green, and +a is red and b* are blue to yellow symbol, which + b is yellow and –b is blue. The experiment was performed in triplicates (Choi *et al*., 2009).

Microbial analyses

Ten grams of each sample were mixed and homogenized with 90 mL sterile sodium chloride solution and the required dilutions were prepared. One milliliter of each dilution was used for culture by pour plate method. Total count and *psychrotrophic* bacteria were counted on Plate Count Agar at 37°C for 2 days and 7°C for 10 days. The results were reported as log CFU[2]/g (Javadian *et al*., 2017).

Mold and yeast test

Dilution of the sample was first prepared in Peptone Water broth, then transferred to a plate containing DRBC[3] medium. Plates were aerobically incubated at 25°C for 5 days (ISIRI, 2008)

Inoculation and enumeration of *Clostridium botulinum* to ham samples

Approximately 10^8 CFU/mL of *Clostridium botulinum* were added to the ham samples. The samples were then massaged to ensure complete mixing of the bacterium with the hams and placed in specially filled coatings in the baking chamber. At least 10 hams were considered for each treatment. All treatments were packed in zippered nylon bags and stored at refrigerator temperature (4 ± 1°C) during the experiment. For bacterial count at each sampling time, 1 g of sample was mixed with 9 mL of physiological serum and suspended for half an hour. Depending on the sample, the dilutions range from 10^2 to 10^4. One milliliter of diluted sample was poured into the petri dish and then 10–15 mL of SC agar medium

[2]colony-forming unit
[3]Dichloran Rose bengal Agar

at 44–47°C was added to the petri dish and thoroughly mixed. After solidification of the medium, about 10 mL of the same medium was poured into the petri dish. Plates were then placed in an anaerobic jar and incubated at 37°C for 22 h. At the end of incubation, all plates containing less than 150 colonies were selected and the black colonies on each plate indicating the probability of *C. botulinum* were counted (ISIRI, 1994).

Statistical analysis

All experiments were performed in a completely randomized design as triplicates and the result was reported as mean ± SD. Statistical analysis of treatments was performed by ANOVA using SPSS software. Significant mean differences were determined by the Duncan test at 0.05 level and charts were plotted using Microsoft Excel software.

Results and Discussion

Proximate factors

The results of moisture content (Table 1) in different treatments showed that replacement of oil with IF increased moisture content ($P < 0.05$). But, two treatments that used natural preservatives had no significant effect on moisture content ($P > 0.05$). The lowest values were observed in control treatments (1 and 2) and the highest moisture content was observed in treatments 3, 4, and 5 (treatments containing IF). This is due to the high water absorption properties of the fiber and also because the fiber improves the stability of the emulsion (Choi *et al*., 2010). Hydrocolloids are also very important factors in bonding with water and keeping it in the product. So, the increase in moisture after adding fiber is obvious (Sahin *et al*, 2005).

Results of fat content (Table 1) showed that replacement of oil with IF reduced fat levels. The highest fat content was observed in control (1 and 2) treatments and the lowest fat content was observed in treatments 3, 4, and 5 (treatments containing IF) ($P < 0.05$). Menegas *et al*. (2013) by replacing IF in chicken sausage fermented with corn oil (45% fat) produced a low-fat sausage with 25% fat.

Results of protein content (Table 1) in different sausage treatments showed that replacement of oil with IF had no significant effect on protein content. All treatments had no significant difference ($P > 0.05$). Menegas *et al*. (2013) reported that the use of IF in fermented chicken sausage had no significant effect on protein content.

Results of ash content (Table 1) in different sausage treatments showed that replacement of oil with IF increased the ash content. The lowest values were observed in control treatments (1 and 2) and the highest amounts of ash were observed in treatments 3, 4, and 5 (treatments containing IF). Increasing ash by adding IF may be due to the fact that IF has significant amounts of ash ($P < 0.05$). Cegiełka and Tambor (2012) reported that adding IF to Polish chicken burger increased burger ash content.

Texture analysis

According to the results (Figure 1a), replacement of oil with inulin increased the firmness values. The lowest values were observed in control treatments (1 and 2) and the highest values were observed in treatments 4 and 5 (treatments containing IF) ($P < 0.05$). Adding fiber seems to increase the ability of the protein to bind to meat. Thus, it creates a firmer and coherent texture and prevents deformation of the sausage (Álvarez *et al*., 2012). By definition, elasticity refers to the rate of return of the sample to its original state after removal of the deformation force. Elasticity is inversely related to the degree of firmness of the treatment. In fact, the formation of a stable network structure increases the elasticity (Zhou *et al*., 2010). According to the results (Figure 1b), the highest values were observed in control treatments (1 and 2) and the lowest values were observed in treatments 3, 4, and 5

Table 1. Proximate factors of different treatments of sausage.

Treatment/Proximate factors	Moisture (%)	Fat (%)	Protein (%)	Ash (%)
1	61.94 ± 0.10^{b}	19.64 ± 0.37^{a}	18.29 ± 0.32^{a}	1.73 ± 0.02^{b}
2	62.00 ± 0.43^{b}	19.78 ± 0.40^{a}	17.94 ± 0.43^{a}	1.78 ± 0.02^{b}
3	67.89 ± 0.36^{a}	14.70 ± 0.40^{b}	18.17 ± 0.32^{a}	2.09 ± 0.02^{a}
4	67.75 ± 0.43^{a}	14.95 ± 0.30^{b}	18.18 ± 0.32^{a}	2.11 ± 0.03^{a}
5	67.54 ± 0.44^{a}	15.11 ± 0.14^{b}	17.91 ± 0.31^{a}	2.03 ± 0.03^{a}

^{a,b,c}Different small letters in the same column represents significant difference ($P < 0.05$) (T1: control, T2: control +MAP, T3: FI 5% +MAP, T4: FI 5% +GE 0.5%+ MAP, T5: FI 5% +GE 0.5% +N 0.5%+ MAP).

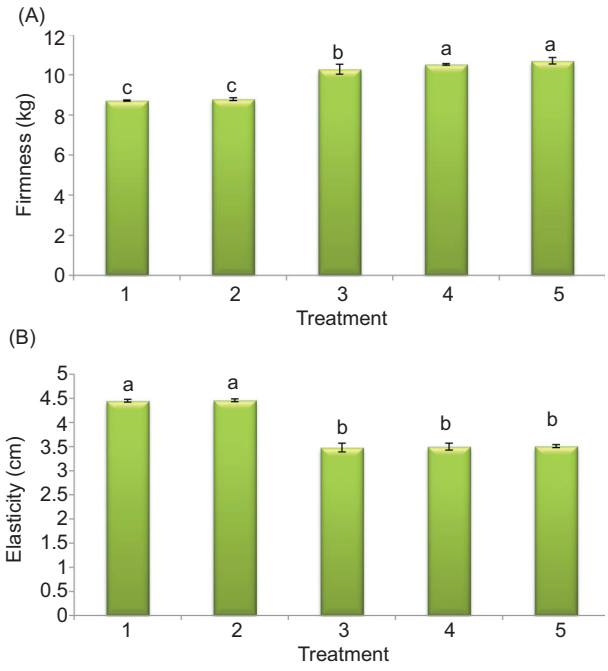

Figure 1. Texture analysis of different treatments of sausage [firmness (A) elasticity (B)] (T1: control, T2: control +MAP, T3: FI 5% +MAP, T4: FI 5% +GE 0.5%+ MAP, T5: FI 5% +GE 0.5% +N 0.5%+ MAP).

(treatments containing IF). Amina *et al*. (2014) examined the effect of adding 5%, 10%, and 15% apple waste fiber to meat nugget texture. Apple fiber increased texture firmness and reduced nugget elasticity.

Cooking loss

Cooking sausage efficiency depends on cooking temperature, cooking time (Kim and Chin, 2007), ingredients, and fat content in the product (Huang *et al*., 2005). The results of cooking loss (Figure 2) in the present study showed that with increasing time the values of cooking loss increased in all treatments. Replacing the oil with IF reduced the cooking loss. The highest values were observed in control (1 and 2) on all days of storage and the lowest values were observed in treatment 3 (treatments containing IF) ($P < 0.05$). Loss of cooking is associated with the amount of fat and water-holding capacity (Eldemery, 2010). Adding dietary fiber to meat products appears to improve water-holding capacity and improve emulsion, thereby increasing cooking efficiency (Choi *et al*., 2010). Various researchers have suggested this as the capacity to bind to water in dietary fiber that improves tissue properties (Choi *et al*., 2010, 2015). Eldemery (2010) also reported that adding orange fiber to meat burger formulation decreased burger cooking loss and with increasing concentration, lower cooking loss were observed.

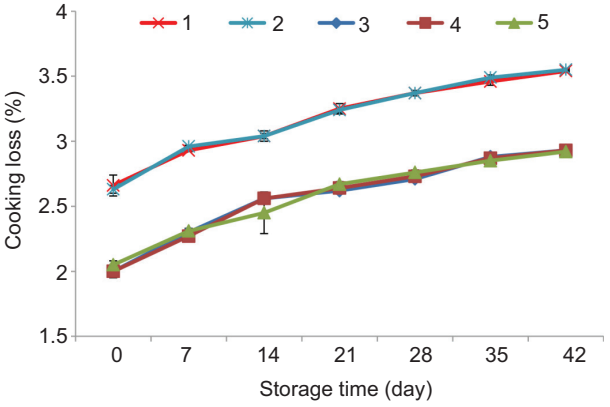

Figure 2. Cooking loss of different treatments during storage) T1: control, T2: control +MAP, T3: FI 5% +MAP, T4: FI 5% +GE 0.5%+ MAP, T5: FI 5% +GE 0.5% +N 0.5%+ MAP).

Peroxide value

According to the results of the present study (Figure 3a), the peroxide value increased in all treatments with increasing time ($P < 0.05$); the increase of peroxide value in meat products was also reported by other researchers (Javadian *et al*., 2017; Valipour *et al*., 2017). The use of MAP slowed the increase in peroxide value, so that the lowest values of peroxide were observed in the nitrite-containing treatments with the modified atmosphere ($P < 0.05$). The high level of carbon dioxide used in MAP prevents the growth of aerobic bacteria. The highest level of carbon dioxide in MAP has been reported up to 50% (Silbande *et al*., 2018). The lowest values were observed on all days of treatment after treatment 2, in treatments 1 and 5 ($P < 0.05$). The lower peroxide value in the inulin treatment is due to the lower fat content in these treatments and thus to the reduction of oxidative spoilage. Also, using two preservatives together more effectively decreased the peroxide value. Plant extracts contain phenolic compounds. Polyphenols are capable of trapping free radicals, especially proxy radicals, which are one of the key intermediate chain reactants, thereby terminating the cycle of oxidative spoilage reactions. Thus, the effect of treatments containing extracts had a higher effect on increasing the peroxide value (Fidan *et al*., 2019). The reason for the lower peroxide value after addition of NI can be attributed to the effect of bacteriocin on the decrease in lipolytic bacteria (such as Pseudomonas species) (Dehbandi *et al*., 2014). Dehbandi *et al*. (2014) also reported that the use of NI slowed the peroxide value in the Kilka fish surimi during storage.

The acceptable level of peroxide in meat for human consumption is 5 (Yanar, 2007). In relation to sausage at the

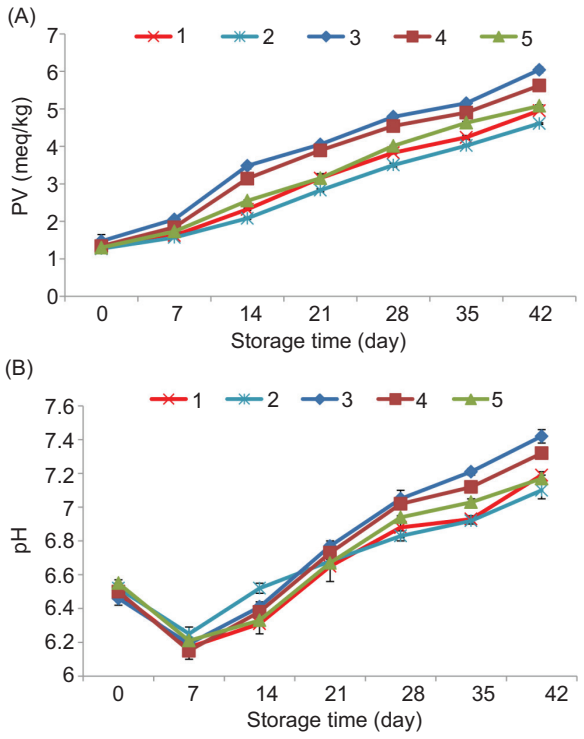

Figure 3. Changes in peroxide value (PV) (A) and pH (B) of different treatments during storage (T1: control, T2: control +MAP, T3: FI 5% +MAP, T4: FI 5% +GE 0.5%+ MAP, T5: FI 5% +GE 0.5% +N 0.5%+ MAP).

end of the storage period, the peroxide value was at an acceptable level in all samples except treatments 3 and 4.

pH value

The pH value of food affects microbial flora composition. In fish, gram-negative and proteolytic microorganisms are dominant. In many foods of animal origin, such as poultry, meat, fish, and dairy products, pH reduction occurs during storage due to chemical changes such as post-slaughter changes in meat or processing. If the food storage time is increased and the microorganisms proliferate, the pH will rise and will be contaminated with the infection and food born microorganisms. Results of sausage pH values (Figure 3b) in the present study showed that pH values first decreased and then increased. The initial decrease in pH may be due to the activity of lactic acid bacteria and acidification of the environment. But, the increase in pH after storage time is due to the natural pattern of spoilage of meat products that is related to the activity of spoilage by microorganisms (Kim *et al*., 2011). The use of modified atmosphere slowed the pH value. High levels of carbon dioxide reduce bacterial growth and result in slowing the increase in pH compared to the control treatment. Overall, the lowest pH values were observed in nitrite + modified atmosphere treatment. Using two preservatives more effectively decreased the pH value. Overall, the lowest values were observed on all days of treatment after treatment 2 in treatments 1, 5 ($P < 0.05$). The latter treatments had no significant difference ($P > 0.05$). The low pH of the sausages treated with the extract is due to its antioxidant properties and the phenolic compounds present in the extract that can protect the sausages from the function of the internal proteases and thus inhibit protein breakdown and production of amines (Fan *et al*., 2008). The mechanism of NI is due to its antibacterial properties and reduction in the capacity of bacteria for oxidative deamination of nonprotein nitrogen compounds (such as ammonia and trimethylamine), thereby reducing the pH-increasing process (Dehbandi *et al*., 2014).

Color test

The color index L indicates the brightness symbol (black to white). So, more the L, lighter the meat. The color index a is the indicator of the color change from green to red. The color index b represents the color change from blue to yellow. Results of color index showed that replacement of nitrite with preservative reduced color index L and a, and increased color index b. Sodium nitrate and nitrite are used to create a bright red (pink) and prevent turbidity of meat products, as well as antimicrobial preservatives, to create a special flavor.

So, it seems natural to replace nitrite with other compounds and change its color. According to the results of using modified atmosphere, the color index L decreases (Figure 4A). Overall, the highest values were observed at the end of the storage period after the control treatments in treatment 5. There was no significant difference between the two latter treatments ($P < 0.05$). This indicates that the oxidation process is decreased. In fact, the brightness values of the sausage samples are correlated with the peroxide values. As the number of peroxides increases, the brightness decreases and the samples darken. In fact, it can be stated that the use of three preservatives, prevents oxidation of pigments and act as a chemical preservative such as nitrite.

The color index a (Figure 4B) also decreased in all treatments. Generally, one of the causes of oxidation in meat products is the presence of compounds such as myoglobin and hemoglobin, which in the presence of metals, such as iron, act as peroxidants. Thus, it is one of the factors affecting the oxidation of oxymyoglobin (light red) to metmyoglobin (brown color) during the storage of meat products. Therefore, as a result of redox oxidation, color index a is reduced (Jin *et al*., 2007).

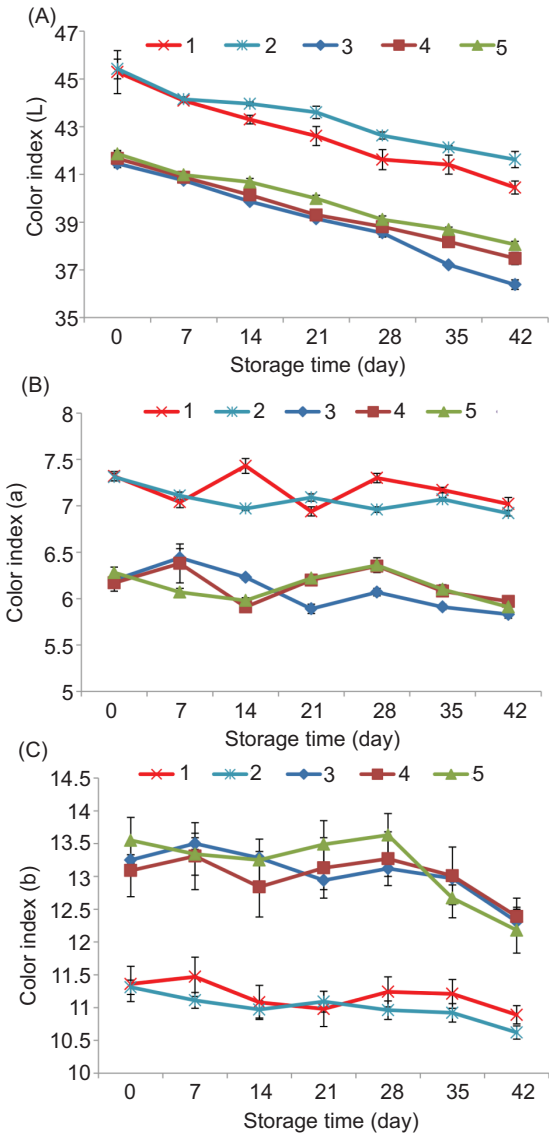

Figure 4. Changes in color index L, b, and a (A, B, and C, respectively) of different treatments during storage (T1: control, T2: control +MAP, T3: FI 5% +MAP, T4: FI 5% +GE 0.5%+ MAP, T5: FI 5% +GE 0.5% +N 0.5%+ MAP).

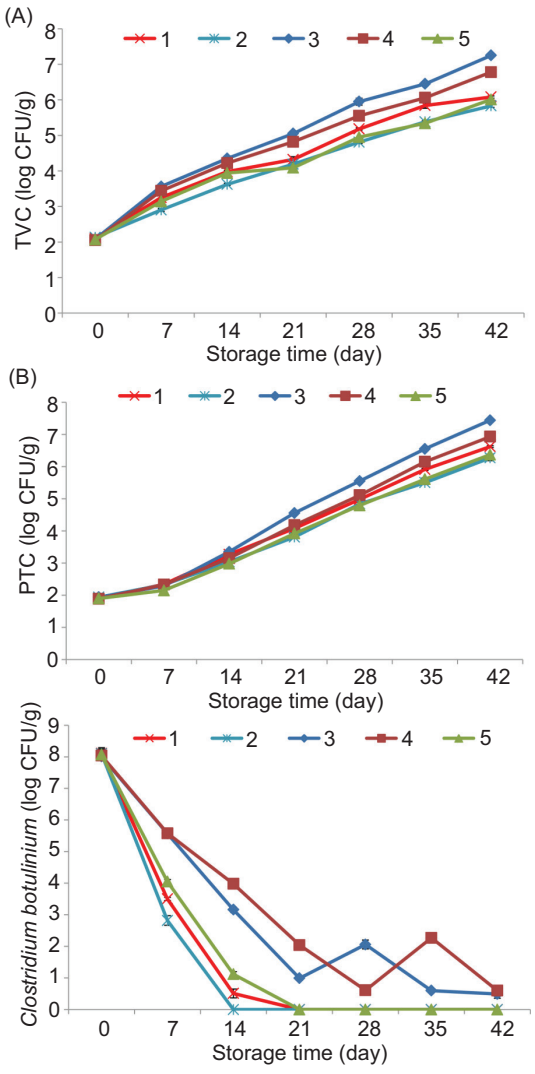

Figure 5. Changes in total viable count (TVC) (A), psychrotrophic bacteria count (PTC) (B), and *Clostridium botulinum* (C) of different treatments during storage (T1: control, T2: control +MAP, T3: FI 5% +MAP, T4: FI 5% +GE 0.5%+ MAP, T5: FI 5% +GE 0.5% +N 0.5%+ MAP).

The color index b (Figure 4C) decreased and increased during the storage period, which was consistent with the results of Giatrakou *et al*. (2010), who also reported that yellow index changes in meat products did not have a specific pattern and can change during storage by product type, formulation, and form of packaging.

Total and psychrotrophic bacteria

The results of total count bacteria (TVC) (Figure 5A) and psychotrophic bacteria (PTC) (Figure 5B) were somewhat consistent ($P < 0.05$) and were increased during storage time ($P < 0.05$). MAP decreased the growth of TVC and PTC so that the lowest values were observed in nitrite + modified atmosphere treatment (treatment 2). Gases used in modified atmosphere, such as carbon dioxide, have antimicrobial activity, and their mechanism is to dissolve in the water content of the food and produce carbonic acid, which enters the cell membrane of the microorganism and after ionization disrupts intracellular electrical balance and ultimately causes bacterial death (Hematian *et al*., 2012). Use of two preservatives together more effectively slowed down the growth of TVC. The lowest values were observed on all days of treatment 2 and then in treatment 1 and treatment 5 ($P < 0.05$), and similar results were observed with PTC bacteria, indicating a positive effect of the two preservatives as a substitute for nitrite in sausages. The lower TVC and PTC in the extract-containing treatments can be

due to phenolic compounds such as cineol. Phenolic compounds in plant extracts destroy the microorganisms and cause liposaccharides to exit and increase the permeability of the cytoplasmic membrane to ATP[4]. ATP release leads to the termination of cell energy storage and cell death (Burt, 2004). NI causes a gap in the plasma membrane and the cytoplasmic components secreted in the surrounding plasma space, and the ability of the compounds to transfer to the plasma membrane is impaired, and after ATP hydrolysis, cell death occurs. The anionic lipid present in the membrane is an active site for NI binding (Li *et al*., 2016).

In the present study, no mold or yeast was observed.

Clostridium botulinum

Clostridia are bacteria that occur in the environment and on the flora of the intestines of humans and animals (Wagner *et al*., 2006). Results of *C. botulinum* (Figure 5C) showed that with increasing time, *C. botulinum* decreased in all treatments. According to the results of the MAP, the process of growth of *C. botulinum* was slowed so that on day 14 the lowest value of the bacterium was observed in control + MAP treatment (T2). The use of the preservative effectively inhibited the bacterium as no treatment was observed on day 21 due to the antimicrobial activity of the extracts. Phenolic compounds in plant extracts destroy microorganisms, resulting in the release of liposaccharides and increased permeability of the cytoplasmic membrane to ATP. ATP withdrawal results in the depletion of cell energy storage and cell death (Burt, 2004). *C. botulinum* is a gram-positive bacterium, and the fact that NI is effective against gram-positive bacteria but not effective against gram-negative bacteria has been well established. NI is specifically active against vegetative cells and heat-resistant spores of Bacillus, Clostridium, and Listeria monocytogenes. The mechanism of NI antimicrobial activity against the vegetative walls of cells is binding to the cytoplasmic membrane. NI is a cationic antimicrobial that binds to electrons with a negative charge through electrostatic bonding. After binding, NI enters the membrane and creates a temporary small cavity. This process causes the rapid release of ions, amino acids, and cellular ATP (Vongsawasdi *et al*., 2012).

Conclusion

The results of the present study showed that addition of IF had a positive effect on a physicochemical and texture of sausages. Also, the use of two natural preservatives combined with modified atmosphere had almost similar chemical and microbial properties to conventional sausage treatment (containing nitrite). Among the treatments containing natural preservatives, the best results were observed in treatment 5 (5% oil, 5% IF + 0.5% GE + 0.5% NI + MAP). Based on the results, a functional product with properties, such as fiber and natural antioxidants, low fat, and nitrite-free, can be produced.

[4]Adenosine triphosphate

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Characterization of functional fish ham produced from Silver carp (*Hypophthalmichthys molitrix*) surimi enriched with natural antioxidant and vegetable fiber

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Abstract

In this study, the effect of grape pomace (GE), orange peel extract (OE), and nisin (N) with modified atmosphere packaging (MAP: 70% CO_2 + 30% N_2) was investigated on the quality and shelf life of ham produced from low fat Silver carp surimi (containing inulin fiber [FI] and salatrim [S]) kept in the refrigerator with T1: control (containing nitrite), T2: control + MAP, T3: GE 0.5%+ FI 5% +OE 0.5% +N 0.5%, T4: S 5%+ OE 0.5%+ GE 0.5% +N 0.5% + MAP, T5: FI 2.5%+ S 2.5%+ GE 0.5%+ OE 0.5%+ N 0.5%+ MAP. The texture characteristics of ham at the beginning of storage, cooking loss, chemical indices (peroxide value, pH, color index), and microbial (total count bacteria, *psychrotrophic* bacteria, mold and yeast, *Clostridium botulinum*) during 42 days storage in the refrigerator (4 ± 1°C) were evaluated. The results of the tests were analyzed according to Duncan's by SPSS software with 95% confidence. The results showed that inulin and salatrim fibers have a positive effect on the texture and cooking loss of ham and inulin had a better effect, so the value of cooking loss in treatment 3 was 1.62% and control treatment was 2.09%. Using three natural preservatives along with MAP could slow down the oxidative spoilage, microbial and color index changes in ham. These combinations also inhibited the growth of *C. botulinum*. In most tests examined, treatment 3 showed no significant difference with treatment 1 (ham containing nitrite) ($P > 0.05$), so this treatment shows that natural additives (nitrite replacement) improve the quality properties of low-fat ham.

Keywords: fat replacement; fish ham; inulin fiber; nisin; plant extracts

Introduction

Seafood is one of the most important sources of protein and have a considerable role in the health of consumers. Surimi is one of the minced fish products, which has been a traditional method of fish preservation. In general, surimi refers to minced and washed fish, which can be used for a variety of food products such as fish sausage, fish ham, fish cake, fish burger, and other products (Jin *et al*., 2017; Ozpolat and Patir, 2015; Shabanpour *et al*., 2007; Zhang *et al*., 2020). One of the meat products is ham which is very common in different parts of the world. These products have low oxidative stability and are sensitive to fat rancidity during storage. Therefore, some additives are used to control microbial load and oxidative changes in meat products. One of the main ways of processing meat products is the use of nitrate and nitrite. These compounds increase the shelf life and also prevent the spoilage of products during storage (Riazi *et al*., 2016; Nogueira *et al*., 2019). Salt and nitrite are also used to increase the shelf life and taste of the meat. Due to the chemical structure of nitrite in sausage and ham, its carcinogenic potential and also in response to consumer demand for natural products, reducing nitrite utilization

or replacing all or part of it in meat products with natural compounds have been considered (Shu *et al*., 2020). Here are some of the compounds that have antioxidant and antimicrobial activity as nitrite substitutes (Araújo *et al*., 2019).

Citrus (*Citrus reticulata*, Blanco) is one of the most important fruits in the world. It contains minerals, phenolics, pectin, and dietary fiber, and is rich in vitamins B, A, and C; as a result, they have nutritional and medicinal properties. Nearly a hundred industries use citrus to produce their products (Drosou *et al*., 2015). One notable point in the citrus industry is the added value of these products through the production of by-products such as citrus peel. These are rich in flavones, polymethoxylates, and phytochemicals, which are rare in other plants, resulting in recent years, special attention has been paid to the use of citrus peel. Grape pomace (GE) is one of the many wastes (5000 tonnes per year) produced by juice factories, a rich source of several valuable compounds such as citric acid, tartrate, dietary fiber, and phenolic compounds. Anthocyanin (malvidin and penonidine), flavonol (quercetin and meristine), and phenolic acids are the major phenolic compounds and flavan-3-L, catechin, and epi-catechin, and gallic acid are the predominant phenolic compounds in GE (Batpho *et al*., 2017).

Bacteriocin–nisin (N) is from group A antibiotics with 34 amino acids that have a ring structure. In the nisin structure, there is a part called the hinge area which is capable of disassembling the ring systems and is characterized by its flexibility. Nisin has no inhibitory effect on gram-negative bacteria, yeasts, and fungi (Siroli *et al*., 2016). This bacteriocin was first synthesized by *Lactococcus lactis* in England in 1928 by Rogers and Whitier and was reported by the Food and Agriculture and World Health Organization (FAO/WHO) in 1969, for its low toxicity to humans, as a GARS[1] and food preservative. Since 1987, it has been used as a permitted additive in food and dairy products (Hematian Sourki *et al*., 2012).

Packaging is another way to increase food shelf life. Modified atmospheric packaging (MAP) is used for the shelf life of fresh (non-frozen) food. The use of vacuum, MAP, and high CO_2 packaging is easily feasible for processed meats, but high levels of CO_2 will have negative effects on product quality, especially texture changes and increased blood loss (Ashraf *et al*., 2011; Ghosh and Dash 2020).

Fat is one of the important constituents that affect the sensory properties of food products, including flavor, color, texture, oral sensation, and overall sensory satisfaction. In general, an increase in fat and oil increases the frying ability and reduces the fragility of the tissue. However, its reduction results in a firm and gummy texture with low moisture content (Akalin and Erisir, 2008). On the other hand, a high intake of fats and oils can increase blood triglycerides, leading to cardiovascular disease and heart failure, so their consumption should be reduced. Inulin is a non-digestible carbohydrate-containing natural fructooligosaccharides and 1–2 glucopyranose residues. It has dietary fiber properties and due to its specific health and technological properties, there is a great interest in its use. The characteristic of inulin as a mimetic lipid is related to its ability to bind to water molecules and form a gel-like network. Inulin also makes the mouth feel greasy. This property has been used in the production of fat-free yogurt, chocolate, imitated cheeses, ice cream, and low-fat fermented sausages, and the results indicate no change in the organoleptic properties of the products (Ognean *et al*., 2006; Shi *et al*., 2020). Another fat replacement is salatrim. Salatrim (derived from small and large molecules of triacylglycerides) is a generic name for a family of triglycerides that contains a mixture of at least one short-chain fatty acid (mainly C4:0, Cs:0, C2:0) and at least one long-chain fatty acid (mostly stearic acid Cus: 0) that locates randomly on glycerol. This triglyceride has the physical properties of fat, but only contains 5 calories per gram instead of 9 calories per gram of natural fat (Surendra Babu *et al*., 2018)

Menegas *et al*. (2013) reported that fermented chicken sausages formulated with standard amounts of corn oil, reduced amounts of oil, and reduced amounts of oil containing inulin as a partial oil substitute remained stable and had no substantial loss of physical, chemical, microbiological, or sensory attributes during storage at 4°C for 45 days.

In this study, we evaluated the effect of natural antioxidants as a nitrite substitute and the use of fat substitutes to reduce oil in ham formulations produced from Silver carp surimi as well as MAP for the increased shelf life of the ham.

Material and Methods

Raw materials

At first, 30 kg of Silver carp were caught from the farms (1 hectare) and transported to the laboratory with ice boxes (0°C), followed by washing and peeling. After this step, the underlying meat was removed without contact with the viscera and then it was ground first through a 10 mm plate and then through a 5 mm plate in a meat grinder (MKG1300P, Panasonic, Japan).

[1]Generally Recognized as Safe.

Preparation of treatments

To prepare the surimi, minced meat was washed with drinking water at a temperature below 10°C for 10 min in three stages. Rinsing was performed in 0.2% brine in the third step. The later step caused better dehydration and reduced solubility of sarcoplasmic proteins in the salt solution. Surimi obtained during experiments was stored at refrigerator temperature (Shabanpour *et al*., 2007). For samples preparation (control treatment), the raw materials were weighed and blended to obtain a uniform paste according to the formulations (Table 1). For this purpose, the meat was placed with a third of the ice in the cutter (Talsa, E-46950, EU) and was mixed with the high-speed cutter, followed by nitrate, protein residues with ice, carbohydrates, fillers, vitamin C, and finally, spices were added into the meat. After mixing, the components of the cutter and paste mixture were filled into the wrapper, the ham was incubated in the baking chamber at 85°C for 45 min (Bourne, 2002; Hayes *et al*., 2009). Inulin and salatrim fibers were added to the primary ham formulation as a fat substitute.

In addition, a ranking test previously performed comparing ham samples with inulin fiber and salatrim at different concentrations showed significantly lower acceptability of the samples incorporating 6% or 7% of them when compared to the rest (5% or lower) (data not shown).

Table 1. Formulation and components of ham (control treatment).

Row	Components	%
1	Fish meat (surimi)	55
2	Oil	10
3	Egg yolk	1
4	Starch	1
5	Gluten	1
6	Skim milk	3
7	Wheat	3
8	Spice	1
9	Salt	1.2
10	Garlic	1
11	Sugar	0.5
12	Phosphate	0.4
13	Vitamin C	0.05
14	Nitrite	0.012
15	Ice+ water	20
16	Citrate	1
17	Casein	0.5
18	Sodium glutamate	0.288
19	Glucono Delta Lactone	0.05

After these, with inulin fiber, salatrim concentrations of 5% were chosen as optimal for the following study. Fat decreased by 5%. Also, extracts of orange peel, grape pomace, and nisin in the concentration of 0.5% (concentration approved by sensory evaluators unchanged in the taste of fish ham) were added combined as a substitute for part of nitrite and then, same steps were taken as that of control treatment and finally the treatments were packed into three multilayer flexible pouches (3 and 4 layers) under modified atmosphere (MAP: 70% CO_2 + 30% N_2).

The treatments were kept at refrigerator temperature (4 ± 1°C) for 42 days. On the 0, 7, 14, 28, 35, and 42 days of storage, three hams from each section were randomly selected and tested to determine qualitative parameters (physicochemical and microbiological). All experiments were performed with three replications.

In total, five treatments were studied:

T1: Control treatment (with nitrite)
T2: Control treatment + MAP
T3: GE 0.5%+ FI 5% +OE 0.5% +N 0.5%
T4: S 5%+ OE 0.5%+ GE 0.5% +N 0.5% + MAP
T5: FI 2.5%+ S 2.5%+ GE 0.5%+ OE 0.5%+ N 0.5%+ MAP

Cooking loss test

The cooking loss test was performed according to the method given by Hayes *et al.* (2009) with slight modification. Thirty grams of samples were stuffed into screw-top test tubes and were heated in a steam bath at 70°C for 30 min. The cooked samples were quickly immersed in cool water for 10 min. Cooking loss was determined by weighing individual samples before and after cooking, and the difference was expressed as a percentage of the original weight.

Texture analysis

To measure the texture of the ham, the cubic pieces were cut into 1 × 1 × 1 cm^3 dimensions and subjected to compression test by a texture analyzer with a flat probe profile of 40 × 40 mm and a load of 10 kg. The force required to compress the samples to 70% of their initial height was measured at a constant rate of 200 mm/min (Vural, 2003).

Chemical analyses

Peroxide value

Peroxide values of different treatments were determined according to the Bagheri *et al.* method (2016). Results were expressed in meq oxygen kg^{-1} lipids.

pH value

The pH values of different treatments were measured with a digital pH meter calibrated to pH 4 and 7 standards (Valipour Kootenaie *et al*., 2017).

Color test

The color of the sausage was measured using the Hunterlab color flex colorimeter. The color test results include three Hunter indices a*, b*, and L*; where, L* is a light symbol which is black (0) and white (100), a* is a green-to-red symbol, where -a is green and + a is red, and b* is blue to yellow symbol, where + b is yellow and -b is blue. The experiment was performed in triplicates (Choi *et al*., 2009).

Microbial analyses

Ten grams of each sample was mixed and homogenized with 90 mL sterile sodium chloride solution and the required dilutions were prepared. One milliliter of each dilution was used for culture by the pour plate method. Total count and *psychrotrophic* bacteria were counted on Plate Count Agar at 37°C for 2 days and 7°C for 10 days, respectively. The results were reported as log CFU/g (Javadian *et al*., 2017).

Mold and yeast test

Dilution of the sample was first prepared in Peptone Water broth, then transferred to a plate containing DRBC[2] medium. Plates were aerobically incubated at 25°C for 5 days (ISIRI, 2008).

Inoculation and enumeration of *Clostridium botulinum* to ham samples

Approximately 10^8 CFU/mL of *Clostridium botulinum* was added to the ham samples. The samples were then massaged to ensure complete mixing of the bacterium with the hams and placed in especially filled coatings in the baking chamber. At least 10 hams were considered for each treatment. All treatments were packed in zippered nylon bags and stored at refrigerator temperature (4 ± 1°C) during the experiment. For bacterial count at each sampling time, 1 g of sample was mixed with 9 mL of physiological serum and suspended for half an hour. Depending on the sample, the dilutions range from 10^2 to 10^4. One milliliter of diluted sample was poured into the petri dish and then 10–15 mL of SC agar medium at 44 to 47°C was added to the petri dish and thoroughly mixed. After solidification of the medium, about 10 mL of the same medium was poured into the petri dish. Plates were then placed in an anaerobic jar and incubated at 37°C for 22 h. At the end of incubation, all plates containing less than 150 colonies were selected and the black colonies on each plate indicating the probability of *C. botulinum* were counted (ISIRI, 1994).

[2]Dichloran Rose bengal Agar.

Statistical analysis

All experiments were performed in a completely randomized design with three replications and the result was reported as mean ± standard deviation. Statistical analysis of treatments was performed using SPSS 16.0 software by ANOVA. Significant differences were determined by the Duncan test at the level of 0.05 and figures were drawn using Microsoft Excel software.

Results and Discussion

Texture analysis

According to the results (Figure 1A), the replacement of oil with inulin fiber and salatrim increased the firmness

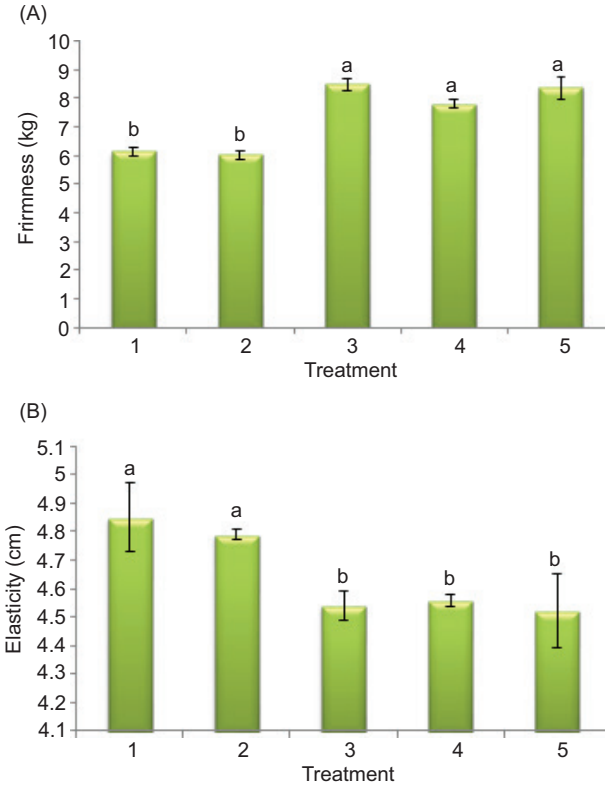

Figure 1. Texture analysis of different treatments of sausage [firmness (A); elasticity (B)] (T1: control, T2: control + MAP, T3: GE 0.5%+ FI 5% +OE 0.5% +N 0.5%, T4: S 5%+ OE 0.5%+ GE 0.5% +N 0.5% + MAP, T5: FI 2.5%+ S 2.5%+ GE 0.5%+ OE 0.5%+ N 0.5%+ MAP). Values with different superscripts are significantly different at $P < 0.05$.

values and the effect of inulin was significantly higher than salatrim. The lowest values were observed in control treatments (T1 and T2) and the highest values were observed in T3 (5% inulin fiber) ($P < 0.05$). The final texture of food products is affected by the composition of the food. The interactions between proteins, starch, and other constituents are important for the final quality of the product. On this basis, compounds used probably enhance the ability of the meat proteins to bind and, as a result, tighten the ham texture. It also appears that lower oil content in T3 affects texture firmness than other treatments (Amina *et al*., 2014).

By definition, elasticity refers to the rate of return of the sample to its original state after removing the deformation force. Elasticity is directly related to the degree of rigidity of the treatment (Amina *et al*., 2014). According to the results (Figure 1B), the highest values were observed in control treatments (T1 and T2) and the lowest values were observed in T3. The results of the present study were consistent with the results of Menegas *et al*. (2013) by applying inulin fiber in chicken sausage fermented with corn oil to increase texture firmness and reduce sausage elasticity.

Cooking loss

The results of cooking loss (Figure 2) in the present study showed that with increasing time, the values of cooking loss increased in all treatments. Replacing the oil with inulin fiber and salatrim reduced the cooking loss. The effect of inulin on the reduction of cooking loss was significantly higher than salatrim. The highest values were observed in control treatments (T1 and T2) on all days of storage and the lowest values were observed in T3 (5% inulin fiber) ($P < 0.05$). In general, the fibers retain moisture during the frying process and are reduced cooking loss. This is due to their ability to form hydrogen bonds with water molecules, that is, to entrap water molecules, which prevents moisture outflow during the frying process (Farajzadeh *et al*., 2013; Marchetti *et al*., 2013). Amina *et al*. (2014) also reported that the addition of apple fiber reduced the cooking time of nugget meat.

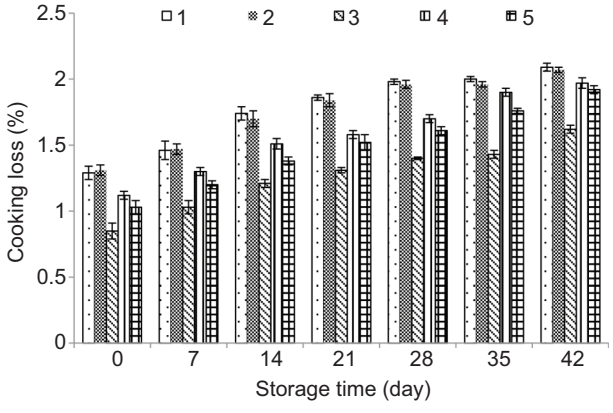

Figure 2. Cooking loss of different treatment during storage (T1: control, T2: control + MAP, T3: GE 0.5%+ FI 5% +OE 0.5% +N 0.5%, T4: S 5%+ OE 0.5%+ GE 0.5% +N 0.5% + MAP, T5: FI 2.5%+ S 2.5%+ GE 0.5%+ OE 0.5%+ N 0.5%+ MAP).

Peroxide value

Lipid oxidation in meat is one of the causes of damage to meat tissue during storage. Oxidation of lipids in meat has a complex mechanism. During this process, in addition to the adverse effects on taste and color, protein solubility is also reduced and eventually nutritional value is declined (Valipour Kootenaie *et al*., 2017).

According to the results of the present study (Figure 3A), the peroxide value increased in all treatments with increasing time (P <0.05), the increase of peroxide value in meat products was also reported by other researchers (Javadian *et al*., 2017; Valipour Kootenaie *et al*, 2017). According to the results, the lowest peroxide values were observed in the nitrite treatments with the MAP during storage ($P < 0.05$). This is due to the high amount of carbon dioxide used in MAP, which prevents the growth of aerobic and anaerobic bacteria.

The highest level of carbon dioxide in the MAP has been reported up to 50% (Silbande *et al*., 2018). The change in peroxide value in the inulin treatment was also slower than salatrim treatment. The lower peroxide value in the inulin treatment was due to the lower fat content which reduced oxidative spoilage. Also, using three preservatives together effectively controlled the increasing trend of peroxide value. The lowest values were observed in all days after T2, in T1 and T3. Most of the time, the latter treatments did not have significant differences ($P <$ 0.05). The antioxidant properties of plant extracts depend on phenolic compounds. Polyphenols are capable of trapping free radicals, especially proxy radicals, which are one of the key intermediate chain reactors, thereby terminating the cycle of oxidative spoilage reactions (Valipour Kootenaie *et al*., 2017). Also, bacteriocin–nisin can decrease peroxide value by decreasing the population of lipase-producing bacteria (such as pseudomonas species) (Dehbandi *et al*., 2014). In total, these three preservatives together with MAP can have similar effect with nitrite on ham.

The permitted level of peroxide value in the aquatic product for human consumption is 5 meq oxygen kg^{-1} lipids (Yanar, 2007). According to the results at the end of the storage period, peroxide value was lower than the acceptable range in all samples except T4.

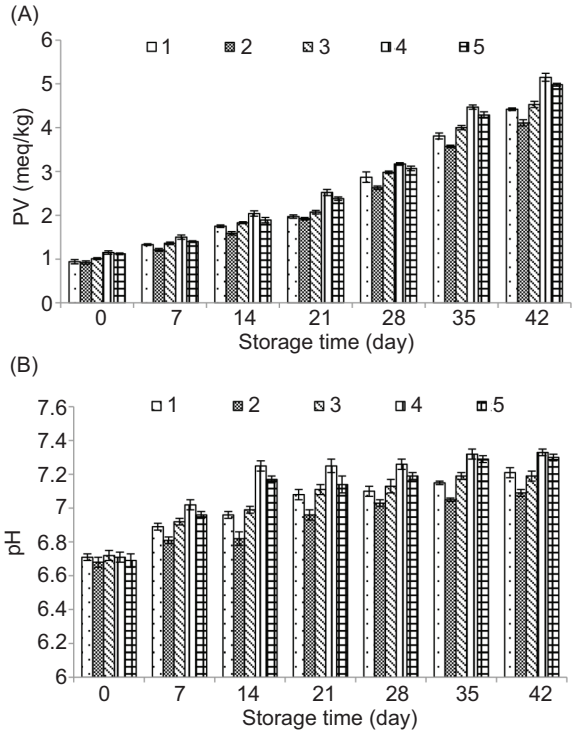

Figure 3. Peroxide value (PV) (a) and pH (b) of different treatment during storage period (T1: control, T2: control + MAP, T3: GE 0.5%+ FI 5% +OE 0.5% +N 0.5%, T4: S 5%+ OE 0.5%+ GE 0.5% +N 0.5% + MAP, T5: FI 2.5%+ S 2.5%+ GE 0.5%+ OE 0.5%+ N 0.5%+ MAP).

pH values

Even during frozen storage that the microorganisms are inactive, the previously produced enzymes and the endogenous enzymes in the meat are active. The results of pH values in ham (Figure 3B), in the present study, showed that with increasing time, pH values increased in all treatments. The increase in pH during storage can also be attributed to the production of volatile nitrogenous bases (such as ammonia and trimethylamine) resulting from the activity of meat-spoilage bacteria (Valipour Kootenaie *et al*., 2017). The use of MAP slowed the pH increasing trend. Since high levels of carbon dioxide reduce bacterial growth, therefore, the pH increasing trend is slower than the control treatment. In general, the lowest pH values were observed in nitrite + MAP treatment (T2) ($P < 0.05$). In general, the lowest values were observed in all days after T2 in T1 and T3 ($P < 0.05$). The latter treatments were not significantly different ($P > 0.05$). The equivalence of pH values in the sample containing extract compared to the nitrite treatments can be related to the antibacterial activity of the extract. As the microbial flora decreases, the number of secondary metabolites produced decreases and the pH increasing trend is slowed (Vilela *et al*., 2016). The use of nisin also influences spoilage bacteria due to its antibacterial properties and reduces the capacity of the bacteria to oxidative deamination non-protein nitrogen compounds (such as ammonia and trimethylamine), thereby reduces the pH-increasing process (Dehbandi *et al*., 2014).

Color test

The color index L indicates the brightness symbol (black to white). So the more L the meat is lighter. The color index a is the indicator of the color change from green to red. The color index b represents the color change from blue to yellow. Results of color index showed that replacement of nitrite with preservatives reduced color index L and a and increased color index b. Sodium nitrate and nitrite are used to create a bright red (pink) and to prevent the darkening of the meat products, as well as antimicrobial preservatives and to create a special flavor. So it seems natural to replace nitrite with other compounds and change its color. According to the results of using modified atmosphere, the color index L decreases (Figure 4A). Overall, the highest values were observed in control treatments. The other three treatments had no significant difference indicating a decrease in the oxidation process in ham. The brightness values of the ham samples are correlated with the peroxide values. As the number of peroxides increases, the brightness decreases and the samples darken. It can be stated that the use of three preservatives prevents the oxidation of pigments and act as a chemical preservative such as nitrite.

The color index a (Figure 4B) also decreased in all treatments. Generally, one of the causes of oxidation in meat products is the presence of compounds such as myoglobin and hemoglobin which in the presence of metals such as iron, act as peroxidants. This is one of the factors affecting the oxidation of oxymyoglobin (light red) to metmyoglobin (brown color) during the storage of meat products. Therefore, as a result of redox oxidation, color index a is reduced (Jin *et al*., 2007).

The color index b (Figure 4C) decreased and increased during the storage period, which was consistent with the results of Giatrakou *et al*. (2010). They reported that yellow index changes in meat products did not have a specific pattern and can change during storage by product type, formulation, and form of packaging.

Total and psychrotrophic bacteria

The results of total count bacteria (TVC) (Figure 5A) and psychrotrophic bacteria (PTC) (Figure 5B) were somewhat consistent ($P < 0.05$) and were increased during storage time ($P < 0.05$). The MAP decreased the growth of TVC and PTC so that the lowest values were observed in

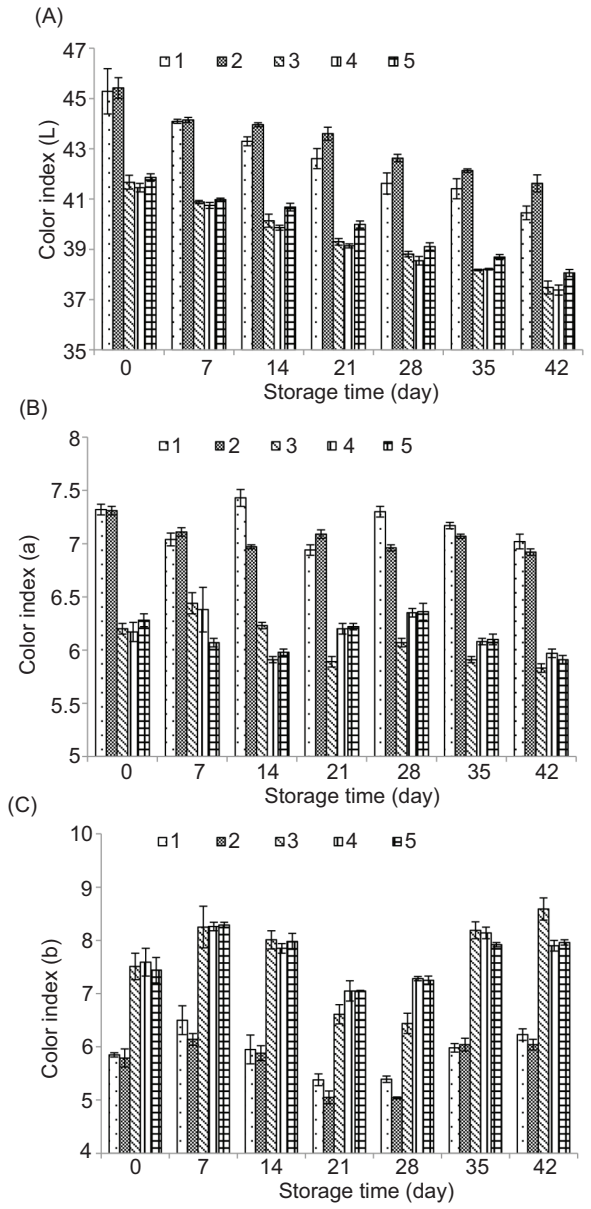

Figure 4. Color index L, b, and a (A, B, and C, respectively) of different treatment during storage period (T1: control, T2: control + MAP, T3: GE 0.5%+ FI 5% +OE 0.5% +N 0.5%, T4: S 5%+ OE 0.5%+ GE 0.5% +N 0.5% + MAP, T5: FI 2.5%+ S 2.5%+ GE 0.5%+ OE 0.5%+ N 0.5%+ MAP).

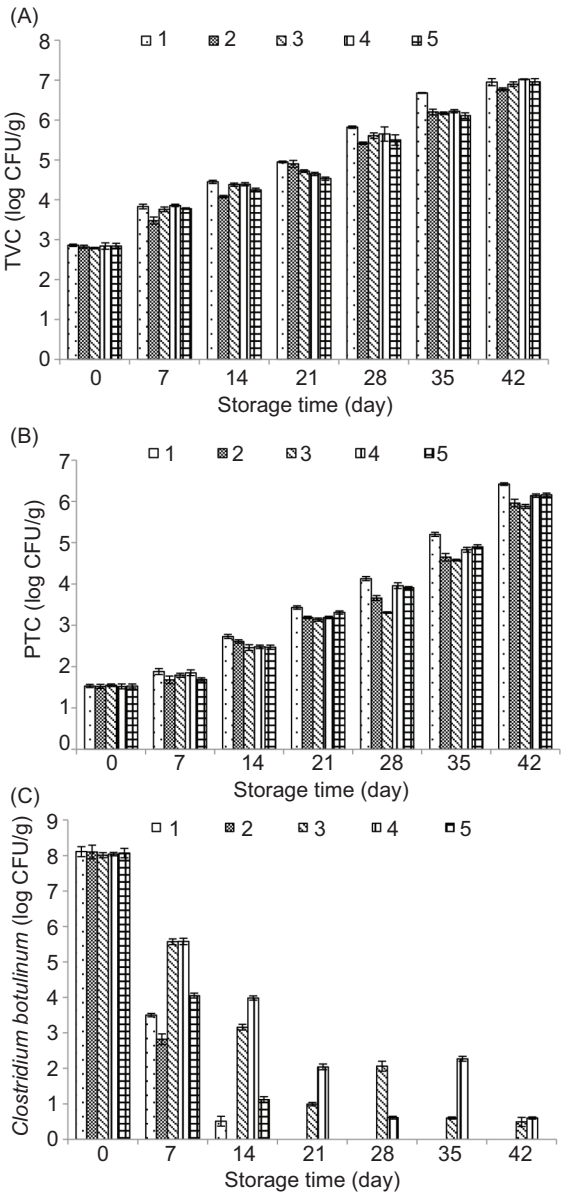

Figure 5. Total viable count (TVC) (A), psychrotrophic bacteria count (PTC) (B), and *Clostridium botulinum* (C) of different treatment during storage period (T1: control, T2: control + MAP, T3: GE 0.5%+ FI 5% +OE 0.5% +N 0.5%, T4: S 5%+ OE 0.5%+ GE 0.5% +N 0.5% + MAP, T5: FI 2.5%+ S 2.5%+ GE 0.5%+ OE 0.5%+ N 0.5%+ MAP).

nitrite + modified atmosphere treatment (T2). Gases used in modified atmospheres such as carbon dioxide have antimicrobial activity and their mechanism is to dissolve in the water of the food and produce carbonic acid, which enters the cell membrane of the microorganism and after ionization, it disrupts the intracellular electrical balance and ultimately causes bacterial death (Ghosh and Dash 2020). The use of three preservatives together more effectively slowed down the growth of TVC. The lowest values were observed in all days of T2 and four other treatments had no significant difference ($P > 0.05$). Similar results were observed with PTC bacteria indicating a positive effect of three preservatives as a substitute for nitrite in sausages. The lower TVC and PTC in the extract containing treatments can be due to phenolic compounds such as cineol. Phenolic compounds in plant extracts destroy the microorganisms and cause liposaccharides to exit and increase the permeability of the cytoplasmic membrane to ATP[3]. ATP release leads to the termination of cell

[3]Adenosine triphosphate.

energy storage and cell death (Burt, 2004). Bacteriocin–nisin also acts by creating gaps in the plasma membrane due to the wide inhibitory spectrum shown on bacteria in vegetative cells, so that cytoplasmic components seep out through the gaps, and after ATP hydrolysis, cell death occurs. The anionic lipid present in the membrane is an active site for nisin binding (Li *et al*., 2016).

In the present study, no mold and yeast were observed.

Clostridium botulinum

Clostridia are bacteria that occur in the environment and on the flora of the intestines of humans and animals (Wagner *et al*., 2006). These bacteria have different resistance to adverse environmental factors. However, it has been shown that sulphite-reducing clostridia can persist against high salt levels of ham and can tolerate subsequent processes such as pasteurization and high water activity (a_w) reactivates them and risks to consumer health (Wagner *et al*., 2006). Therefore, the inactivation of these bacteria using natural preservatives is important. Results of *C. botulinum* (Figure 5C) showed that with increasing time, *C. botulinum* decreased in all treatments. According to the results of the MAP, the process of growth of *C. botulinum* was slowed so that on day 14 the lowest value of the bacterium was observed in control + MAP treatment (T2). The use of the preservative effectively inhibited the bacterium as no treatment was observed on day 21 due to the antimicrobial activity of the extracts. Phenolic compounds in plant extracts destroy microorganisms and result in the release of liposaccharides and increased permeability of the cytoplasmic membrane to ATP. ATP withdrawal results in the depletion of cell energy storage and cell death (Burt, 2004). *Clostridium botulinum* is a gram-positive bacterium, and the fact that nisin is effective against gram-positive bacteria and not effective against gram-negative bacteria has been well established. Nisin is specifically active against the vegetative cells and heat-resistant spores of Bacillus, Clostridium, and Listeria monocytogenes. The mechanism of nisin antimicrobial activity against the vegetative walls of cells is binding to the cytoplasmic membrane. Nisin is a cationic antimicrobial that binds to electrons with a negative charge through electrostatic bonding. After binding, nisin enters the membrane and creates a temporary small cavity. This process causes the rapid release of ions, amino acids, and cellular ATP (Vongsawasdi *et al*., 2012).

Conclusions

The results of the present study showed that the addition of inulin fiber and salatrim had a positive effect on the texture and cooking loss of ham, which had a higher effect of inulin fiber. Also, the use of three natural preservatives combined with MAP in most treatments had almost similar chemical and microbial properties compared to conventional ham treatment (containing nitrite). In all tests, among the treatments containing natural preservatives, the best results were obtained in T3 (5% oil + 5% inulin fiber + 0.5% GE extract + 0.5% orange peel extract (OE) + 0.5% N + MAP), so the results can produce a functional low-fat, nitrite-free, fiber-containing product and natural antioxidants.

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Underutilized horse chestnut (*Aesculus indica*) flour and its utilization for the development of gluten-free pasta

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PAPER

Abstract

There has been a growing demand for the production of gluten-free products due to increased occurrence of celiac disease. Thus, different research groups have been investigating the use of various available materials for the development of these functional products to fulfill customer's needs. Horse Chestnut (Aesculus indica) seeds are underutilized, low-cost, and gluten-free, found in hilly areas of the Himalayan region of Kashmir valley, India. To determine their potential as an alternative to conventional food grains, an investigation was conducted to determine the physicochemical, functional, pasting, and thermal properties of Horse Chestnut (HCN) flour and its compatibility for the development of gluten-free pasta. HCN flour comprised 73.34% carbohydrate, 11.36% protein, 6.34% crude fiber, 3.27% fat, 3.16% ash, 3.15g/g oil absorption capacity, and 4.65% water absorption index. HCN flour showed 505 cp peak, 354 cp trough, 151 cp breakdown, 472 cp final viscosity, and 66.05°C pasting temperature. Transition temperatures (onset, peak, and conclusion) and enthalpy change (ΔH) were 60.12°C, 69.90°C, 81.53°C, and 10.56 J/g, respectively. Pasta prepared from HCN flour using guar gum (0, 0.5, and 1%) was analyzed for color, cooking qualities, and textural and sensory analysis. The present results showed that HCN flour possesses good nutritional quality and has properties comparable to conventional wheat flour. Therefore, HCN-flour-based pasta can act as a nutritious alternative to conventional gluten-free pasta and add variety to the diet of people suffering from celiac disease.

Keywords: celiac disease; cooking; horse chestnut; nonconventional; nutritional; pasta

Introduction

Horse Chestnut (*Aesculus indica*) is abundantly found in hilly areas of the Himalayan region of Kashmir valley, India. The seeds of the tree are harvested during October and November. Horse Chestnut (HCN) seeds are mainly consumed by wild animals and many seeds are wasted. The seeds are kept in running water to reduce the bitterness attributed to the presence of anti-nutrients and then added in powdered form to wheat flour for making *Halwa* (porridge) and *chapattis,* a traditional flatbread (Rajeasekaran and Joginder, 2009). HCN seeds can be used as nutritional supplements because they are nutritionally good and have a distinctive composition of carbohydrate, crude protein, fat, and ash. Oleic acid, linoleic acid, palmitic acid, linolenic acid, arachidic acid, and myristic acid are the major fatty acids present in the HCN seed. The chief minerals present in HCN include potassium, phosphorus, calcium, sulfur, iron, copper, zinc, and manganese (Majeed *et al.*, 2010). The seeds

also have several medicinal uses like in the treatment of fever, viral diseases, skin infections, and cardiovascular disorders (Kaur *et al*., 2011). HCN seeds are rich in polyphenols, flavonoids, fiber, and essential minerals (Majeed *et al*., 2010).

Pasta, a traditional delicacy, is a popular staple food used throughout the world because of its low cost, longer shelf life, and ease of preparation. Commonly, wheat flour is used in the preparation of pasta (Kaur *et al*., 2012). Pasta food products are produced by extruding feed mixture in an extruder equipped with different dyes that determine the shape of the final product. The pasta products are then dried and packed. It has been recognized as an ancient means of nourishment and versatile food from nutritive and gastronomic perspectives (Fuad and Prabhasankar, 2010). Pasta is considered a superior medium for the incorporation of nutrients as per the reports of Food and Drug Administration and World Health Organization (Pasqualone *et al*., 2016). The selection of flour for pasta development depends on water absorption and water solubility indexes and the acceptability and cooking parameters of the developed pasta (Rudra *et al*., 2020). The behavior of starch and protein in pasta during cooking is such that starch swells and solubilizes but protein gets coagulated during cooking (Hager *et al*., 2012). Best-quality pasta has acceptable firmness with low solid loss, and stickiness (Phongthai *et al*., 2017). Gluten as the structure builder promotes viscosity, elasticity, extensibility, and cohesiveness in pasta products (Lazaridou *et al*., 2007). Gluten helps to maintain the shape of bakery products and pasta due to the extensibility and elastic dough behavior with resilience and textural properties acceptable to consumers (Phongthai *et al*., 2017). However, due to the amplified dominance of celiac disease and other health issues such as gluten allergy, nonceliac gluten sensitivity, and other gluten-related disorders, demand for gluten-free pasta products has increased (Dib *et al*., 2018; Ozgoren and Yapar, 2019). To avoid these disorders, grains other than wheat may be used, either individually or in combined formulation, to replace gluten in food products. The only possible treatment for individuals having celiac disease is to consume foods devoid of gluten (Witczak *et al*., 2017). However, gluten-free products have low nutritional quality, and defective textural and sensory property due to crumbly and fragile dough formation (Cai *et al*., 2016). Hence, to counterbalance the absence of gluten, fortification with gums, proteins, and different flours are considered (Foschia *et al*., 2017; Padalino *et al*., 2016). To replace gluten and to maintain the dough structure and texture of the final product, various types of protein and hydrocolloids are used in gluten-free products (Foschia *et al*., 2017). Hydrocolloids, such as xanthan gum, guar gum, hydroxypropyl methylcellulose, and carboxymethyl cellulose used as an addictive in gluten free products can improve their textural characteristics (Lorenzo *et al*., 2018; Palavecino *et al*., 2017). To improve the quality attributes like mouthfeel, texture, consumer acceptability, and shelf-life of gluten-free products, diverse research has been done using alternative components like hydrocolloids, emulsifiers, starches, and nongluten proteins (Capriles *et al*., 2016; Kumar *et al*., 2019). The worldwide market for gluten-free food products increased up to 83% with an estimated annual growth rate of 12.3% for pasta products until 2022 (Chauvin, 2019). Gluten-free products are not only consumed by celiac patients but also by other individuals as well (Bresciani *et al*., 2021). Researchers have been motivated by United Nations for the Agenda 2030 to research healthy formulations for bakery and pasta products and the use of functional agro-industrial by-products for fortification in foods (Simonato, 2021).

Because of its good nutritional profile and gluten-free nature, HCN flour may represent a potential nonconventional alternative to conventional flours. Only limited information is available regarding HCN flour and there are no reports about the production of gluten-free pasta from HCN four. Therefore, it is essential to characterize the flour to explore possible utilization in food industry and to optimize the ingredients for the development of quality pasta from HCN flour. So, in the present study, characterization of HCN flour with respect to physicochemical, functional, thermal, and pasting properties, and development and characterization of pasta were carried out.

Materials and Methods

Raw materials

Fully matured Horse Chestnut seeds were obtained from Horse chestnut trees located at Anantnag, Jammu & Kashmir, India, cleaned and stored at 5°C till used. Hard wheat was procured from Punjab Agriculture University (Ludhiana). The chemicals and reagents were procured from Sigma-Aldrich.

Flour preparation

Horse Chestnut flour was prepared with slight modification to the method of Singh *et al.* (2013). The kernels obtained after manual dehulling of HCN seeds were sliced and then dried in a tray dryer at 60°C. The slices before drying were treated to reduce the anti-nutritional factors in the previous work of RAFIQ *et al.* (2016). The dried seed slices were ground in a laboratory-type grinder to obtain HCN flour. Both wheat and HCN flours were sieved using a 60-mesh sieve. HCN and wheat flour were then analyzed for various properties.

Physicochemical characteristics of HCN flour

Color analysis

Flour color analysis was done in triplicates, with Color Flex Spectro-colorimeter (D-25, Ruston, USA), and color values (L*, a*, and b*) were determined.

Proximate analysis

Standard methods were employed to analyze the flour samples for moisture, protein, ash, fiber, and fat content (AOAC, 2006), while the carbohydrate content was determined using the difference method.

Bulk density

An accurately weighed sample was poured into a graduated cylinder. Tapping during the filling was done for uniform packing. Bulk density was calculated using the below equation:

$$\text{Bulk density } (g/cm^3) = \frac{\text{Weight of the sample } (g)}{\text{Volume of sample } (cm^3)}$$

True density

The method followed by Raigar and Mishra (2015) was used to determine true density. An accurately weighed flour sample (1 g) was taken and poured into a burette filled with toluene. The equation given below was used to calculate true density:

$$\text{True density } (g/cm^3) = \frac{\text{Weight of flour sample } (g)}{\text{Rise in toluene level } (cm^3)}$$

Functional properties

Water absorption capacity

Water absorption capacity (WAC) of the flour was determined per Vázquez-Luna *et al.* (2019) with slight modifications. Approximately, 5 g of the sample was mixed with 70 mL of water for 1 min and then held at room temperature for 30 min, followed by centrifugation (3000 × g for 15 min). After centrifugation, the sediment obtained was weighed and WAC was calculated:

$$\text{WAC}(g/g) = \frac{\text{Weight of sediment}}{\text{Weight of flour sample}}$$

Oil absorption capacity

The method followed by Khan and Saini (2016) was used to determine the oil absorption capacity (OAC) of the sample. Five grams of flour and 75 mL of oil were mixed and agitated for 30 min and then centrifuged for 10 min at 3000 × g. Afterward, oil was drained while the retained sample was weighed to calculate OAC as shown in the below equation:

$$\text{OAC } (g/g) = \frac{\text{Weight of residue}}{\text{Sample weight}}$$

Emulsion properties

The emulsion activity and stability of samples were calculated by using the method of Du *et al.* (2014). Sample (3.5 g) and water (50 mL) were agitated for 30 s using the high setting homogenizer (Yorco, India). Afterward, groundnut oil (25 mL) was added and then again homogenized for 30 s, followed by another addition of 25 mL groundnut oil and then homogenized for 90 s. The emulsion obtained was then centrifuged for 5 min at 1100 × g.

$$\text{Emulsion activity } (\%) = \frac{\text{Volume of emulsified layer}}{\text{Emulsion volume before centrifugation}} \times \text{Emu}$$

After calculating the emulsion activity, emulsion stability was evaluated by heating the flour emulsion samples (15 min at 85°C), then cooling, followed by centrifugation for 5 min at 1100 × g. The percent emulsion activity that remained after heating can be expressed as the emulsion stability.

Foaming properties

The foaming properties, viz., foaming capacity (FC) and foaming stability (FS), were observed with minor modifications to the method followed by Jia *et al.* (2021). Sample (3 g) and distilled water (50 mL) were mixed, and the dispersion was homogenized for 3–5 min. The mixture was then instantly poured into the measuring cylinder and the increase in volume percent upon whipping was calculated. The percentage of volume increase was the foaming capacity and the change in the foam volume after 1 h of storage was recorded as the foaming stability.

FC and FS were calculated using the below mentioned formulas:

$$\text{FC}(\%) = \frac{V_2 - V_1}{V_2} \times 100$$

$$\text{FS}(\%) = \frac{V_3}{V_2 - V_1} \times 100$$

where, V_1 = volume prior to whipping (mL),
V_2 = foam volume after whipping (mL), and
V_3 = foam volume (mL) after holding

Least gelation concentration (LGC)

The method of Khan and Saini (2016) was used to determine the gelation properties of the flour samples. Two

percent, 4, 6, 8, 10, 12, 14, 16, 18, and 20% (w/v) of flour suspensions in 5 mL of water were boiled for 1 h, cooled in cold water, and kept at 4°C for 2 h.

Pasting characteristics

Rapid Visco Analyzer (TecMaster, Warriewood, Australia) was used for the determination of the pasting profile. A programmed heating and cooling cycle (taking sample 3 g, 14% mb; and distilled water 25 mL) was used for generating the viscosity profile. Experimental conditions include holding the samples for 1 min at 50°C, heating to 95°C at 12°C/min cooling rate, holding for 2.5 min at 95°C), cooling to 95 to 50°C at 12°C/min heating rate, and then holding for 2 min at 50°C.

Thermal properties

A differential scanning calorimeter (DSC821, Mettler Toledo, Switzerland) was involved in determining thermal properties of the sample. The samples were prepared with flour (3mg, dwb) and water (7 µl), sealed, and kept undisturbed at room temperature for 1 h. The sample was heated at 20–120°C at a rate of 10°C/min.

Pasta preparation

Different pasta formulations (control sample (durum wheat); native sample (HCN flour + 0% gum); pasta formulation containing HCN flour and 0.5 and 1% guar gum, respectively) were mixed using Hobart benchtop mixer (5KPM50, USA) for 1 min at low speed followed by conditioning for 5 min with distilled water (30 mL). The sample was fed to a single screw extruder (Model: Dolly La Monferrina Italy) of 7 mm die diameter. Pasta was then transferred to cabinet drier for 4 h set at 60°C, reducing the moisture content to 8%, and then packed in high-density polyethylene pouches.

Characterization of pasta

Color parameters

Color parameters of pasta were determined using a color spectrophotometer (CM-3600d, Konica Minolta, Bremen, Germany). Color analysis was done in terms of L*, a*, and b* values.

Cooking properties

Cooking time was evaluated by using the AACC (2000) method. Pasta sample (10 g) was cooked in water (300 mL). The optimum time of cooking is the point of disappearance of the inner white core of pasta and was observed by squeezing the pasta between glass plates. The weight of the cooked pasta after being drained for 2 min was taken as the cooked weight (g). The cooking loss was calculated by evaporation of cooking water to dryness at 110°C overnight.

Water uptake

Water uptake is the difference between the weight of cooked and uncooked samples, and is expressed as uncooked pasta weight percentage (Çabuk and Yilmaz, 2020). The investigation indicated the absorption of water during the cooking process.

Cooking loss

The method followed by Teterycz *et al*. (2020) was employed for cooking loss determination. Sample (10 g) was cooked in boiling water (300 mL) and then cooking water was dried overnight (105°C). The residue left after drying was weighed and then cooking loss was calculated.

Texture

For determination of texture, the samples were cooked till optimum cooking time, and then drained with 10 min rest time. Four pasta strands were taken and placed under the compression plate of pasta firmness/stickiness rig. The resulting force-time curve was used to determine firmness, cohesiveness, springiness, adhesiveness, chewiness, and gumminess.

Sensory evaluation

Panel comprising 12 trained members have done the sensory evaluation of pasta samples; evaluated the attributes like firmness, slipperiness, chewiness, surface adhesiveness, appearance; and determined the total score for each pasta sample. Perceived intensities were scored on a 5-point scale.

Statistical analysis

The experimental data was statistically analyzed by One-way ANOVA using triplicate readings for all experiments except sensory evaluation where 12 observations were averaged.

Results and Discussion

Physiochemical properties of HCN flour

The desired color characteristics for the flour are low chroma (a*) value and high lightness (L*) value to meet the consumer preference. The color parameters and nutritional composition of HCN and wheat flour are given in Table 1. Results showed that the L* value of the flours was almost similar; however, significant difference

Table 1. Physicochemical characteristics.

Physicochemical	Horse Chestnut flour	Wheat flour
Color		
L*	92.07 ± 0.49[a]	92.85 ± 0.28[a]
a*	3.47 ± 0.21[a]	2.03 ± 0.07[b]
b*	13.70 ± 0.26[a]	9.69 ± 0.15[b]
Moisture content (%)	9.71 ± 1.23[b]	11.35 ± 0.54[a]
Protein (%)	7.78 ± 1.19[b]	11.36 ± 1.10[a]
Crude fat (%)	3.27 ± 0.39[a]	1.40 ± 0.57[b]
Crude fiber (%)	6.34 ± 0.22[a]	1.67 ± 0.14[b]
Ash (%)	3.16 ± 0.05[a]	0.88 ± 0.03[b]
Carbohydrate (%)	69.74[b]	73.34[a]
Energy value (Cal/100g)	327.51[b]	346.84[a]
Bulk density (g/mL)	0.64 ± 0.12[a]	0.51 ± 0.10[a]
True density (g/mL)	1.25 ± 0.24[a]	1.42 ± 0.12[a]

Results presented are mean values and the superscripts significantly differ in row ($P < 0.05$).

was observed in a* and b* values. This variation in the color parameters may be ascribed to a difference in the natural pigment content inherent to HCN and wheat flours depending on the origin of the plant (Drakos *et al*., 2017). Nutritional composition of HCN and wheat flour also varied significantly (Table 1). Moisture content of HCN flour was low (9.71%) compared to wheat flour (11.35%). Similarly, protein and carbohydrate content of HCN flour was lower than wheat flour. However, HCN flour showed significantly higher values for ash, fat, and crude fiber content than wheat flour. High ash content of HCN flour could be related to high mineral content of the HCN seeds being rich in nitrogen, potassium, phosphorus, sulfur, calcium, iron, etc. (Majeed *et al*., 2010). The bulk density did not differ significantly in both the flours (Table 1). Similar results were observed for the true density of HCN flour (1.25 g/mL) and wheat flour (1.42 g/mL).

Functional properties of HCN flour

Water absorption capacity

WAC describes the interaction of flour and water under a limited quantity of water. The WAC of flour plays a significant part in food research as it affects other determining properties of food material. HCN flour showed significantly lower values of WAC, 2.21 g/g as compared to wheat flour, 4.65 g/g (Table 2). Hydrophilic behavior and gel-forming ability of starch, and protein quality are the determining factors that affect the WAC of flour (Al-Farga *et al*., 2016). Higher number of hydrophilic components in flour is responsible for its elevated WAC (Njintang *et al*., 2008). The variation in the WAC behavior of the flours could be related to difference in the composition of the flour.

Table 2. Functional characteristics.

Properties	HCN flour	Wheat flour
WAC (g/g)	2.22 ± 0.12[b]	4.65 ± 0.08[a]
OAC (g/g)	3.24 ± 0.15[a]	1.10 ± 0.05[b]
Foaming capacity (%)	20.2 ± 1.17[b]	31.40 ± 0.71[a]
Foaming stability (%)	22.32 ± 2.41[b]	54.05 ± 5.52[a]
Emulsion activity (%)	58.69 ± 0.31[b]	61.01 ± 1.33[a]
Emulsion stability (%)	62 ± 0.27[b]	68.32 ± 0.12[a]

WAC, Water absorption capacity; OAC, Oil absorption capacity. Results presented are mean values and the superscripts significantly differ in row ($P < 0.05$).

Oil absorption capacity

OAC capacity is an important property of the flour, which defines its application in various food formulations and determines its capacity to entrap oil which affects flavor retention and mouthfeel of the developed product (Khattab and Arntfield, 2009). OAC of flour is important for new product development and storage stability. Significantly higher values of OAC of 3.15 g/g in HCN flour than 1.10 g/g in wheat flour was observed (Table 2). Interaction of nonpolar side chains present in the flour with oil through hydrocarbon side chains affect their OAC (Adebowale and Lawal, 2004). Flour particle size, starch content, and occurrence of nonpolar amino acids are other factors that affect the OAC of flour (Chau *et al*., 1997; Ofori *et al*., 2020). Also, the protein type and content contribute to oil-retaining properties of the foods (Ravi and Sushelamma, 2005).

Foaming properties

The foaming properties are essential parameters for the formulation of food products. Results related to foaming capacity and stability of HCN, and wheat flour is given in Table 2. Carbohydrate and protein content play an important role in determining the foaming properties of flour (Sreerama *et al*., 2012). Formation of interfacial layer by proteins is responsible for foaming, keeps air bubbles in suspension, and slows down coalescence rate (Adebowale and Lawal, 2004). HCN flour and wheat flour showed foaming capacity of 20.20 and 31.40%, respectively. Low-protein and high-fat content of HCN flour is responsible for lower foaming capacity of HCN flour (Siddiq *et al*., 2010). Formation of the protein-fat complex may influence the foaming properties. Foam stability for Horse Chestnut flour (22.32%) was also lower than wheat flour (54.05%). Good foam stability is most likely associated with soluble proteins' surface activity

in continuous water phase while the presence of highly ordered globular proteins in the flour which resist surface denaturation could be related to low foamability (Du *et al*., 2014).

Emulsion properties

The ability of protein to form a stable emulsion is known as emulsion activity, and it gives information about the ability of protein to occupy the interfacial layer of oil–water emulsion while the emulsion stability is related to the strength of an emulsion to stress, provided by the proteins (Singh *et al*., 2010). Emulsion capacity and stability values of HCN and wheat flour are significantly different (Table 2). Emulsion activity and stability of HCN flour was lower (58.69 and 62%) than wheat flour (61.01 and 67.87%), which might be related to the difference in quality and content of protein. Proteins having several polar side chains make them more hydrophilic, thereby increasing the protein–water interactions, and thus affecting their emulsification properties (Yan *et al*., 2020). The high emulsion stability depends on the globular nature of proteins in the flour. The emulsifying properties of different foods vary due to varied composition and processing conditions and the capacity of protein to form and stabilize emulsions (Adebowale *et al*., 2005).

Least gelation concentration

LGC is expressed as the quantity of flour required per volume to obtain a gel. Results related to LGC of flour samples are presented in Table 3. Flour which has lower value of LGC has greater ability to form gels and low concentration of flour is required for gel formation. Higher concentration (6 g/100 mL) of HCN flour was required for initial gel formation than wheat flour (4 g/100 mL). A firm gel was obtained when using 12% of HCN flour and 10% of wheat. The difference in the gel-forming ability among various types of flours is related to different ratios of components like carbohydrates, proteins, and lipids (Drakos *et al*., 2017). The lower LGC value of wheat flour might be related to high protein and starch content, which are responsible for gel formation (Joshi *et al*., 2015).

Table 3. Least gelation concentration.

Concentration	HCN Gel	Wheat Gel
2%	No	No
4%	No	Weak
6%	Weak	Weak
8%	Weak	Weak
10%	Weak	Firm
12%	Firm	Firm
14%	Firm	Firm
16%	Firm	Hard
18 %	Hard	Hard
20%	Hard	Hard

Pasting characteristics

Pasting profile of wheat and HCN flour is depicted in Figure 1. Pasting properties play a significant part in the selection of raw materials for industrial use as a thickener, binder, etc. Rigidity of starch granules which affects swelling of starch granules and leaching of amylose in solution determine the pasting properties of the flour (Leewatchararongjaroen and Anuntagool, 2016). Compared to wheat flour (pasting temperature: 83.36°C), the pasting temperature of HCN flour was low (66.05°C). It could be the starch in HCN flour that is less resistant to swelling that is responsible for the low PT of Horse Chestnut flour. The pasting temperature has also been related to the amount of protein present. Shevkani *et al*. (2015) have reported high pasting temperature with higher amount of protein in rice flour. Peak viscosity (PV) which indicates the degree of granule swelling was 505 cP for HCN flour and 1215 cP for wheat flour. The low peak viscosity could be due to high fat content in HCN flour that produces more fluid system and subsequently low viscosity. Trough viscosity was 354 cP (HCN flour) and 1005 cP (wheat flour) and depicts a decline following PV owing to swollen starch granules disruption upon high temperature and shear. Breakdown viscosity was 151 cp for HCN flour and 210 cp for wheat flour. The low breakdown viscosity indicates higher resistance to shearing at high temperature. Final viscosity (FV) was 472 cP for HCN flour and 1560 cP for wheat flour. Besides this, setback viscosity (SBV) of HCN flour (118 cp) was considerably lower than that of wheat flour (301 cP). The important factors that affect setback and final viscosities are polymerization or reordering of leached amylose and linear amylopectin (Abu *et al*., 2006). The difference in pasting parameters of the flours could be linked to the occurrence of components such as proteins, minerals, lipids, etc., which can bind with starch, thereby affecting the pasting parameters of the flour (Ocheme *et al*., 2018). The protein and lipid components of the flour affect the pasting properties through amylose–protein and amylose–lipid interactions (Yu *et al*., 2012). Due to the protective effect of protein molecules on the integrity of starch granules, viscosity of corn starch paste decreased (Singh *et al*., 2014). Difference in the viscosity of wheat flour pastes is correlated to variation in the proportion of polymeric or monomeric proteins among wheat flours (Singh *et al*., 2016).

Thermal properties of HCN flour

Thermal properties are related to gelatinization behavior described as granular disruption, loss of

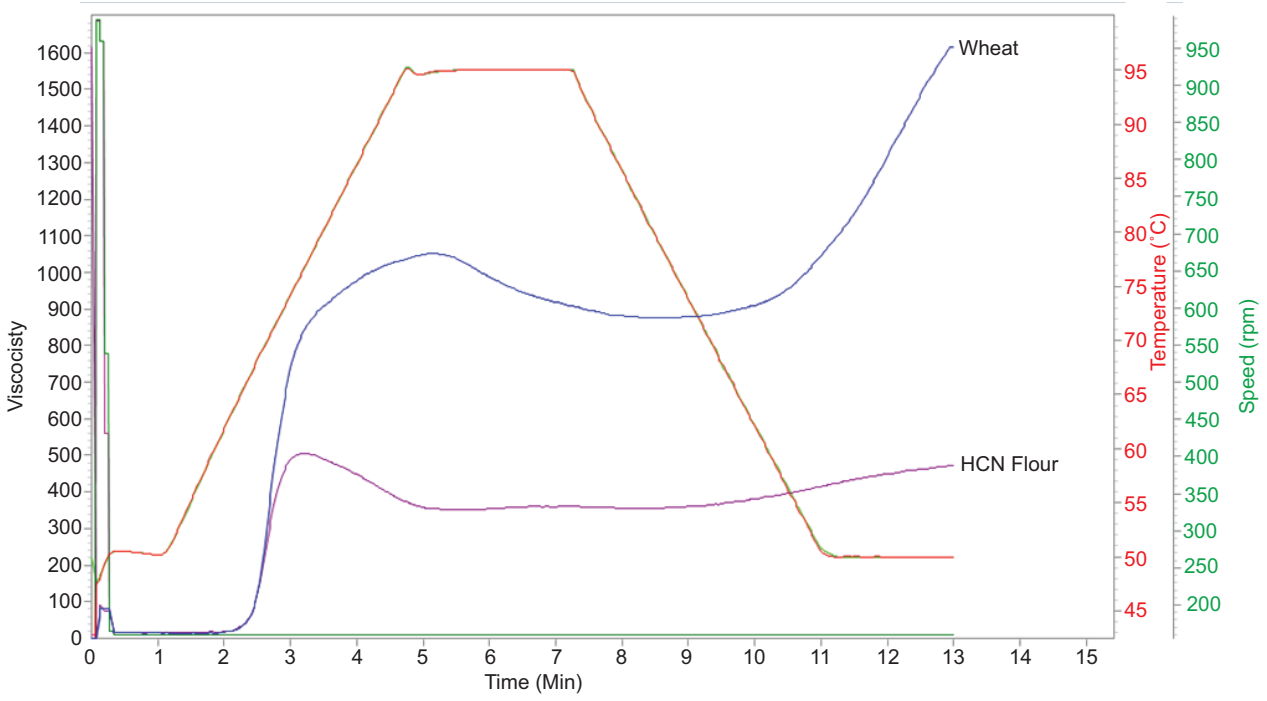

Figure 1. Pasting profile of wheat and HCN flour.

double helical and crystalline structure of starch (Rincón-Londoño *et al.*, 2016). Table 4 shows the results related to onset, peak, and conclusion temperatures, and enthalpy of gelatinization (ΔH) of HCN and wheat flour. Transition temperatures for HCN flour were T_o = 60.12, T_p = 69.90, and T_c = 81.53 °C) while for wheat flour transition temperatures were high (T_o = 78.32, T_p = 81.12, and T_c = 86.34°C). Starch granule size, their form and distribution, and the bonding between micellar network of the granule and other flour components are responsible for variation in transition temperatures of flours (Xu *et al.*, 2019). Enthalpy of gelatinization of 10.56 J/g was observed for HCN flour while for wheat flour, it was 13.21 J/g. Variation in the shape of starch granules, and proportion of large and small granules are the factors that affect the enthalpy of gelatinization (Kaur and Singh, 2005). The decreased gelatinization enthalpy can also be due to the disruption of crystalline regions upon depolymerization of amylose and breakdown of amylopectin.

Table 4. Thermal characteristics.

Parameters	Horse Chestnut flour	Wheat flour
Onset Temp. (°C)	60.12 ± 0.11^b	78.32 ± 0.98^a
Peak Temp. (°C)	69.90 ± 0.23^b	81.12 ± 0.12^a
Conclusion Temp. (°C)	81.53 ± 0.17^b	86.34 ± 0.18^a
ΔH (J/g)	10.56 ± 0.41^b	13.21 ± 0.63^a

Results presented are mean values and the superscripts significantly differ in row ($P < 0.05$).

Color characteristics of pasta

Customer acceptance depends on the color of the product and the most desirable attribute of pasta quality is the bright yellow color (Petitot *et al.*, 2010). Color values (L*, a*, and b*) of raw and cooked Horse Chestnut pasta, and control were significantly different (Table 5). The L* value for Horse Chestnut pasta was less than the control pasta sample. The differences in color parameters between control and HCN pasta samples could be related to the inherent color of the flour and the pigments present. The addition of gum in pasta formulation at different levels showed no significant effect on pasta color. Similar effect of hydrocolloids on color characteristics of developed noodles was observed by Shere *et al.* (2020) Color values of cooked and uncooked pasta were significantly different which could be related to the leaching of pigments on cooking.

Cooking characteristics of pasta

The cooking characteristics of pasta are influenced by numerous phenomena taking place during cooking, viz., hydration, starch gelatinization, and interaction with nonstarchy constituents. Cooking parameters for the pasta samples including cooked weight, water uptake, solid loss, and cooking time are given in Table 6. The

Table 5. Color characteristics of raw and cooked pasta

Sample	Raw pasta			Cooked Pasta		
	L*	a*	b*	L*	a*	b*
A	75.40 ± 2.05^{a}	1.30 ± 0.34^{b}	20.73 ± 1.2^{a}	70.60 ± 1.68^{a}	0.04 ± 0.01^{a}	19.34 ± 0.76^{a}
B	62.85 ± 1.36^{b}	1.49 ± 0.40^{a}	18.09 ± 1.83^{b}	55.84 ± 1.46^{b}	0.04 ± 0.03^{a}	16.10 ± 0.70^{b}
C	61.41 ± 1.90^{b}	1.35 ± 0.32^{b}	17.49 ± 1.31^{c}	56.17 ± 2.01^{b}	0.03 ± 0.03^{a}	15.50 ± 0.72^{c}
D	61.82 ± 1.41^{b}	1.38 ± 0.29^{ab}	18.88 ± 0.53^{b}	56.65 ± 1.61^{b}	0.02 ± 0.02^{a}	15.20 ± 1.61^{c}

Results presented are mean values and the superscripts significantly differ in column ($P < 0.05$).
Sample A: control (durum wheat); Sample B: HCN seed flour; Sample C: HCN seed flour + 0.5% gum addition; Sample D: HCN seed flour + 1% gum addition.

Table 6. Cooking properties of pasta.

Parameters	Sample (g)	Cooking time (min)	Cooked wt. (g/100g)	Solid loss (g/100g)	Water uptake (g/100g)
Sample A	5	5.1 ± 0.20^{a}	13.49 ± 0.86^{a}	6.9 ± 0.42^{c}	137 ± 7.21^{a}
Sample B	5	4.2 ± 0.25^{bc}	8.57 ± 0.12^{c}	16.8 ± 1.43^{a}	115 ± 2.65^{c}
Sample C	5	4.5 ± 0.40^{ac}	10.30 ± 0.90^{b}	10.6 ± 0.52^{b}	125 ± 3.61^{bc}
Sample D	5	4.8 ± 0.56^{ab}	12.39 ± 0.38^{a}	8.13 ± 0.45^{c}	132 ± 6.56^{ab}

Sample A: control durum wheat; Sample B: native Horse Chestnut flour; Sample C: 0.5% gum addition; Sample D: 1% gum addition. Results presented are mean values and the superscripts significantly differ in column ($P < 0.05$) ($n = 6$).

cooking time, cooked weight, and water uptake increased linearly ($P < 0.05$) with gum addition in pasta formulation. Results observed are in accordance with findings of Sozer *et al*. (2007). The enhancement in water uptake with the addition of gum might be related to the accessibility of carboxyl and hydroxyl groups in gum structure, thereby increasing the water uptake of the pasta sample (Gull *et al*., 2018). In addition, higher water uptake occurs during extended cooking time, as extra water diffuses and interacts with both protein and starch matrices. Besides this, increased affinity for water occurs because of a surge in polar amino-acid groups during cooking owing to protein denaturation (Alonso *et al*., 2000), leading to subsequent increase in water uptake for pasta. The cooked weight of HCN pasta samples increased from 9.57/5 g to 12.39g/5 g with an increase in the concentration of gum used. The rise in cooked weight may be related to water-binding and water-holding capacity of the gum used for the preparation of pasta (Widelska *et al*., 2019). Cooking loss is regarded a vital indicator of pasta quality. Cooking loss of pasta samples significantly decreased ($P < 0.05$) with an increase in the addition of gum concentration from 0 to 1% in the pasta formulation. The decrease in cooking loss might be due to the addition of guar gum, which improved the protein network, formed a matrix with the proteins where starch granules get entrenched, and thus decreased the tendency of solid loss (Chauhan *et al*., 2017; Martin-Esparza *et al*., 2018). HCN pasta made with 1% gum showed comparable cooking parameters to that of the control sample. For good-quality pasta, an acceptable cooking loss is around 8% (Dick and Youngs, 1988). Hence, the HCN-flour-based pasta containing 1% gum has a cooking loss in the acceptable range and could be considered as good quality. A desirable firmness and cooking resistance, low cooking loss, and stickiness are characteristics of high-quality pasta (Lucisano *et al*., 2012). Studies have shown lowest cooking loss with improved physical and textural properties of gluten-free pasta prepared with 0.6% of xanthan gum concentration (Milde *et al*., 2020). Kamsiati and Herawati (2019) observed cooking loss of 5.060% and hardness of 27.530N in cassava macaroni with guar gum addition of 0.25%.

Textural properties of pasta

The textural assessment of pasta is an important criterion to estimate its quality and acceptance by consumers (Milde *et al*., 2020). Textural attributes of the developed pasta are presented in Table 7. The use of gum showed significant effects ($P < 0.05$) on the various textural attributes of the tested pasta samples. Firmness of the native HCN pasta sample (5.44N) was lower than the control sample (2.307N). Moreover, with the addition of gum, the firmness of HCN pasta samples increased from 2.307N to 4.297N, which might be related to an increase in the binding between protein and starch (Duda *et al*., 2019). Adhesiveness of HCN pasta (native) was higher than control pasta while adhesiveness of HCN pasta prepared with 1% gum addition was comparable with control. Pasta adhesiveness decreased significantly ($P < 0.05$) with increased gum concentration in the pasta formulation. The adhesiveness is associated with the amount of starch

Table 7. Textural properties of cooked pasta.

Parameters	Sample A	Sample B	Sample C	Sample D
Hardness (N)	5.44 ± 0.63^{a}	2.307 ± 0.10^{d}	3.139 ± 0.22^{c}	4.297 ± 0.42^{b}
Adhesiveness (Ns)	−0.390 ± 0.02^{c}	−0.202 ± 0.07^{a}	−0.354 ± −0.27^{b}	−0.398 ± 0.09^{c}
Cohesion	0.699 ± 0.07^{a}	0.470 ± 0.02^{c}	0.584 ± 0.07^{b}	0.695 ± 0.08^{a}
Springiness %	85.572 ± 2.34^{a}	53.558 ± 1.92^{b}	84.577 ± 3.45^{a}	88.060 ± 3.71^{a}
Gumminess	387.654 ± 4.23^{a}	110.460 ± 3.65^{d}	187.047 ± 4.27^{c}	304.454 ± 2.81^{b}
Chewiness	331.724 ± 4.45^{a}	59.160 ± 3.45^{d}	158.199 ± 2.45^{c}	268.101 ± 4.45^{b}

Sample A: control durum wheat; Sample B: native Horse Chestnut flour; Sample C: 0.5% gum addition; Sample D: 1% gum addition. Results presented are mean values and the superscripts significantly differ in row ($P < 0.05$) (n = 6).

Table 8. Sensory evaluation of pasta.

Parameters	Sample A	Sample B	Sample C	Sample D
Slipperiness	3.67^{a} ± 0.82	1.83^{c} ± 0.98	3.00^{b} ± 1.10	3.53^{a} ± 1.75
Firmness	4.00^{a} ± 1.26	1.50^{c} ± 0.84	2.50^{b} ± 1.22	3.33^{a} ± 0.82
Chewiness	3.50^{a} ± 1.97	2.67^{b} ± 1.52	3.00^{a} ± 1.51	3.57^{a} ± 1.26
Surface adhesiveness	3.50^{a} ± 1.22	1.13^{c} ± 1.03	2.50^{b} ± 1.52	3.40^{a} ± 1.02
Appearance	4.33^{a} ± 0.82	2.67^{c} ± 1.51	3.17^{b} ± 1.60	4.00^{a} ± 1.67
Overall sensory score	4.5^{a} ± 0.26	1.63^{c} ± 0.13	2.94^{b} ± 0.10	4.43^{a} ± 0.17

Sample A: control durum wheat; Sample B: native Horse Chestnut flour; Sample C: 0.5% gum addition; Sample D: 1% gum addition. Mean ± S.D. with different superscripts in a row differ significantly ($P < 0.05$) (n = 12).

and gelatinization of starch. Besides, addition of gum results in the development of continuous protein network, which reduces pasta adhesiveness. Padalino *et al.* (2013) and Widelska *et al.* (2019) also observed that the hydrocolloid addition to the gluten-free pasta formulation led to the decrease in adhesiveness. Other textural attributes including cohesiveness, springiness, gumminess, and chewiness also increased with the addition of gum in pasta formulation. Mirhosseini *et al.* (2015) observed a similar kind of improvement in textural attributes with the addition of polysaccharide gums to gluten-free pasta formulations. HCN-flour-based pasta made with 1 % gum show comparable textural properties with that of the control sample.

Sensory quality of pasta

The prepared pasta samples were evaluated for appearance, firmness, chewiness, surface adhesiveness, and slipperiness by sensory panellists using a 5-point scale. The mean values of sensory attributes for pasta samples are shown in Table 8. Sensory score of native HCN pasta (Sample B) was significantly low compared to control sample (sample A), which could be related to the difference in composition and properties of their corresponding flours (Bolarinwa and Oyesiji, 2021). Among the HCN-flour-based pasta samples, Sample B (0% gum) showed a minimum score for sensory attributes (appearance, firmness, chewiness, slipperiness, and adhesiveness) while sample D (1% gum) showed the highest score. No significant difference was observed in the sensory parameters of Sample A (control) and Sample D. Overall, sensory score of Sample D was comparable with that of the control sample. Enhancement in the textural properties of pasta with the addition of gums in pasta formulation led to an improvement in sensory quality. Shere *et al.* (2020) also observed that gum addition enhanced the overall sensory quality of the developed instant noodles.

Conclusions

The results provide an insight about the physicochemical, functional, pasting, and thermal properties of HCN flour, and the quality characteristics of pasta made from HCN flour. The results showed significant difference between the various properties of wheat and HCN flour. Difference in the ratio of flour constituents (protein, starch, etc.) could be the reason for the difference in properties of the two flours. Cooking, textural, and sensory characteristics of HCN-flour-based pasta samples showed that good-quality pasta comparable with commercial

wheat-based pasta could be prepared from HCN flour. The study would serve as a template about the derived properties of HCN flour. Furthermore, gluten-free pasta could be made from HCN flour using appropriate proportion of gum in the production process. In addition, the study suggests that HCN flour could be used as a substitute for conventional flours for the development of gluten-free pasta. Further research should be carried out to determine the possible utilization of HCN flour in the development of other food products.

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Conflict of Interest

The authors state no conflict of interest.

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Cooking methods affect eating quality, bio-functional components, antinutritional compounds and sensory attributes of selected vegetables

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Abstract

The study aimed to evaluate the effect of boiling, air-frying and microwave methods of cooking on the phytochemical and antinutritional activity of some vegetables. Total phenolic content was the highest in kale (9.70 mg GAE/g) using air frying and in carrot using microwave (9.15 mg GAE/g) and boiling (5.16 mg GAE/g) methods. The cooking of vegetables for 15 min of air frying depicted a significant increase in total flavonoids. Oxalate content in vegetables were significantly reduced by air frying, tannins by boiling and saponin in microwave cooking. A significant decrease in oxalate content was observed in kale by air frying and boiling methods, in carrot by microwave cooking, and reduction in tannins in tomatoes by air frying and boiling methods.

Keywords: antinutrients, beans, cooking, kale, phytochemicals, spinach

Introduction

In recent years, consumption of food with bioactive ingredients with nutraceutical potential has increased in consumer diet. Vegetables are one of the natural foods associated with numerous healthy ingredients and are recommended as a part of a healthy lifestyle for end users. Vegetables, consumed as raw, cooked or in processed form, are parts of fresh plants which provide nutrition to humans (Groch, 2008). Consumption of vegetables in the human diet is associated with health benefits because of immunoregulatory and antioxidant properties to fight inflammatory, allergic, atherogenic, microbial, thrombotic, cardiovascular diseases, cancer and diabetes (Rashmi and Negi, 2020). Vegetables are a rich source of fibers, vitamins, minerals and phenolic components (Septembre-Malaterreb *et al.*, 2018). The presence of bioactive nutrients in vegetables is associated with health benefits to fight chronic diseases (Eliassen *et al.*, 2012; Pojer *et al.*, 2013). Phytochemicals, such as flavonols, isoflavones, phenolic acids, flavones, and carotenoids, present in vegetables have demonstrated antioxidant, anti-inflammatory and antitumor effects (Septembre-Malaterreb *et al.*, 2018). Besides phytochemicals, there are secondary metabolites such as antinutrients with a detrimental effect on nutrients and sensory properties of vegetables. Antinutrients most commonly present in vegetables are lectins, tannins, phytic acids, saponins, phytates, glucosinolates, cyanogens and phytoalexins, with reduced bioavailability of essential components and inhibition of digestive enzyme activity (Septembre-Malaterreb *et al.*, 2018; Kaur *et al.*, 2015).

Kale, a leafy and nutritionally important vegetable of the family Brassica, is consumed in different parts of the world (Singh *et al.*, 2009). Kale is a good source of ascorbic acid, carotenoids, flavonoids and polyphenol-rich compounds with health-promoting phytochemicals having reduced

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risk of heart disease, cancers and diabetes (Chenard *et al.*, 2015; Ferioli *et al.*, 2013; Korus and Lisiewska, 2011).

Spinach is a nutritious leafy vegetable containing a good amount of vitamins, dietary fiber, minerals and polyunsaturated fatty acid, a therapeutic ingredient used against arthritis, obesity, cancer, osteoporosis and anemia (Bassey and Khan, 2015; Patricia *et al.*, 2014).

Beans belong to the family Fabaceae and are rich in proteins, starch, dietary fiber, minerals and vitamins (Siddiq *et al.*, 2010). Beans have been associated with many health benefits because of dense and rich ingredients such as flavonoids, phenolic acids and isoflavones (Aparicio-Fernandez *et al.*, 2005; Granito *et al.*, 2008; Lin *et al.*, 2008; Chung *et al.,* 2008).

Carrots are one of the most important vegetables with a potentially rich source of carbohydrates, minerals and carotenoids with anticarcinogenic, antioxidants, and immune-boosting properties (Fiedor and Burda, 2014; Tanaka *et al.*, 2012).

Tomato is a globally cultivated fleshy vegetable with dense and rich nutritive components, including vitamins, minerals and phytochemicals that impart health benefits (Frusciante *et al.*, 2007). Tomato usage in the diet has been related to the reduction of inflammatory risk processes, cancer, cardiovascular disease, hypertension, diabetes and obesity (Adams *et al.*, 2011.

Vegetables with health beneficial ingredients are primarily consumed after processing using different cooking methods with aim of enhancing palatability, digestibility and taste. Cooking treatment induces physical and chemical changes, structure, nutritional quality and texture of vegetables (Rehman *et al.*, 2003; Tepe and Ekinci, 2021). It also induces cell wall permeability and increases the extraction of phytochemicals and reduction in antnutrients (Rodrıguez-Amaya, 1999). A significant loss of vitamins and phytochemicals has been observed during the cooking of vegetables that varies with different cooking methods (Lin and Chang, 2005). Different cooking methods, such as boiling, microwave, steaming, griddled, frying, and baking, affect the structural and nutritional profile of vegetables (Fabbrin and Crosby, 2016; Zhan and Hamauzu, 2004). Oven cooking, air frying, boiling, steaming, microwave treatment, steaming and vacuum cooking are different methods of cooking used for different vegetables to enhance their nutritional profile, bioactive potential, reduce the antinutritional ability and improve sensory acceptability (Fratianni *et al.*, 2021; Nartea *et al.*, 2021; Rana *et al.*, 2021; Rinaldi *et al.*, 2021; Salamatullah *et al.*, 2021; Sun and Ling, 2021). Domestic methods of cooking, such as boiling, air frying and microwave cooking, have increased with an added interest in the phytochemical and antioxidant activity of cooked vegetables. The present study proposes to use optimum time by applying boiling, air frying and microwave methods of cooking, and their effect on phytochemical and antioxidant activity of kale, spinach, beans, carrot and tomatoes.

Material and Methods

Materials

Fresh kale (*Brassica oleracea* var. acephala L.), spinach (*Spinacia oleracea L.*), beans (*Phaseolus vulgaris* L.), carrot *(Daucus carota* L.) and tomatoes (*Solanum lycopersicum* L.*)* were purchased from the vegetable market of Kashmir Valley. After proper removal of post-harvest nonedible parts, the edibles were soaked in clean water (25°C, pH 7), dried and diced into uniform slices. The dried vegetables were subsequently divided into four parts and treated with boiling, air frying and microwave cooking method.

Cooking Treatments

Boiling: Vegetable samples (100 g) were boiled at 100°C in a stainless steel pan with 150-mL distilled water for 2, 4 and 6 min until it becomes tender.

Air frying: Vegetable samples (100 g) were fried at 200°C in an air fryer with 5 mL of hot refined oil for 7.5, 10 and 15 min.

Microwave: Vegetable samples (100 g) were micro-cooked in 150 mL of water at 800-W power for 1, 2.5 and 5 min.

The cooking was followed by an afterward tempering period of a few minutes at room temperature. The tempered samples were subjected to homogenization in a blender. The homogenized samples were packed in polyethene pouches, sealed and stored at -20°C for further analysis.

Proximate analysis

The proximate composition of vegetable samples was determined according to standard procedures of the Association of Official Analytical Chemists (AOAC, 2002) for moisture (method 925.09), fat (method 920.39), protein (method 955.04), crude fiber (method 962.09) and ash content (method 991.43).

Phytochemical and antioxidant activity of vegetables

Preparation of extract

Homogenized vegetable samples, 2 g, were extracted with 80% methanol (50 mL). Then centrifugation of

samples was performed at 2,200 rpm for 15 min at room temperature. The decanted supernatant was stored at 4°C for determining total phenolic content, total flavonoid content and antioxidant activity of vegetable samples.

Total phenol content

The total phenolic content of vegetable samples was determined according to the method demonstrated by Singleton *et al.* (1999). A sample extract of 0.2 mL was treated with Folin Ciocalteu reagent (1 mL). After 3 min, the reaction mixture was added with 20% sodium carbonate solution (0.80 mL) and stirred properly and incubated for 2 h at ambient temperature. The absorbance value of the reagent mixture was measured at a wavelength of 725 nm using an ultraviolet-visible spectroscopy (UV/VIS) (UV-1700 Spectrophotometer, Japan). The results were expressed as mg GAE/g using gallic acid as standard.

Total flavonoid content

The total flavonoid content of cooked vegetables was measured by the method described by Ebrahimzadeh *et al.* (2008). A sample extract of 2 mL was mixed with 10% aluminium chloride (100 µL), followed by the addition of 1 N potassium acetate (100 µL). The reagent mixture was diluted with 2.8 mL of distilled water. The sample mixture was incubated at room temperature for 30 min and absorbance was measured at 415 nm. The total flavonoid content was expressed as quercetin equivalent (mg QE/g of sample) using quercetin as standard.

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity

The DPPH free radical assay was used to determine the antioxidant activity of cooked vegetables as per the method described by Mulla and Ahmed (2019). Sample extract, 100 µL, was used for antioxidant activity and was added with 1 mL of 0.01% DPPH reagent. The sample mixture was stirred properly and incubated for 30 min in the dark. Absorbance was measured at 517 nm after 30 min using a spectrophotometer. The antioxidant activity using free radical scavenging was measured as follows:

$$\text{Antioxidant acitivity (\%)} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100 \quad (1)$$

where Abs control = absorbance of control at 30 min, and Abs sample = absorbance of sample solution at 30 min.

Antinutritional component analysis of cooked vegetables

Oxalate content

The oxalate content of cooked vegetables was determined using the method described by Ghoora *et al.* (2020) with minor modifications. Cooked vegetable sample (1 g) was treated with 75 mL of 3-M sulfuric acid, stirred constantly for 1 h, followed by filtering through Whatman filter paper No. 1; 25-mL filtrate aliquot was titrated with potassium permanganate ($KMNO_4$ 0.05 M) solution till faint pink color appeared as endpoint. Titration with 1 mL of 0.05-M $KMNO_4$ is related to 2.2 mg of oxalate (Chinma and Igyor, 2007) and expressed as mg/g of oxalate.

Tannin content

The tannin content in vegetables was analyzed according to the standard procedure of AOAC (2002) with minor modifications. The cooked vegetable sample (3 g) was extracted with distilled water for 4 h and filtered for tannin analysis. Tannin extract amounting to 25 mL was treated with 25 mL of indigo solution. The mixture was diluted with 750 mL of distilled water and titrated with 0.1-N $KMNO_4$ solution until the blue color of the solution changed to green color. Finally, tannin content in samples was calculated using the following equation:

$$\text{Tanin (mg/g)} = (V - V_0) \times 0.004157 \times 250 \times 100/g \times 25, \quad (2)$$

where V and V_o are volume of 0.1-N $KMNO_4$ solution for the titration of sample and blank (without the sample), respectively; 0.004157 is the tannin factor.

Saponin content

Saponin content in cooked vegetables was determined by the method described by Obadoni and Ochuko (2001). Cooked vegetables (3 g) were mixed with 20% aqueous ethanol (30 mL) with constant stirring for 3 h on a hot plate. The sample of ethanol mixture was filtered and concentrated, followed by the addition of 10-mL diethyl ether in a separating funnel with vigorous shaking. The separated aqueous part was used and treated with n-butanol (20 mL). The n-butanol extract was washed twice with 5% aqueous NaCl (10 mL). The remaining solution was heated in a water bath. Finally, the concentrated sample formed after evaporation was dried on a dry bath to a constant weight. The saponin content was expressed using the following equation:

$$\text{Saponin (mg/g)} = \frac{W_2 - W_1}{W} \times 100, \quad (3)$$

where W_1 is the weight of the evaporating disk, W_2 is the weight of the disk and sample and W is the weight of the sample.

Sensory analysis of cooked vegetables

The sensory evaluation of cooked vegetables was performed by 15 semi-trained panellists having the basic knowledge of sensory analysis, nutritional interest and

willingness to participate. The assessment was exercised for color, flavor, taste texture and overall acceptability score using a 5-point hedonic rating scale to generate the following measurable sensory scores: Excellent for a score of 5, Good for a score of 4, Average for a score of 3, Fair for a score of 2 and Poor for a score of 1.

Statistical analysis

The experimental data were expressed as mean and standard deviation in triplicate using SPSS Version 16. Phytochemical and antinutritional analyses were performed using one-way ANOVA, followed by Duncan's multiple range tests at a 5% level of significance.

Results and Discussion

Proximate composition of fresh vegetables

The nutrition analysis of fresh vegetables selected for different cooking methods is presented in Table 1. The selected vegetables differed significantly in nutritional composition. The moisture content of fresh vegetables ranged from 84 to 83 g/100 g. Tomatoes depicted the highest moisture content (91.60 g/100 g), followed by carrot (89.30 g/100 g), kale (88.60 g/100 g), beans (81.80 g/100 g) and the lowest in spinach (81.97 g/100 g). The selected fresh vegetables were low in protein content, ranging from 0.86 to 3.93 g/100 g. Beans reported the highest protein content of 3.93 g/100 g and the lowest was determined in carrot (0.86 g/100 g). Low fat content was reported in all vegetables, ranging from 0.20 to 0.60 g/100 g. Fiber content in fresh vegetables ranged from 1 to 10 g/100 g, and higher content was observed in spinach (10.00 g/100 g), followed by beans (5.00 g/100 g), kale (4.00 g/100 g), carrot (3.00 g/100 g) and the lowest in tomatoes (1.00 g/100 g). Ash content in selected fresh vegetables was the highest in spinach (2.00 g/100 g), followed by kale (1.60 g/100 g), carrot (1 g/100 g), tomatoes (0.80 g/100 g) and the lowest in beans (0.10 g/100 g). Carbohydrate content in selected fresh vegetables varied from 2.05 to 8.97 g/100 g, with the highest reported in beans (8.97 g/100 g) and the lowest in kale (2.05 g/100 g). Variations in the nutrient composition of selected vegetables could be due to different agro-climatic conditions (Singh *et al.*, 2001). Results of the nutritional composition of fresh vegetables demonstrated similar results as reported by Naz *et al.* (2018) and Rani and Fernando (2016).

Effect of cooking methods on phytochemicals of vegetables

Total phenolic content

Boiling, air frying and microwave cooking methods depicted increase in the level of total phenolic content of selected vegetables (Figure 1). Among raw vegetables, total phenolic content was reported the highest in tomatoes (2.03 mg GAE/g), followed by carrot (1.97 mg GAE/g), beans (1.69 mg GAE/g), spinach (1.19 mg GAE/g) and the lowest in kale (1.13 mg GAE/g). Boiling time from 2 to 8 min significantly increased the total phenolic content from 4.01 to 5.00 mg GAE/g for kale, 3.32 to 4.30 mg GAE/g for spinach, 2.07 to 4.01 mg GAE/g for beans, 1.76 to 5.60 mg GAE/g for carrot and 2.99 to 3.63 mg GAE/g for tomatoes. The air frying of selected vegetables from 7.5 to 15 min reported a significant increase in total phenolic content. Increase in total phenolic content was reported in: kale from 1.60 to 9.70 mg GAE/g, spinach from 4.84 to 7.47 mg GAE/g, beans from 6.14 to 8.04 mg GAE/g, carrot from 2.82 to 5.12 mg GAE/g and tomatoes from 5.31 to 6.58 mg GAE/g. Microwave cooking also demonstrated increase in total phenolic content with respect to processing time (1, 2.5, and 5 min). The total phenolic content of selected vegetables demonstrated an increasing trend with increase in processing time from 1 to 5 min in microwave cooking. Increase in the total phenolic content of selected vegetables in 1 to 5 min was reported in: kale from 4.31 to 7.78 mg GAE/g, spinach from 4.45 to 7.24 mg GAE/g, beans from 5.20 to 7.17 mg GAE/g, carrot from 2.77 to 9.15 mg GAE/g

Table 1. Proximate composition of vegetables.

	Proximate composition (g/100 g)				
	Kale	Spinach	Beans	Carrot	Tomatoes
Moisture	88.60 ± 0.10	81.97 ± 1.00	81.80 ± 0.01	89.30 ± 0.10	91.60 ± 1.00
Ash	1.60 ± 0.02	2.00 ± 0.10	0.10 ± 0.69	1.00 ± 0.15	0.80 ± 0.01
Fat	0.60 ± 0.01	0.63 ± 0.01	0.20 ± 0.39	0.20 ± 0.39	0.40 ± 0.01
Protein	3.15 ± 0.02	2.97 ± 0.01	3.93 ± 0.02	0.87 ± 0.01	0.96 ± 0.01
Crude Fiber	4.00 ± 0.10	10.00 ± 0.10	5.00 ± 0.10	3.10 ± 0.10	1.00 ± 0.15
Carbohydrate	2.05 ± 0.02	2.43 ± 0.01	8.97 ± 0.01	5.53 ± 0.02	5.24 ± 0.02

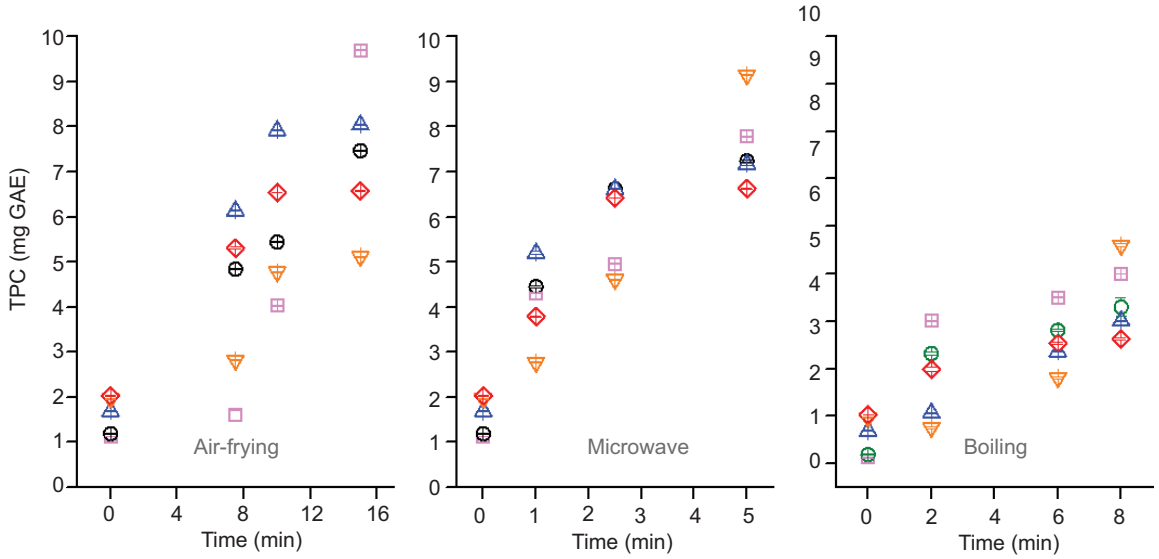

Figure 1. Effect of different cooking methods on the phenolic content (mg GAE/g) of kale (□), spinach (○), beans (△), carrot (▽) and tomatoes (◇).

and tomatoes from 3.79 to 6.63 mg GAE/g. Use of different cooking methods indicated significant improvement in phenolic content of selected vegetables as compared to raw vegetables; however, the use of microwave as a cooking medium significantly increased the total phenolic content of selected vegetables than boiling and air frying methods. The increasing trend of total phenolic content in selected vegetables was due to the release of polyphenols from intracellular complexes during cooking, inactivation of polyphenol oxidase (PPO) enzyme (Oboh, 2005), release of bioactive compounds because of the breakdown of cell walls (Ferracane *et al.*, 2018), and increase in free flavonols (Stewart *et al.*, 2000). Similar findings were also reported in cooked vegetables by Geetha *et al.* (2018), Hossain *et al.* (2017) and Saikia and Mahanta (2013).

Total flavonoid content

The total flavonoid content of selected vegetables is evidenced in Figure 2. Fresh vegetables contained 0.05–0.10 mg QE/g of total flavonoids, and the highest flavonoids were reported in spinach and the lowest in tomatoes and kale. Boiling, air frying and microwave cooking methods reported an increasing trend in total flavonoids content in selected vegetables. The boiling method of cooking with respect to the duration of 2, 6 and 8 min significantly increased the total flavonoids content of vegetables. With increasing boiling time from 2 to 6 min, flavonoids content in: kale was increased from 0.63 to 1.33 mg QE/g, spinach from 0.15 to 0.78 mg QE/g, beans from 0.12 to 0.33 mg QE/g, carrot from 0.04 to 0.27 mg QE/g and tomatoes from 0.38 to 0.61 mg QE/g. The air frying method of cooking demonstrated a significant increase in flavonoid content in kale, spinach and beans than the boiling and microwave methods of cooking. Selected vegetables cooked for 7.5, 10 and 15 min by air frying method depicted a similar trend of increased flavonoid content. The air frying method with an optimized duration of 7.5–15 min reported an increase in flavonoid content from: 1.35 to 2.41 mg QE/g for kale, 0.96 to 2.23 mg QE/g for spinach, 0.38 to 1.42 mg QE/g for beans, 0.04 to 0.19 mg QE/g for carrot and 0.25 to 0.31 mg QE/g for tomatoes. Microwave cooking for a short duration of 1, 2.5 and 5 min also depicted a similar trend of increased flavonoid content in kale, spinach, beans, carrot and tomatoes (Figure 2). In microwave cooking, at a processing time of 5 min, kale had the highest flavonoid content (0.97 QE mg/g), followed by spinach (0.95 QE mg/g), tomatoes (0.79 QE mg/g), carrot (0.52 QE mg/g) and the lowest in beans (0.44 QE mg/g). A significant increase in the flavonoid content of selected vegetables by different cooking methods is attributed to extracted flavonoid compounds of vegetable matrix and changes in the structure of cell walls of vegetables during cooking (Wachtel-Galor *et al.*, 2008). Variations in the flavonoid content of selected vegetables because of different cooking methods and processing time could be due to differences in the extraction of flavonoids in vegetables, heating medium and duration of cooking. Similar results of increased total flavonoid content in vegetables were reported by Geetha *et al.* (2018), Mazzeo *et al.* (2011) and Saikia and Mahanta (2013).

Antioxidant activity

The reported antioxidant activity of fresh vegetables was the highest in tomatoes (25.90%), followed by carrot (14.81%), beans (11.11%), kale (7.40%) and the lowest in

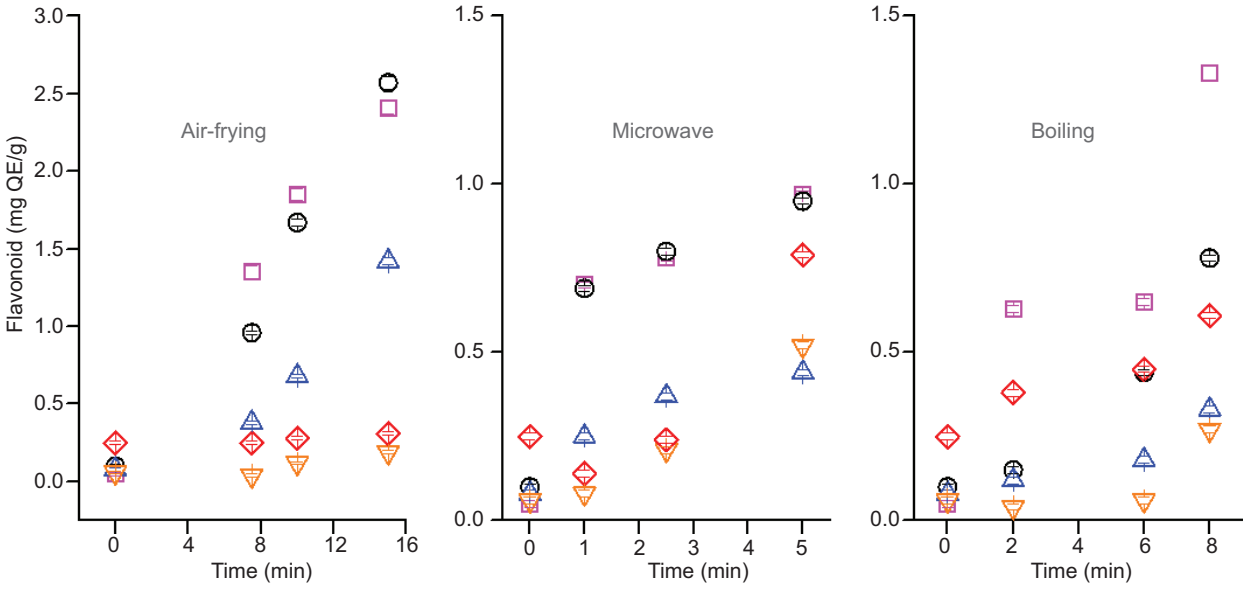

Figure 2. Effect of different cooking methods on the flavonoid content (mg QE/g) of kale (□), spinach (○), beans (△), carrot (▽) and tomatoes (◇).

spinach (3.70%) (Figure 3). The boiling method significantly increased the antioxidant activity of selected vegetables with respect to the optimized cooking time of 2, 6 and 8 min. The boiling method of cooking for 2–8 min reported a significant increase in the antioxidant activity of kale (18.50–75.10 %), spinach (32.90–85.96%), beans (27.03–51.85%), carrot (42.22–83.70%) and tomatoes (38.51–97.20%). Antioxidant activity in selected vegetables differed significantly with respect to optimized time (7.5, 10 and 15 min) in the air frying method of cooking. Antioxidant activity significantly increased from: 7.40% to 28.14% for kale, 37.03% to 55.10% for spinach, 27.40% to 43.70% for beans, 51.80% to 64.40% for carrot, and 35.88% to 67.93% for tomatoes. The microwave method of cooking established a similar trend of increased antioxidant activity in selected vegetables with respect to optimized processing time (1, 2.5 and 5 min) as compared to fresh vegetables. The optimized cooking time of 5 min demonstrated higher antioxidant activity, with the highest of 92.50% reported in carrot, followed by 91.40% for beans, 79.25% for tomatoes, 62.50% for spinach and 54.07% for kale. An increase in the antioxidant activity of selected vegetables because of different cooking methods could be due to the Maillard reaction (Saikia and Mahanta, 2013), secondary metabolites (Tayebeh *et al.*, 2021), the release of antioxidant compounds from disrupted cell walls, inhibition of oxidative enzymes and reduced oxidation of antioxidants (Morales and Babel, 2002), and increased phytochemicals during cooking (Zeb *et al.*, 2018). Similar findings were reported by Agamy (2016), Hossain *et al.* (2017), and Mirzaei *et al.* (2014).

Effect of cooking methods on antinutritional components of vegetables

Oxalate content

Boiling, air frying and microwave cooking evidenced a decrease in the level of oxalate content in selected vegetables as compared to fresh ones (Figure 4). The highest oxalate values in raw vegetables were observed in tomatoes (6.60 mg/g), followed by spinach (5.72 mg/g), beans (3.96 mg/g), kale (1.58 mg/g) and the lowest in carrot (1.41 mg/g). Cooking of selected vegetables by boiling significantly reduced oxalate content with respect to the optimized processing time of 2, 6 and 8 min. Reduction in oxalate content was observed in 2–8 min of boiling, varying from 0.88 to 0.22 mg/g for kale, 3.10 to 1.44 mg/g for spinach, 4.10 to 2.42 mg/g for beans, 4.10 to 2.42 mg/g for carrot and 5.50 to 4.40 mg/g for tomatoes. Air frying with optimized processing time (7.5, 10 and 15 min) also decreased the oxalate content of selected vegetables. Reduction in oxalate content at 15 min of processing was observed for kale (0.20%), 1.32 mg/g for spinach, 0.44 mg/g for beans, 0.46 mg/g for carrot and 1.54 mg/g for tomatoes. The microwave cooking method at a processing time of 1–5 minutes reduced oxalate content from: 0.77 to 0.44 mg/g in kale, 2.42 to 0.88 mg/g in spinach, 4.62 to 2.86 mg/g in beans, 0.98 to 0.32 mg/g in carrot and 6.38 to 4.35 mg/g in tomatoes. In general, cooking methods reduced the oxalate content of vegetables as compared to raw vegetables; however, air-frying and boiling presented significantly higher reductions than microwave cooking. The significant reduction in oxalate content in selected vegetables could be due to leaching

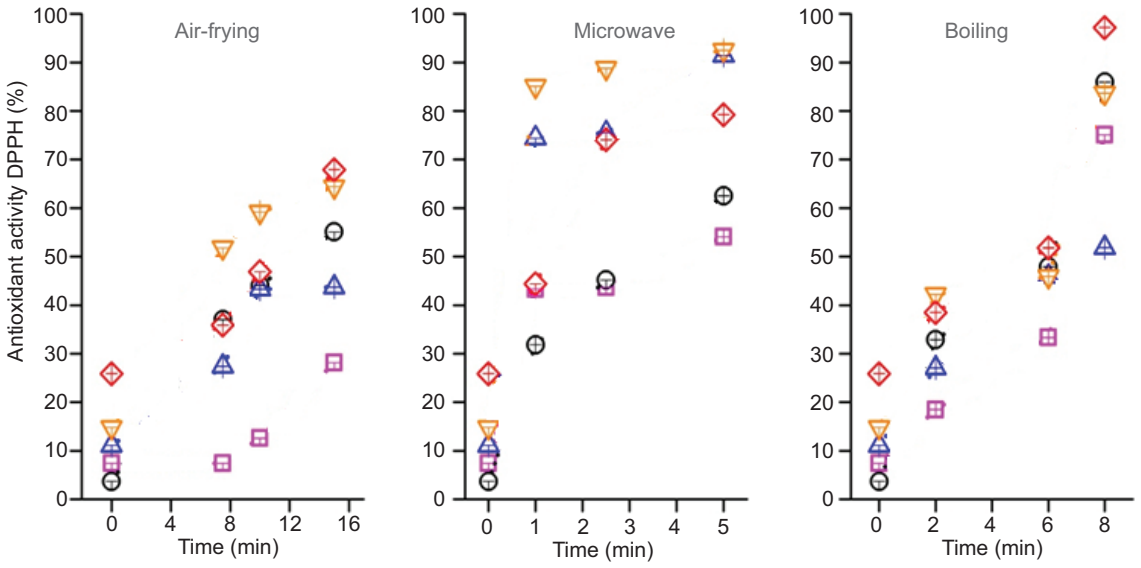

Figure 3. Effect of different cooking methods on the antioxidant activity of kale (□), spinach (○), beans (△), carrot (▽) and tomatoes (◇).

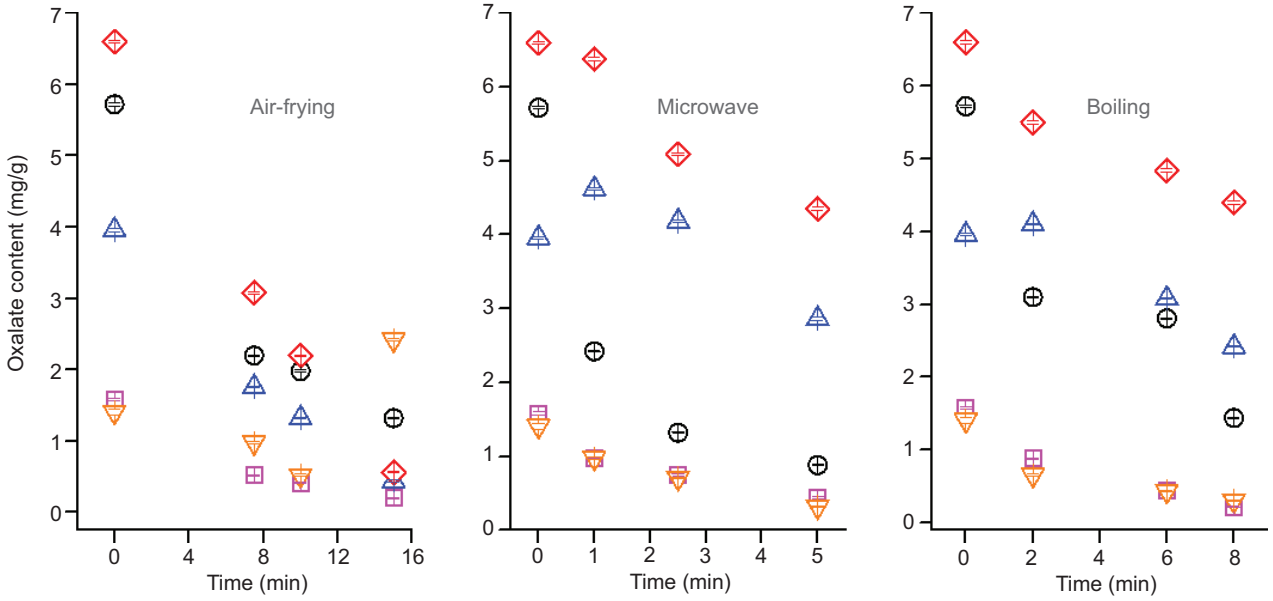

Figure 4. Effect of different cooking methods on the oxalate content of kale (□), spinach (○), beans (△), carrot (▽) and tomatoes (◇).

out of soluble oxalate from disrupted tissues during the cooking process (Catherwood *et al.*, 2007; Yadav and Sehgal, 2003). Reduction in oxalate content in cooked vegetables was also observed by Agiriga and Siwela (2018), Akhtar *et al.* (2014) and Hemmige *et al* (2017).

Tannin content

Cooking of vegetables by boiling, air frying and microwave methods significantly reduced ($p < 0.05$) the tannin content as compared to fresh vegetables (Figure 5). The tannin content in fresh vegetables ranged from 6.30 to 15.4 mg/g. The highest tannin content was observed in beans (15.4 mg/g), followed by carrot (10.2 mg/g), spinach (9.20 mg/g), tomatoes (8.30 mg/g) and kale (6.30 mg/g). The tannin content in cooked vegetables reduced significantly with respect to the optimized processing time for boiling (2, 4 and 6 min), air frying (7.5, 10 and 15 min) and microwave cooking (1, 2.5 and 5 min). The reduced tannin content in selected vegetables at 15 min of boiling was observed as 0.30 mg/g for kale, 1.20 mg/g for spinach, 1.20 mg/g for beans, 1.0 mg/g for carrot and 0.1 mg/g for tomatoes. However, air frying for 15 min and

microwave for 5 min, respectively, recorded 2.0 mg/g and 0.1 mg/g for kale, 1.30 mg/g and 4.10 mg/g for spinach, 2.60 mg/g and 1.20 mg/g for beans, 1.50 mg/g and 1.20 mg/g for carrot and 1.0 mg/g and 1.50 mg/g for tomatoes. The reduction of tannins in cooked vegetables could be related to the thermal degradation of tannins during the process of cooking (Miglio *et al*, 2008). Similar results in vegetables were also reported by Alajaji and El-Adawy (2006) and Hemmige *et al.* (2017).

Saponin content

Cooking vegetables by boiling, air frying and microwave methods significantly reduced the saponin content of vegetables as compared to fresh ones (Figure 6). Saponin content in fresh vegetables ranged from 19.0 to 32.10 mg/g. The highest saponin content in raw vegetables was observed in spinach (32.10 mg/g), followed by kale (24.50 mg/g), beans (22.30 mg/g), tomatoes (20.30 mg/g) and carrot (19.0 mg/g). The saponin content of cooked

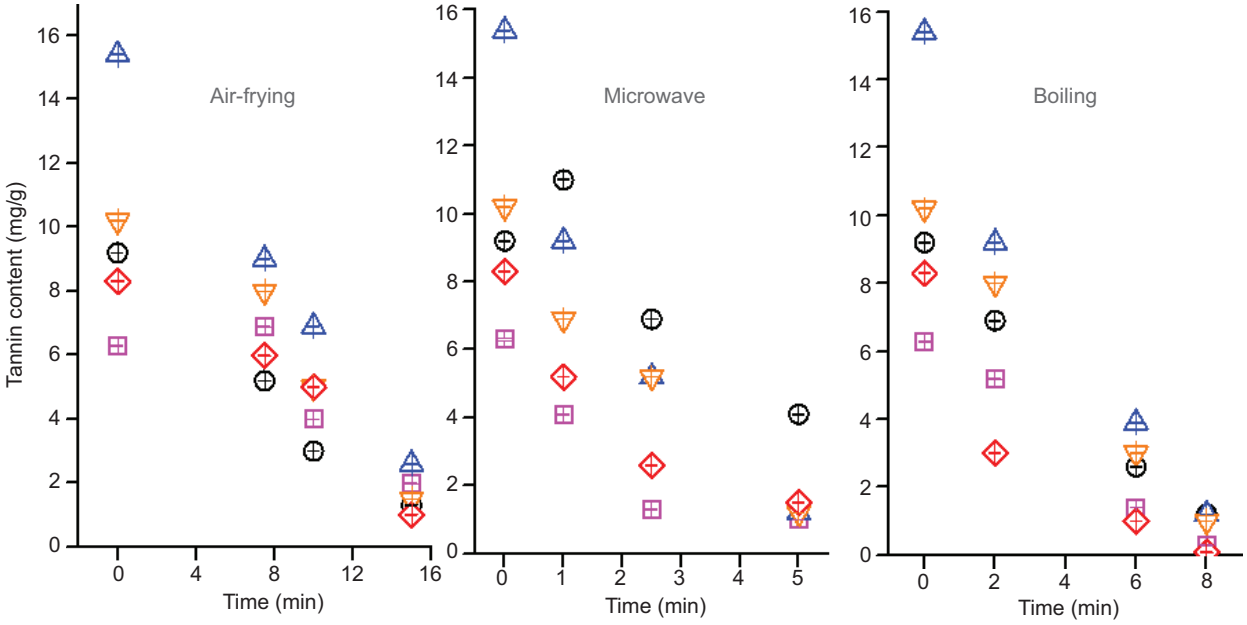

Figure 5. Effect of different cooking methods on the tannin content of kale (□), spinach (○), beans (△), carrot (▽) and tomatoes (◇).

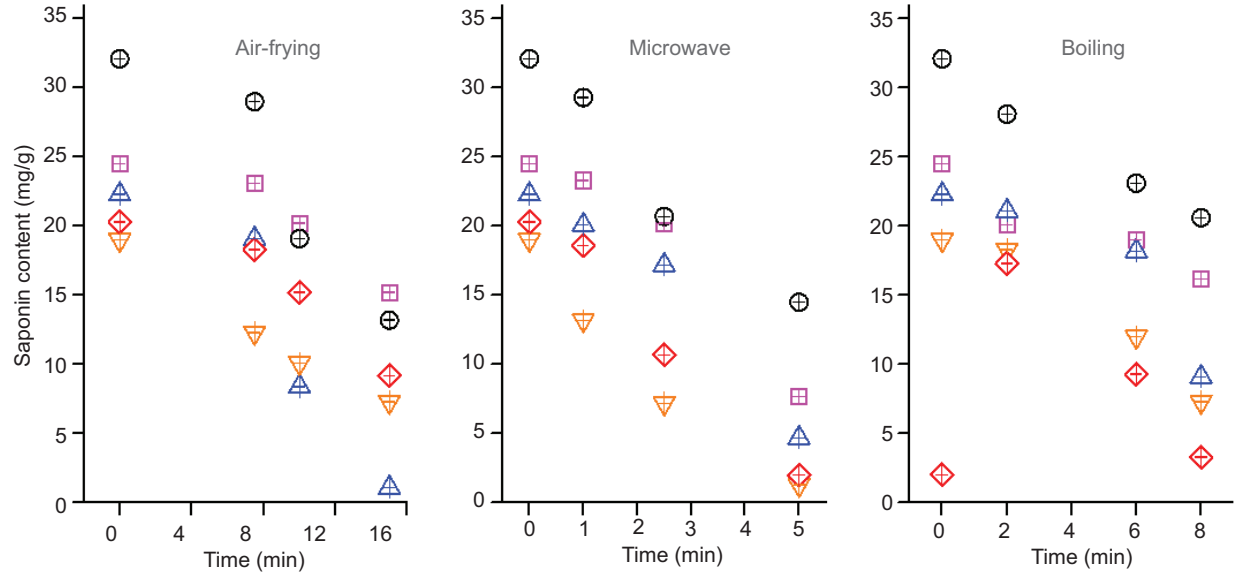

Figure 6. Effect of different cooking methods on saponin content of kale (□), spinach (○), beans (△), carrot (▽) and tomatoes (◇).

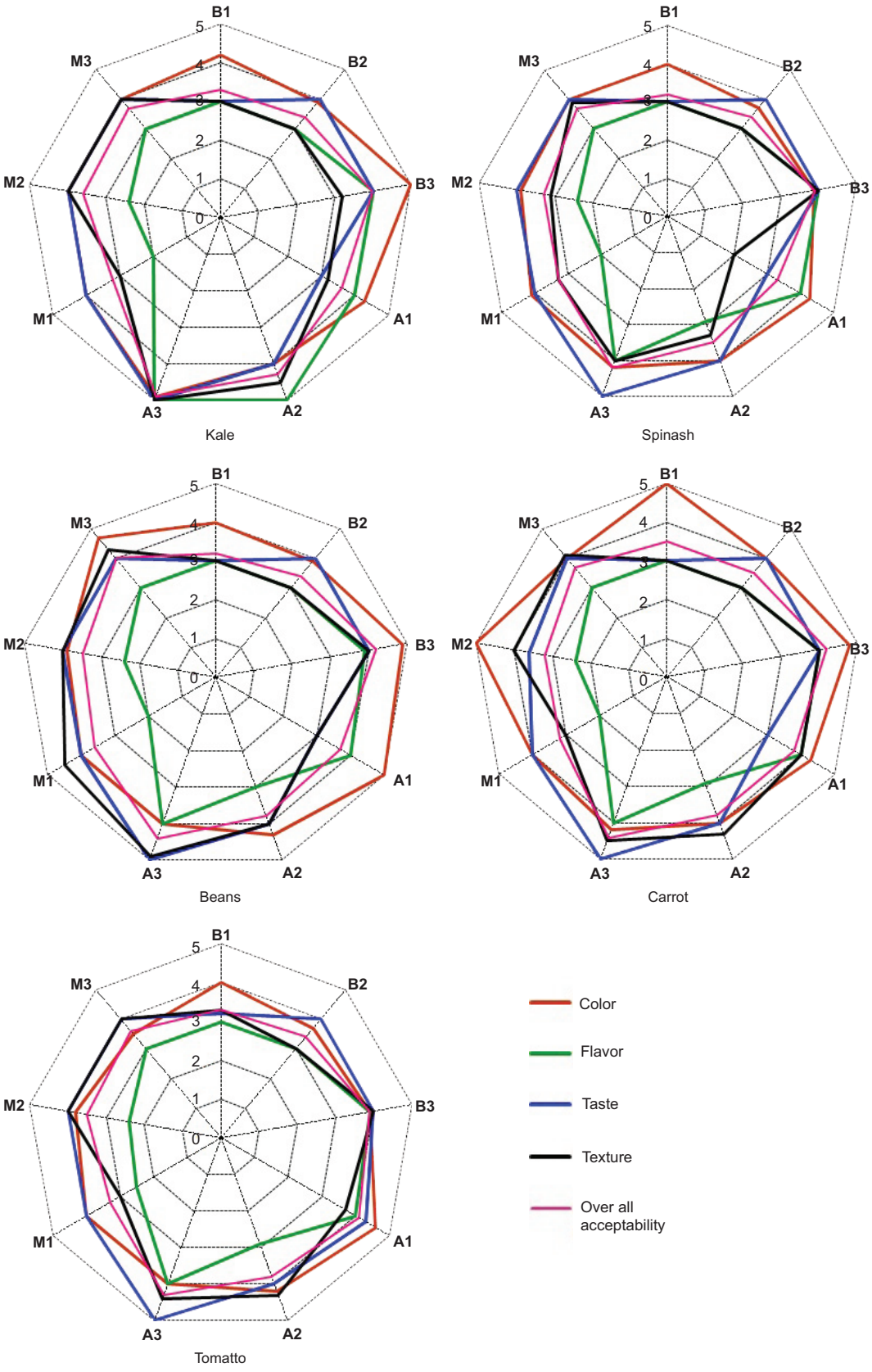

Figure 7. Effect of different cooking methods on the sensory evaluation of vegetables. A1, A2 and A3 are air frying method of cooking vegetables for 7.5, 10 and 15 min; B1, B2 and B3 are boiling method of cooking vegetables for 2, 6 and 8 min; and M1, M2 and M3 are microwave method of cooking vegetables for 1, 2.5 and 5 min.

vegetables is significantly reduced with boiling, air frying and microwave cooking. Boiling significantly reduced saponin content with an increase in cooking time from 2 to 6 min. Reduction in saponin content at 6 min of processing time in cooked vegetables was 16.20 mg/g for kale, 20.60 mg/g for spinach, 9.10 mg/g for beans, 7.30 mg/g for carrot and 3.30 mg/g for tomatoes. In air frying, saponin content reduced significantly at the processing time of 7.5, 10 and 15 min. Reduction in saponin content of cooked vegetables at 15 min of air frying was 15.20 mg/g for kale, 13.2 mg/g for spinach, 1.10 mg/g for beans, 7.30 mg/g for carrot and 9.20 mg/g for tomatoes. Microwave cooking also decreased saponin content with an optimized cooking time of 1, 2.5 and 5 min. A higher level of reduction in the saponin content of cooked vegetables at 5 min of processing was as follows: 7.70 mg/g for kale, 14.50 mg/g for spinach, 4.70 mg/g for beans, 1.30 mg/g for carrot and 2.0 mg/g for tomatoes. However, microwave and air frying demonstrated a significantly higher reduction in saponin content in cooked vegetables than by the boiling method. Reduction of saponin content could be related to thermal degradation associated with heat supplied to cooked vegetables. This was also observed by Alajaji and El-Adawy (2006), Badifu and Okeke (1992), Hemmige *et al.* (2017) and Ilelaboye *et al.* (2013).

Sensory evaluation of boiling, air frying and microwave in cooked vegetables

The sensory evaluation of vegetables is presented in Figure 7. Cooking of vegetables by boiling, air frying and microwave method improved the sensory property of cooked vegetables, besides increasing bioactive compounds and reducing antinutritional factors. Boiling, air frying and microwave cooking significantly improved the color, flavor, taste and texture of cooked vegetables with an increased duration of optimized cooking time. In boiling, the highest overall acceptability based on color, flavor, taste and texture was observed at 8 min of cooking for beans (4.2) and carrot (4.2), followed by kale (4.0) and the lowest in the case of spinach (3.9) and tomatoes (3.9). In air frying, the highest overall acceptability observed at 15 min of cooking was as follows: kale (4.9), beans (4.4), carrot (4.4), tomatoes (4.3) and the lowest in spinach (4.2). However, microwave cooking at 5 min established higher overall acceptability for beans (4.0), followed by carrot (3.7), kale (3.7), spinach (3.7) and the lowest for tomatoes (3.6). The sensory evaluation based on 5-point hedonic scale depicted higher consumer acceptability for air frying than boiling and microwave cooking. Significant improvement in sensory attributes of cooked vegetables by boiling, air frying and microwave cooking could be due to enhancement in color, taste and flavor, and retention of texture during cooking (Nunn *et al.*, 2006). The sensory scores of cooked vegetables by boiling, air frying and microwave cooking were in the acceptable range, with a high recommendation of processes for preparing semi-processed vegetables with good consumer acceptance. Similar results of sensory evaluation of cooked vegetables have been reported by Şengul *et al.* (2014), Soomro *et al.* (2018) and Sultana *et al.* (2008).

Conclusion

Vegetables are a rich source of vitamins and phytochemicals, and their consumption in daily diet, besides providing nutrition, helps to fight chronic diseases. Cooked vegetables such as kale, spinach, beans, carrot and tomatoes are a rich source of phytochemicals and antioxidants as compared to fresh ones, which are lower in antinutrients. The cooking of vegetables by boiling, air frying and microwave methods manifests significant improvement in bioactive components. Cooking vegetables significantly increases their phytochemical and antioxidant activity. Boiling, air frying and microwave cooking of vegetables significantly decrease their antinutritional components, thus decreasing the bioavailability of nutrients in vegetables. The stated methods of cooking of selected vegetables increased phenolic content and flavonoids but reduced saponin, tannin and oxalate contents. Phytochemicals had a positive relationship with the cooking time for all three cooking methods. Consumer acceptability of cooked vegetables was highest for the air frying method of cooking as compared to boiling and microwave cooking.

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Replacement of meat by mycoproteins in cooked sausages: Effects on oxidative stability, texture, and color

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Abstract

Processed meat is one of the most consumed products worldwide. Naturally, production of proteins with animal origins includes limitations such as costs, energy, time, and environmental problems. Thus, replacement of meats by alternative biomaterials such as mycoproteins can be promising. Mycoproteins with hyphal morphologies, including branches and lengths, have close structures to meat and can be a potential alternative for meat products. Therefore, the major objectives of this study included complete replacement of sausage meats by mycoproteins and comparing characteristics of the novel formula with those of meat. In general, physicochemical, microbial, nutritional, and mechanical characteristics of the formulas were assessed. Results showed that the mycoprotein substitution improved the nutritional and health effects due to the higher valuable protein and lower lipid contents. Besides, it had a high content of essential amino acid and unsaturated fatty acid, compared to meat sausage. Absence of yeasts, molds, *Salmonella* spp., *Eshrichia* (*E.*) *coli*, and *Staphiloccocus* (*S.*) *aureus* verified the effectiveness of the heat treatment and also the effectiveness of the hygienic procedures in both samples. With regard to phycicochemical properties, more contents of moisture and lipids in sausages containing mycoprotein were linked to further water binding capacity (WBC) ($P < 0.05$) and oil binding capacity (OBC) in them, compared to beef samples. Besides, the mycoprotein sample had lower ($P < 0.05$) values of carbohydrates, ash, and pH, compared to the beef sample. In contrast, beef sausages had better textural characteristics, such as hardness, cohesiveness, gumminess, and springiness indexes, compared to mycoprotein sausages. Higher water and OBC values of the mycoproteins led to the filling of the protein interstitial spaces as well as decreasing of the textural attributes. Thus, it resulted in the use of less oil and water in mycoprotein formulations. In conclusion, mycoproteins can be addressed as appropriate replacements for meats in sausages.

Keywords: meat alternatives; mycoproteins; nutritional values; sausages; textural properties

Introduction

In recent decades, the world population has significantly increased from 2.6 to 8 billion individuals (Gabriel *et al.*, 2014). If the global population grows at the recent rate, it may reach 9 billion individuals by 2042, which could pose serious problems in providing food to all (Upadhyaya *et al.*, 2016). Approximately, 1 billion people globally will not be able to properly access food sources with sufficient energy and proteins (Godfray *et al.*, 2010). This can result in serious medical problems such as defective immune system and stunted growth. In contrast, high

consumption of meat products can pose serious health problems as well (Qian *et al*., 2020). Research and development of meat replacements majorly focuses on the production of products that imitate the physical characteristics of meat such as appearance, taste, and texture, as well as providing its nutritional values. Muscle products, such as chicken and steaks, minced products, such as burgers and nuggets, and emulsion products, such as Frankfurter and Mortadella sausages, are the major meat replacements (Kyriakopoulou *et al*., 2021). However, excessive meat consumption can significantly affect the global climate change (Hashempour-Baltork *et al*., 2020b). Thus, the quest for novel substitutes for animal proteins requiring less financial resources, energy, and time consumption can be promising. One of the best meat substitutes includes mycoproteins with relatively similar textures to meat (Upadhyaya *et al*., 2016). The common source of mycoproteins is usually *Fusarium* (*F.*) *venenatum*, a filamentous fungus generally recognized as safe (GRAS) (Hashempour-Baltork *et al*., 2020b). Generally, mycoproteins contain 10 g of carbohydrates, 13 g of fats, 25 g of fibers, and 45 g of proteins, as well as various vitamins, carotenes, minerals, and essential amino acids (EAA) in 100 g of dry matter (Finnigan *et al*., 2019). Recent studies on human volunteers have demonstrated that biological values of proteins from mycoproteins are similar to biological values of proteins from milks.

Commonly, sensory characteristics, such as texture, taste, and overall appearance of the final products, are critical for their overall acceptance. Fusarium biomass is virtually odorless and tasteless and is appropriate to imitate the consistency and taste of regular meats. Comparing mycoproteins with textured soya and poultry muscle tissues, recent studies using microscopy techniques have shown that large cable-like fibers (especially in vegetable protein textures) result in rubbery textures, which are unfavorable when chewing. Technically, further fibrous and rubbery eating qualities occur in poultry and mycoprotein products due to their tight packaging laminations, compared to those that occur in products of vegetable protein origins (Hashempour-Baltork *et al*., 2020b). Ideally, mycoproteins can be good replacements for meat since their dry weights include nearly 50% of proteins, similar to grilled steaks. However, the fungi include lower fat quantities (~13%) than those steaks do. Furthermore, this is a vegetable fat with cholesterol alternative (ergosterin) and good fiber content (~25%), which are increasingly accepted by the health-conscious people (Finnigan *et al*., 2017). Based on the literature, mycoproteins can mitigate the problem of food unavailability worldwide. Moreover, even routine production of mycoproteins would need only lower water resources and occupy less land (Hashempour-Baltork *et al*., 2020b). The use of mycoproteins can limit foodborne diseases and lower blood cholesterol. Toxin analysis and allergy assays have shown no general concerns (Hashempour-Baltork *et al*., 2020a). However, a little information is available on various formulations for the replacement of food meats by mycoproteins. Hence, the major objective of the current study was to compare physicochemical, microbial, nutritional, and mechanical characteristics of sausages containing mycoproteins with those containing meat.

Materials and Methods

Preparation of sausages

Sausage samples (40% red meat) were prepared in three replications in a famous meat production factory, Tehran, Iran. Frozen beef samples were defreezed at 4°C for 16 h before use. Then, beef samples were minced twice using laboratory mincer (Model MK-G1800, Panasonic, Japan) equipped with 6–10 mm steel plates. Mycoprotein masses were provided by Ghazabon Paya, Iran. Two sausages of mycoproteins and meats were prepared for 4 kg batters (Table 1) based on the guidelines from meat producers. Formulations of both samples were mostly similar, with differences in meat and mycoprotein contents.

To prepare sausages, minced beef/mycoprotein was transferred into a bowl chopper (Robot Coupe Model R-10, France) and mixed slowly with the dry ingredients, except spices. Ice was continuously added to the mixture in the chopping process to control the temperature. Then, oil and spices were added to the mixture, respectively. The total time of mixing was 10 min while the final temperature of the batters was set below 12°C. Then, the batters were stuffed into impermeable cellulose casings using a hydraulic piston-type stuffer. Then, the sausages were cooked at 76°C for 60 min using a steam chamber and then cooled down to a final temperature of 10°C using ice-water bath and stored at 4°C overnight (Kamani *et al*., 2019). In general, each sausage type was prepared in two batches. Totally, two sausages from each batch were chosen for further analysis.

Table 1. Sausage ingredients.

Ingredient	Content (% w/w)
Meat/mycoprotein	40
Sunflower oil	10
Ice	20
Mixed spices	3.5
Soy protein isolate	5
Gluten	10
Flour	10
Salts	1.5

Physicochemical characteristics

Proximate pH, moisture, protein, lipids, carbohydrate, ash, peroxide value, water holding capacity (WHC), oil binding capacity (OBC), and water binding capacity (WBC) of the two sample types were assessed using official methods (AOCS, 2017).

Microbial characteristics

The count of microorganisms, *Escherichia* (*E.*) *coli*, *Salmonella* spp., *Staphylococcus* (*S.*) *aureus*, *Bacillus* (*B.*) *cereus*, *Clostridium* (*C.*) *perfringens*, yeasts, and molds were enumerated based on the ISO protocols (ISO, 2013).

Nutritional characteristics

Vitamins

Briefly, 10 g of the ground samples were weighed using a 50-mL glass beaker. Then, 20 mL of fresh 5% (w/v) metaphosphoric acid solution were added to the vessel and mixed well. The mixture was then homogenized by stirring at room temperature (RT) for 2 min. The homogenate was centrifuged at 3000 rpm for 5 min; then, the upper solution was filtered using Albet no. 1305 filter papers and re-filtered using 0.45-µm Millipore filters for liquid chromatography (LC) analysis (Valls *et al*., 2001). Then, stock solutions of 100 µg mL^{-1} vitamin B_5 (pantothenic acid), vitamin B_9 (folic acid), vitamin B_2 (riboflavin), and vitamin B_7 (biotin) (Sigma-Aldrich, St. Louis, MO, USA) were prepared and stored in 4 °C until use. The vitamin content of the sample was assessed using the standard curve, and high-performance liquid chromatography (HPLC) (Waters, USA) was used for the assessment of vitamin B according to Sasaki *et al*. (2020). The method included the use of Capcell Pak C18 SG120 HPLC Column (250 nm × 4.6 mm with 5-µm particle sizes) (Osaka Soda, Japan), gradient elution of phosphate buffer-acetonitrile (pH 3), ion-pairing reagent (mobile phase) with 1.0 mL min^{-1} flow rate and UV detection (210 nm). The vitamin B compound was separated within 60 min. The value of 0.01 µg g^{-1} was set as the detection limit.

Amino acids

Briefly, 10 g of dried sausage sample were hydrolyzed using 1 N hydrochloric acid (50 mL) based on an original protocol by Czauderna *et al*. (2003). Amino acid (AA) standards were purchased as a cell-free AA mixture from Sigma-Aldrich, St. Louis, Missouri, USA. The sample was then centrifuged (10,000 g) to collect the hydrolysate. One-hundred microliters of this hydrolysate were carefully injected into the HPLC instrument (Waters, USA) at 40°C. The HPLC instrument was equipped with Zorbax Eclipse-AAA Column (4.6 mm × 150 mm, 5 µm) (Agilent Technologies, USA) as well as a fluorescence detector. Furthermore, sodium dihydrogen phosphate (NaH_2PO_4) solution (40 mmol l^{-1}) was used in the instrument as Mobile Phase A and acetonitrile:methanol:water solution (45:45:10 v/v/v) as Mobile Phase B.

Fatty acids

Generally, the Folch method (chloroform:methanol 2:1 v/v) was used for the sample lipid extraction (Folch *et al*., 1957). Fatty acid methyl esters (FAME) were methylated based on the European Official Methods of Analysis (Godfray *et al*., 2010; Hashempour-Baltork *et al*., 2018). The FAME were analyzed using gas chromatography (GC) (Agilent 6890, Agilent Technologies, USA) equipped with capillary column (30 m per 0.25 mm ID, 0.25-µm film thickness) and flame ionization detector (FLD) (Thermo TR-5, ThermoFisher Scientific, USA). The instrument used helium (He) as the carrier gas with 0.2 mL min^{-1} flow rate based on a method described by Hashempour-Baltork *et al*. (2017). Fatty acid (FA) was identified by comparing the sample and the reference methyl ester chromatograms (Sigma-Aldrich, USA).

Mechanical characteristics

Texture profile analysis (TPA) was performed using Stable Micro Systems Texture Analyzer Model TA.XT Plus (Stable Micro Systems, UK). The analyzer was equipped with a 50-kg load cell. The sample was cut into pieces of 25 mm and axially fixed on the platform. Two-cycle compression assay was carried out with up to 50% of the strain compression of the original height using steel probes. Return speed, distance, and contact force were 2 mm s^{-1}, 50 mm, and 20 g, respectively. The various attributes of the food, including cohesiveness, hardness (N), adhesiveness (N.S), springiness (%), gumminess, chewiness, and springiness, were assessed (Kamani *et al*., 2019).

Statistical analysis

Descriptive data of the study were recorded as mean ± SD (standard deviation) using SPSS Software v.17 (IBM Analytics, USA). Duncan's test was used for the comparison of means and different letters represent significant statistical differences ($P < 0.05$). For each sample of each test, three replicates were used.

Results and Discussion

Physicochemical assessments

Proximate analyses of sausages formulated with mycoproteins and beef are shown in Table 2. Results of moisture contents demonstrated that moisture in sausages formulated with mycoproteins significantly was higher ($P < 0.05$) than moisture in sausages formulated with beef. Protein and lipid contents seemed to increase with the substitution of mycoproteins in the formulation of sausages. Higher contents of moisture and lipids were linked to further WBC ($P < 0.05$) and OBC in mycoprotein samples, compared to beef samples. Based on the results, mycoprotein formulation included lower values of carbohydrates, ash, and pH, verified by the previous studies (Hashempour-Baltork *et al*., 2020). The low pH in mycoprotein samples was associated with low pH of mycoproteins (4.7) in comparison to pH of meat (5.6). In another report, use of mycoproteins significantly increased nutritional values of fish sausages (e.g., ash, carbohydrate, fat, and protein) ($P < 0.05$) (Bahmani and Movanes, 2021). In this study, the pH of fish sausages enriched with mycoproteins increased during storage (Bahmani and Movanes, 2021). No significant difference ($P < 0.05$) was generally reported between the two formulations of sausages in WHC and peroxide values ($P > 0.05$). Characterization of these two sausage samples indicated that lesser oil and water should be used in the formulation due to the higher WBC and OBC values in mycoproteins than beef. In addition to higher OBCs, higher proteins and lower carbohydrates could be used in mycoprotein samples as appropriate meals for obese people. These findings verified previous findings, which addressed mycoproteins as healthy nutritious proteins (Finnigan *et al*., 2019).

Microbial assessments

Microbiological analysis was carried out on Day 1 after cooking to understand heat behaviors of mycoproteins and compare microbial patterns of the samples. Absence of yeasts, molds, *Salmonella* spp., and *E. coli* verified the effectiveness of the heat treatment in both samples. Due to the absence of *S. aureus*, the hygienic procedures seemed to be effectively preventive. The number of *B. cereus* and *C. perfringens* were similarly reported to be less than 10 cfu g^{-1} in both samples. Researchers demonstrated that the presence of NaCl and phosphate might inhibit the bacterial growth (Kim *et al*., 2021). Moreover, a similar report has been published on increased load of *Pseudomonas* spp. in fish sausages enriched with mycoproteins during refrigerated storage (Bahmani and Movanes, 2021).

Nutritional assessments

Vitamins

Levels of vitamins B_2, B_5, B_7, and B_9 were assessed in both samples (Table 3). Contents of vitamin B_9 showed no significant difference ($P < 0.05$) between the samples. For other vitamins, mycoprotein sausages significantly achieved higher scores ($P < 0.05$). According to Hashempour-Baltork *et al*. (2020), who comprehensively compared the vitamin B content in meats and mycoproteins, contents of riboflavin, niacin, pyridoxine, and pantothenic acid was 9, 14, 5, and 10 μg g^{-1} in mycproteins and 0.018, 0.5, 0.052, and 0.35 μg g^{-1} in meats. These differences were seen in the formulated products as well.

Table 3. The level of vitamin B group in mycoprotein/beef sausages (μg/g).

Treatment	Vit B2	Vit B5	Vit B7	Vit B9
Mycoprotein sausage	3.31 ± 0.5[a]*	0.02 ± 0.05[a]	8.81 ± 1.45[a]	0.48 ± 0.05[a]
Beef sausage	1.51 ± 0.5[b]	0.01 ± 0.04[b]	2.57 ± 1.5[b]	0.51 ± 0.03[a]

*Mean ± SD, Different letters represent significant differences ($P < 0.05$).

Table 2. Proximate analysis and pH of beef sausage.

Treatment	Moisture (%)	Protein (%)	Lipid (%)	Carbohydrate (%)	Ash (%)	pH	Peroxide (mEq/kg)	WHC** (%)	WBC** (mL/g)	OBC** (mL/g)
Mycoprotein-sausage	59 ± 1.9[a]*	12.5 ± 1.0[a]	13.9 ± 1.2[a]	9.8 ± 1.1[b]	2 ± 0.5[b]	5.7 ± 0.01[b]	1.2 ± 0.2[a]	54 ± 2.5[a]	0.98 ± 0.09[a]	0.37 ± 0.08[a]
Beef sausage	47.5 ± 1.5[b]	11.6 ± 1.1[b]	8.9 ± 1.3[b]	10.9 ± 1.2[a]	3 ± 0.4[a]	6.5 ± 0.01[a]	1.3 ± 0.2[a]	55 ± 3.3[a]	0.79 ± 0.1[b]	0.3 ± 0.08[b]

*Mean ± SD, Different letters represent significant differences ($P < 0.05$).
**WHC: water holding capacity, WBC: water binding capacity, OBC: oil binding capacity.

Table 4. The amino acid profile in mycoprotein/beef sausages (µg/g).

Amino acid	Content in mycoprotein-sausage (%g per 100 g protein)	Content in beef-sausage (mg per 100 g protein)
L-Alanine	4.84 ± 0.99^{b*}	14.90 ± 0.59^{a*}
L-Arginine	6.74 ± 0.07^{a}	6.0 ± 0.18^{a}
Aspartic acid	5.25 ± 0.03^{b}	15.20 ± 0.10^{a}
L-Cystine	11.21 ± 0.15^{b}	96 ± 0.05^{a}
L-Glutamic	12.92 ± 0.52^{b}	29.02 ± 0.12^{a}
Glycine	4.15 ± 0.23^{b}	9.05 ± 0.35^{a}
L-Histidine	3.20 ± 0.75^{a}	1.10 ± 0.85^{b}
L-Isolucine	5.90 ± 0.63^{a}	3.00 ± 0.53^{b}
L-Leucine	7.80 ± 0.35^{a}	5.01 ± 0.33^{b}
L-Lysine	7.50 ± 0.61^{a}	3.0 ± 0.66^{b}
L-Methionine	1.90 ± 0.80^{a}	1.2 ± 0.11^{b}
L-serine	5.35 ± 0.23^{a}	1.04 ± 0.17^{b}
L-Theronine	13.75 ± 0.77^{a}	11.95 ± 0.95^{b}
L-Tyrosine	4.35 ± 0.97^{a}	4.950 ± 0.907^{a}
L-Valine	5.90 ± 0.70^{a}	4.10 ± 0.07^{b}
Phenyl Alanin	9.78 ± 0.87^{a}	3.15 ± 0.50^{b}
Proline	5.30 ± 0.77^{b}	17.1 ± 0.60^{a}
Tryptophan	6.7 ± 0.60^{a}	1.5 ± 0.70^{b}

*Mean ± SD, Different letters represent significant differences ($P < 0.05$).

Amino acids

Based on the nutritional and physiological roles, AAs can be differentiated as EAAs, including valine, tryptophan, threonine, phenylalanine, methionine, lysine, isoleucine, leucine, histidine (essential for infants), arginine (semi-essential), and nonessential amino acids (NEAA), including tyrosine, serine, proline, glycine, glutamine, glutamic acid, cysteine, asparagine, aspartic acid, and alanine (Damodaran and Parkin, 2017). The AA profiles showed that mycoprotein sausages included almost all EAAs, compared to beef sausages (Table 4). These findings were similar to those of other studies, reporting the presence of EAAs in single-cell proteins (SCP) of *F. venenatum* (Hashempour-Baltork *et al*., 2020). In fact, three food categories totally provide 80.9% of daily protein needs of humans (Górska-Warsewicz *et al*., 2018). The significance of protein nutrition includes EAA content, biological value, digestibility, net protein use, and protein efficiency ratio. Hashempour-Baltork *et al*. (2020) compared the quality of the mycoproteins with that of meat proteins and demonstrated that nutritional indices of these two sources were almost similar. Monteyne *et al*. (2020) reported that mycoproteins were good food sources enriched with EAAs.

Table 5. Fatty acid profile of mycoprotein/beef sausage.

Fatty acid	Content in mycoprotein-sausage (% w/w)	Content in beef-sausage (% w/w)
Palmitic (C16:0)	18.8 ± 0.20^{b}	32.4 ± 0.29^{a*}
Stearic (C18:0)	10.90 ± 0.16^{a}	28.3 ± 0.20^{b}
Oleic (C18:1)	24.95 ± 0.11^{a}	9.13 ± 0.365^{b}
Linoleic (C18:2)	25.35 ± 0.15^{a}	21.31 ± 0.353^{b}
α-Linolenic (C18:3)	15.14 ± 0.18^{a}	5.10 ± 0.748^{b}

*Mean ± SD, Different letters represent significant differences ($P < 0.05$).

Fatty acids

The FA composition of the sausage samples are presented in Table 5. The total SFAs in mycoprotein and beef sausages were 29.7 and 60.7% (w/w), respectively. In fact, unsaturated fatty acid (UFA) levels in mycoprotein sausages (65.44) were significantly ($P < 0.05$) higher than UFA levels in beef sausages (35.54% w/w). These contents as well as previous contents highly verified the results, especially for the ratio of UFA to saturated fatty acids (SFA) of 2:1 (Reihani and Khosravi-Darani, 2018). In 2009, Hosseini *et al*. (2009) reported 3.2–3.5:1 ratio of UFA to SFA. Higher consumption of USFAs provides health benefits to patients with cardiovascular diseases (CVD). Naturally, the ratio of polyunsaturated fatty acid (PUFA) to SFA in beef is typically 0.1. However, the ratio decreases with an increase in meat fats (Vahmani *et al*., 2015). Naturally, the ratio reaches 1.44 in mycoproteins. Chicken fat naturally includes 30% of SFAs, 45% of monounsaturated fatty acids (MUFA), and 21% of PUFAs (Hashempour-Baltork *et al*., 2020; USDA, 2008). These values are close to those of mycoproteins (Table 5).

Mechanical assessments

Table 6 represents mechanical properties of the cooked samples. Hardness of mycoprotein sausages was significantly lower than that of meat sausages ($P < 0.05$). Similar decreases were reported for cohesiveness when meat was totally replaced by mycoproteins. It was reported that beef sausages needed a greater force of chewing, compared to that of nonmeat samples. This was possibly due to the occurrence of stronger networks in myofibril proteins, increasing the product resistance to compression. Mycoprotein sausages showed lower values for gumminess and springiness ($P < 0.05$). Lower springiness values were reported by Youssef and Barbut (2011), when soy protein extracts were used as meat alternatives in emulsified meat batters. Kamani *et al*. (2019) recorded lower levels of food hardness, cohesiveness, gumminess, and springiness by replacement of meats by proteins of

Table 6. The hardness, adhesiveness, springiness, cohesiveness, chewiness and gumminess of mycoprotein/beef sausages.

Treatments	Hardness (N)	Adhesiveness (N.S)	Springiness (%)	Cohesiveness	Gumminess	Chewiness
Mycoprotein-sausage	23 ± 1.1[b]*	1.5 ± 0.19[a]	0.49 ± 0.21[b]	0.19 ± 0.02[b]	20.1 ± 1.2[b]	1.0 ± 0.01[b]
Beef-sausage	37.12 ± 1.5[a]	0.5 ± 0.10[b]	0.75 ± 0.2[a]	0.24 ± 0.01[a]	25.4 ± 1.5[a]	2.1 ± 0.2[a]

*Mean ± SD, Different letters represent significant differences ($P < 0.05$).

plant origin in chicken sausages. Researchers concluded that proteins of nonmeat origin could include further fat and water contents, which might fill the protein interstitial spaces and decrease the product springiness (Kamani *et al*., 2019; Youssef and Barbut, 2011). This is also addressed for mycoprotein replacement regarding WBC and OBC (Table 2). Textural analysis demonstrated association of sample meats with lower values of adhesiveness. It could be interpreted that a decrease in meat quantity might lead to a significant decrease in the consistency of the cooked emulsions.

Conclusion

This study was carried out to investigate the appropriateness of mycoproteins as complete substitutes for meat in beef sausages. The results showed that mycoprotein substitution improved nutritional and health effects due to the high-value proteins with EAAs and less lipid content (mostly UFAs). Also, it has high contents of EAA and UFA, compared to meat sausages. Absence of yeasts, molds, *Salmonella* spp., *Eshrichia* (*E.*) *coli*, and *Staphiloccocus* (*S.*) *aureus* verified the effectiveness of heat treatment and also hygienic procedures in samples. Phycicochemical evaluations show higher contents of moisture and lipids in sausages containing mycoprotein due to WBC and OBC, compared to beef samples. Besides, mycoprotein samples had lower values ($P < 0.05$) of carbohydrates, ash, and pH, compared to beef samples. However, mycoprotein sausages achieved lower scores of hardness, cohesiveness, gumminess, and springiness in mechanical assessments, compared to beef sausages. Mycoproteins could hold excessive water and fat caused by higher WBC and OBC, filling the protein interstitial spaces and decreasing the springiness. This suggested less use of oil and water in mycoprotein formulations. This study has provided valuable information for increasing public awareness on the characteristics of mycoprotein products, including nutritional, textural, and formulation characteristics.

This is the first study that substituted meat with mycoproteins in sausages. However, further studies are necessary to optimize mycoprotein sausages using texture improvement ingredients to enhance their gel-forming and textural characteristics. These are currently the major problems in the manufacturing of meat-free sausages. The production of meat alternatives seems necessary due to the preference of consumers for vegetarian diets, and the increasing nutritional awareness of the populace. Like other functional foods that were unknown in the past, after numerous studies and production of various products, today there is a unique response to these products. In the past 2 years, the COVID-19 pandemic has drawn the public attention to food security and meat supply worldwide with further global demands for meat alternatives with plant origins.

Acknowledgment

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Conflict of interest

The authors report no conflict of interest.

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Effect of different thawing methods on quality properties of stuffed pasta (manti)

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RESEARCH ARTICLE

Abstract

Stuffed pasta or 'manti' is a traditional food consumed fondly in Turkey. For consumption, it is produced as fresh, frozen or dried manti. Frozen manti is cooked directly without thawing. The present study investigated the effects of different thawing processes on physicochemical properties and sensory qualities of frozen stuffed pasta (manti). The following five thawing processes were used individually: (1) infrared, (2) microwave (3) infrared-assisted microwave, (4) dry hot air and (5) running water thawing. The storing temperatures of manti found in the market were 10°C and 24.5°C. Consequently, manti samples were thawed up to these temperatures. It was determined that the shortest thawing period (20 sec) was that of infrared-assisted microwave process. The longest process was dry hot-air thawing (420 sec) in manti samples that were thawed until refrigerator (+4°C) and room temperatures (24.5°C). Thawing methods did not change pH, moisture values and weight gain ($p > 0.05$). It was found that sensory qualities and amount of dry matter passing into the water of samples were different ($p < 0.05$). It was concluded that thawing was necessary for frozen manti, and the best method was microwave thawing.

Keywords: manti, qualitative properties, stuffed pasta, thawing methods

Introduction

Some foods are susceptible to spoilage, because the microorganisms that cause spoilage are found in the foods. These foods are generally of animal origin. One of such foods produced is stuffed pasta (manti) with minced meat, which is fondly consumed in Turkey. Manti with minced meat is defined as a traditional food prepared by making a mixture of different types of spices and minced beef, putting it in small pieces of dough, and boiling it in water prior to consumption. The industrial production scheme of manti is shown in Figure 1 (Gökmen et al., 2015; Sitti *et al.*, 2009). Its shelf life is short because of high moisture content and presence of beef. In the thawing process, compared to the freezing process, more damage could occur in the textural properties of products (Zhu *et al.*, 2004). Hence, it is important to keep manti with minced meat under suitable conditions, for which different studies are found out in literature. Manti is dried for this purpose, consumed wet or stored by freezing. Traditional methods (dry hot air), and infrared and microwave methods are employed for drying manti. In addition, vacuum and modified atmosphere packing applications are also carried out after pasteurization of fresh manti. However, the shelf life of manti could not be significantly extended except for drying applications. Both sensory and nutritional losses are observed in dried manti compared to fresh manti. Freezing is another method that could be employed to consume fresh foods. Thanks to the freezing process, shelf life of manti could be extended to store it for about 4–6 months at a storage temperature of –18°C (having international validity) (Günşen and Büyükyörük, 2005). Freezing process is used to extend the shelf life of foods and to slow down and/or stop the activities of microorganisms and enzymes in the structure of foods. During the thawing of frozen

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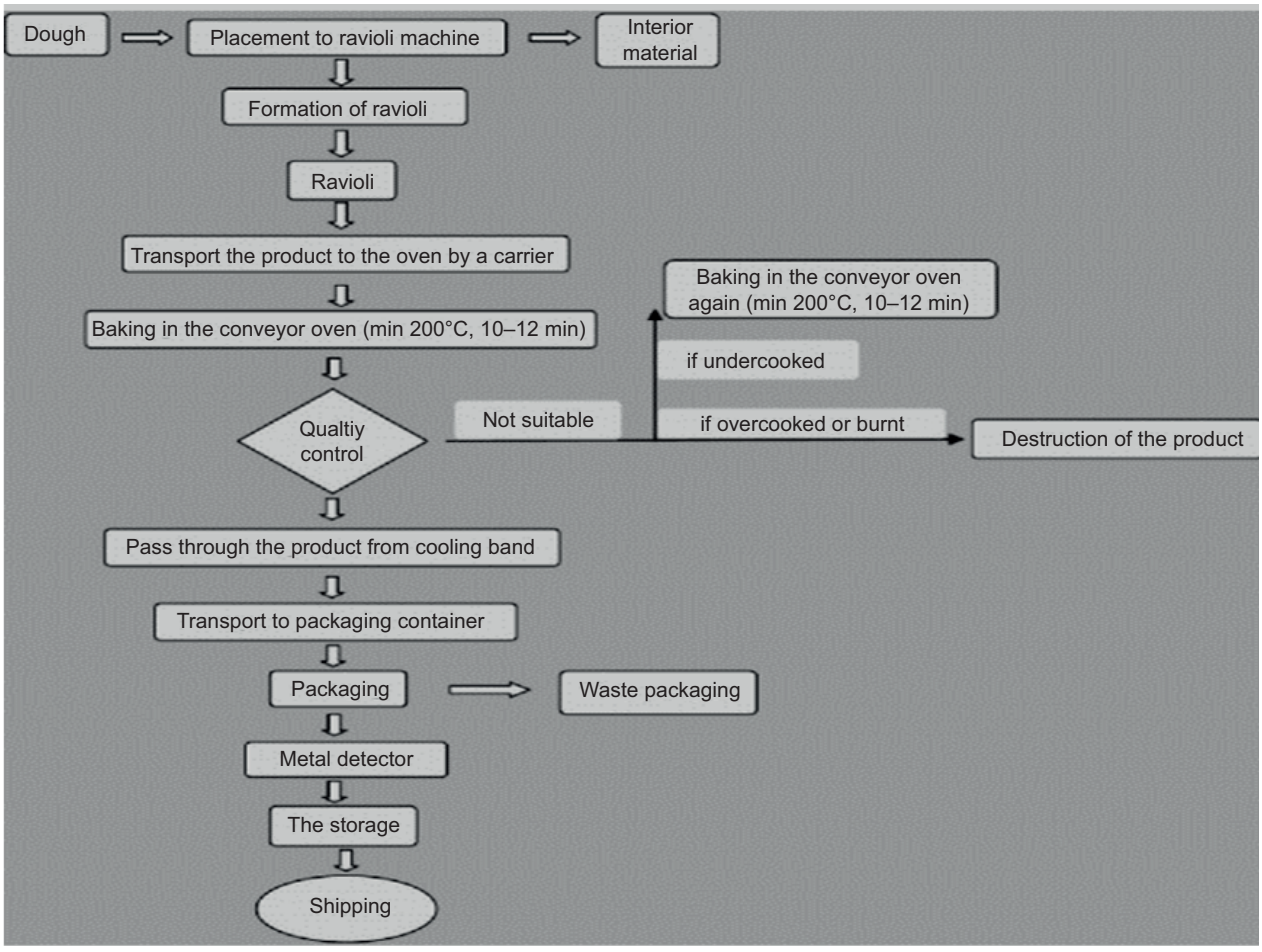


Figure 1. The industrial production scheme of manti.

foods, many physicochemical, sensory and microbiological changes occur depending on the freezing method and time (Atasever and Çubukçı, 2018; Azevedo *et al.*, 2005).

Different freezing methods are used during the thawing of frozen foods. However, after freezing of manti, no other method used for thawing is applied and the frozen manti is cooked directly. Freezing is an important process that protects the quality of food to extend its shelf life. Thawing is a very important stage of frozen foods prior to consumption or processing (Durmaz *et al.*, 2018). Hence, thawing process could determine the most appropriate quality of food. Different thawing processes are available, such as hot-air thawing, high-pressure thawing, low-temperature thawing, running water thawing, vacuum thawing, ultrasonic thawing, microwave thawing, infrared-assisted microwave thawing etc. (Cai *et al.*, 2019; Liu *et al.*, 2020; Xu *et al.*, 2020).

Today, traditional methods based on heat transfer on an industrial scale (based on the use of air or water heat transfer) are applied for thawing. These methods cause water loss, textural defects and negative changes in proteins during the process. In the traditional methods of thawing, product's quality is lost due to temperature differences in the product. Temperature inside the product must be uniform and the process time must be shortened. For these reasons, interest in the dielectric dissolution methods for thawing has increased (Uyar et al., 2015). No study has determined the thawing of frozen noodles and frozen filled pasta (Anonymous 2021a, 2021b, 2021c). Thawing processes determine the final quality of frozen manti; hence, improper thawing process causes irreversible degradation in the quality of frozen manti.

Frozen manti is cooked directly without thawing. Therefore, it is necessary to determine an appropriate process for thawing of frozen manti to preserve the quality of product. Studies are conducted about the effects of thawing methods on the physical, chemical and microbiological properties of meat and meat products. Therefore, in this study, we investigated the effects of hot-air, running water, microwave, and infrared-assisted

microwave thawing processes on physical, chemical, and sensory properties of frozen manti.

Material and Method

Frozen stuffed pasta (manti) (with 50.6% carbohydrate, 3.6% sugar, 8.5% protein, 5% fat, and 2% salt) was purchased from ANEDA GIDA Industry and Trade Limited Company (Guven Mantı), Istanbul, Turkey. Manti samples were packed in polyethylene bags (20 × 22 cm, 450 g per bag) and stored at -18°C.

Equipments and reagents

Following equipments and reagents were used: microwave oven (NN-GD568, Panasonic Appliances Microwave Oven Co. Ltd, Shanghai, China), tap water, pure water, kitchen oven (Bosh Oven Co. Ltd, Shanghai, China), pH meter (Inolab-pH 7110, GER), precision balance, sample containers, frozen raw manti samples, portable thermohygrometer (sauna type) and thermocouple.

Features of raw manti

Ground beef features: it was double-pulled, obtained from leg meat; it contained about 25% fat. Manti shape: bundle type; manti dimensions: 1.5 × 1.5 × 1.5 cm; spices used in manti mixture: salt, black pepper, and mint; manti weight: average 2 g; and starting temperature for manti mortar: -3°C.

Storage conditions

Produced manti samples were stored in nylon nonvacuum bags (brand: Eco Pazar; 40-micron thick; oxygen, carbon dioxide and transparent nylon food bags with high air permeability and 20 × 30 cm in size) at -18 °C.

Thawing methods and time

It was determined that the temperatures at which manti was stored in the market were the best thaw temperatures. In addition, inside temperatures of manti were measured with a thermocouple. The storing temperatures of manti in the market were 10°C and 24.5°C. Manti samples were frozen for 24–48 h. Control samples were not defrosted, and cooked directly. After freezing of manti samples, these were thawed by the following five different thawing processes: in oven (hot-air thawing) at 40°C; running water process—the samples were thawed under 40°C running water; microwave and infrared-assisted microwave thawing processes—the samples were thawed in a microwave oven (NN-GD568, Panasonic). The power and frequency were 1,200 W and 2,450 Hz, respectively.The process was finished when the center temperature of the sample reached 10°C and 24.5°C (Cai *et al.*, 2019; Liu *et al.*, 2020; Xu *et al.*, 2020).

Features of infrared lamp: 60% power, 1,000 watt, bar-type, short wavelength. Features of microwave: 40% power, 240 V supply voltage, and 50 Hz frequency.

Manti features: Distance between the ray source and the product: 25 cm. Amount of manti taken: 10 kg.

Ambient conditions

Ambient conditions included room temperature and 47% relative humidity. In microwave and infrared-assisted microwave processes, the experiment was carried out at room temperature and under atmospheric conditions. The temperature value was determined as 24.2°C under atmospheric conditions.

pH analysis

The pH values of homogenized samples were read with a pre-calibrated pH meter (Inolab-pH 7110, GER) (Gökmen *et al.*, 2019).

Weight gain

Cooking efficiency was measured by the ratio of cooking weight to initial weight. Results were expressed as percentage values (Murphy *et al.*, 1975).

Moisture loss

It was calculated by taking into consideration the initial weights of samples (Murphy *et al.*, 1975).

Dry matter passing into water (DMPW)

It was based on the principle of gravimetric determination of the amount of substance passing into the cooking water during cooking of manti (Gökmen *et al.*, 2019).

Sensory analysis

Sensory analysis of manti samples was performed by a panel of seven trained and experienced sensory analysts.

The following parameters were considered for analysis (Gökmen *et al.*, 2019): 500 g of manti sample was taken into 2 L of drinking water and boiled; the samples were analyzed as shown in Table 1. Manti was cooked when it was on the surface of cooking water. In sensory analysis, the age group was 25–40 years.

Statistical analysis

Three measurements were conducted to test the effects of each thawing process on the physicochemical properties and sensory qualities of frozen manti samples. The analysis was performed with the JMP statistical software, and significant difference ($p < 0.05$) was determined by Tukey's test. Analysis with each thawing process was performed in triplicate. Independent variables were as follows: manti shape, manti weight, microwave power, infrared power, distance between product and ray source, thawing, running water, and oven temperature.

Results

The effects of different tempering methods on the physicochemical properties and sensory qualities (appearance, taste, smell, aroma, mouth feeling, and general evaluation) of manti samples were investigated prior to consumption.

Results of thawing time

Among thawing processes, the shortest dissolution time was observed in the infrared-assisted microwave process, and the longest time was determined in dry hot-air process at 40°C (Table 2).

Results of physicochemical analysis

The weight gain, moisture and pH values of the samples were not found statistically significant ($p > 0.05$). However, a statistical difference ($p < 0.05$) was determined in terms of the amount of dry matter passing into the water (Figure 3). Effects of thawing process and tempering time on weight gain, moisture% and pH values of thawed manti samples were not found. Maximum amount of dry matter passing into water was found in control samples, and minimum amount was found in dielectric processes (microwave, infrared, and infrared-assisted microwave). If the thawing time was extended in dielectric methods, then decrease in the amount of dry substance passing into water in manti was observed. Moreover, no significant relationship was established in other processes.

Results of sensory analysis

Results of sensory analysis demonstrated differences in the sensory parameters (appearance, taste, smell, aroma, mouth feeling, and general evaluation) of samples. Among the sensory parameters, the highest appearance value was found in the microwave process dissolved at 10°C ($p < 0.05$), and no significant difference was found among other processes. In terms of taste and odor parameters, the highest values were obtained in the microwave dissolution processes (at 10°C and 24.5°C). The highest values were established in aroma and mouth feeling parameter analysis of samples that were thawed at room temperature with microwave process. All the values obtained through different processes with the lowest general evaluation parameters in control samples, infrared-assisted microwave, and infrared dissolution (at room temperature and refrigerator temperature) samples were found to be statistically significant ($p < 0.05$) (Figures 4 and 5).

Besides this, the microwave process got the highest scores in the taste parameter of sensory analysis. Thawing time had no effect on taste and aroma parameters. Infrared thawing process got the lowest scores for odor parameter. The effect of thawing time on odor parameter could not be determined. Infrared application got the lowest scores for aroma parameter of sensory analysis. The

Table 1. Sensory analysis form.

Product: Manti with minced meat					
Quality criteria	**Codes**				
Taste					
Odor					
Flavor					
Appearance					
Mouth feeling					
General taste					
Values	1–3 = Very bad	4–5 = Bad	5–6 = Medium	7–8 = Good	9–10 = Very good

microwave thawing process got the highest scores for mouth feeling parameter. The effect of annealing time on mouth feeling and appearance parameters could not be determined. Microwave process received the highest scores for general evaluation parameter of sensory analyses. The annealing time had no effect on aroma parameter. Microwave thawing process got the highest scores for appearance parameter.

Table 2. Thawing processes, temperatures, and time.

Thawing processes	Thawing temperatures and time	
	10°C (sec)	24.5°C (sec)
Microwave medium level	23	33
Infrared	60	155
Infrared-assisted microwave (50–50%)	20	30
40°C Running water	150	420
40°C Dry hot air	240	420

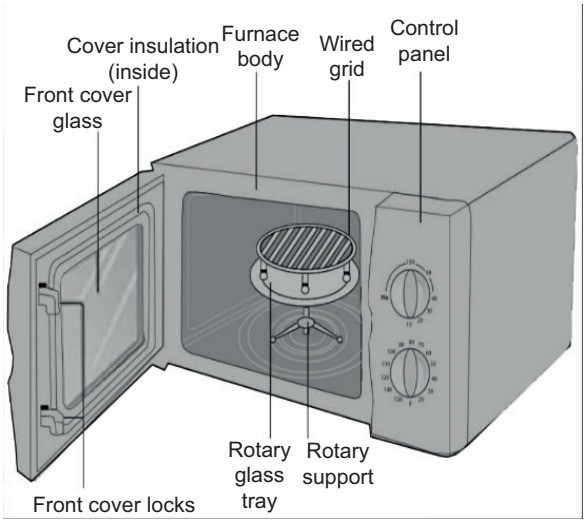
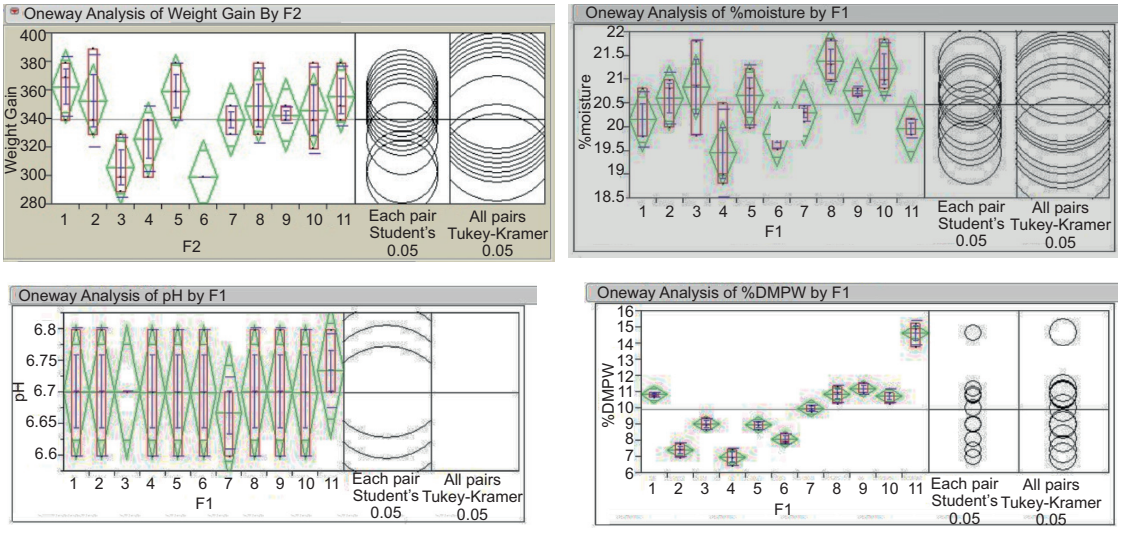


Figure 2. Far infrared (FIR)-assisted microwave oven.

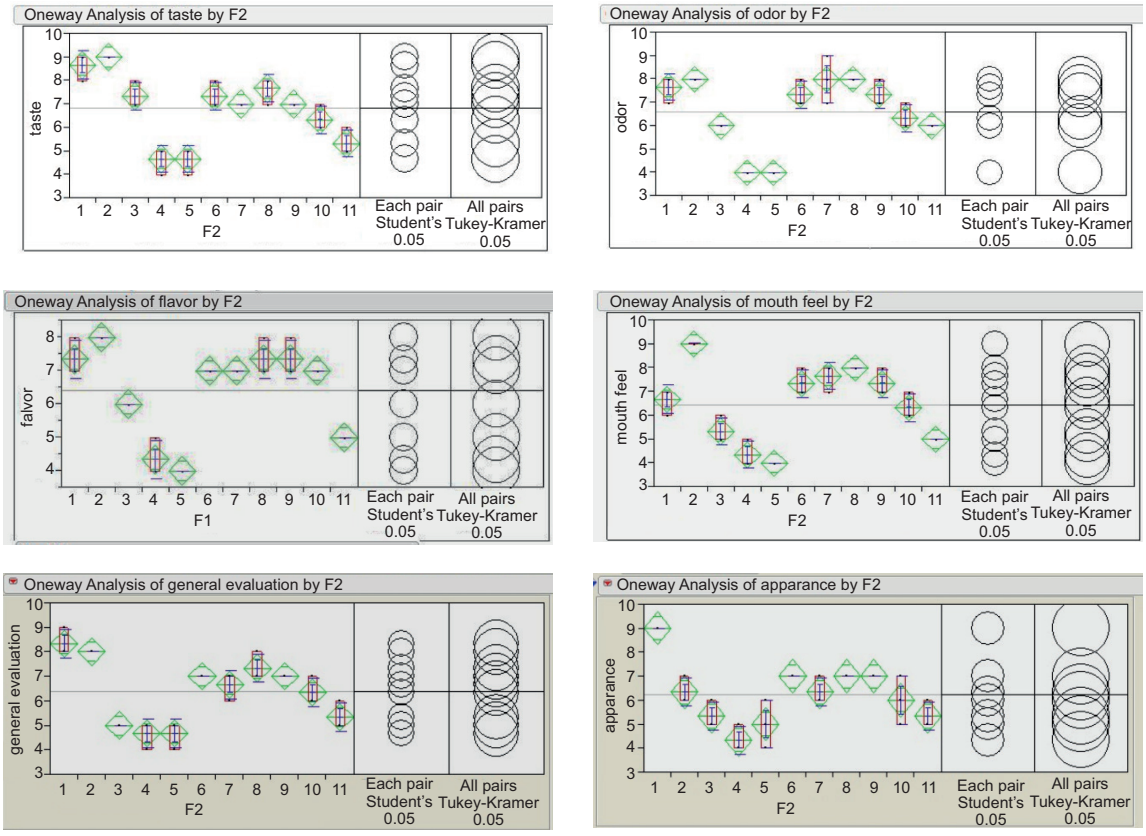
Discussion

In the study, the maximum thawing time was determined for infrared-assisted microwave process whereas the minimum thawing time was obtained for running water process. In a study conducted by Álvarez *et al.* (2005), the shortest dissolution time was determined for an infrared–microwave combination process used to defrost frozen mashed potatoes, compared to infrared and microwave processes used separately. In another study, microwave process had a shorter thawing time compared to the conventional method used for thawing strawberries (Holzwarth *et al.*, 2012). In a study conducted on thawing frozen anchovies and blue fish, it was reported that the dissolution time was minimum in case of microwave thawing and maximum for running water thawing (Alberio *et al.*, 2012). It was determined that the obtained results were compatible with the results obtained in literature. No significant differences ($p > 0.05$) were observed between samples for weight gain, pH values and moisture content. The most effective factor for weight increase was starch gelatinization and the water-holding capacity of dough. It was assumed that the annealing processes performed had no effect on the rheological properties of manti dough. It was reported that pH values of fresh manti were close to neutral depending on the processing method applied (Sitti, 2011).



1. Microwave medium-level, 23 sec, 2. microwave medium-level, 33 sec, 3. Infrared, 60 sec, 4. infrared, 155 sec, 5. infrared-assisted microwave (%50–50), 20 sec, 6. infrared-assisted microwave (%50–50), 30 sec, 7. 40°C running water, 150 sec, 8. 40°C running water, 420 sec, 9. 40°C dry hot air, 240 sec, 10. 40°C dry hot air, 420 sec, 11. control.

Figure 3. Results of physicochemical analysis.



1. Microwave medium-level, 23 sec, 2. microwave medium-level, 33 sec, 3. Infrared, 60 sec, 4. ınfrared, 155 sec, 5. ınfrared-assisted microwave (%50–50), 20 sec, 6. ınfrared-assisted microwave (%50–50), 30 sec, 7. 40°C running water, 150 sec, 8. 40°C running water, 420 sec, 9. 40°C dry hot air, 240 sec, 10. 40°C dry hot air, 420 sec, 11. control.

Figure 4. Results of sensory analysis.

Thawing processes in case of frozen manti did not cause any significant change ($p > 0.05$) in its pH value. A study has reported that the pH values of manti samples stored at refrigerator temperature and packed in vacuum and modified atmosphere increased with increase in storage time, but initially there was no significant change in the pH values of samples (Sitti, 2011). In this study, since manti samples were stored for a short duration at -18°C, the probability of biochemical reactions was very low, and their pH values depicted no change. In addition, a very short duration of thawing processes reduced the possibility of biochemical reactions. Therefore, there was no significant change in pH values, and the values obtained were compatible with those found out in literature.

An important parameter for the quality of manti is the amount of dry matter allowed into water. As this value increases, the quality of manti decreases (Sitti, 2011). According to the results, the DMPW values of control samples were found to be high as well as statistically significant ($p < 0.05$). Therefore, it was concluded that the short-term thawing of manti instead of cooking it directly would decrease the amount of dry matter transferred into water, and thus its cooking quality would be preserved. After thawing of frozen manti, its cooking improved the sensory quality of samples. The obtained results were found to be statistically significant ($p < 0.05$). Direct cooking the frozen manti caused sudden changes in the temperature of manti, and therefore, the amount of starch passing to the cooking water increase and the manti dough sticks This happened because the sudden change in temperature caused excessive gelatinization of starch, and the lattice structure of starch particles embedded in proteins was damaged, resulting in the emergence of starch particles (Gökmen *et al.*, 2015).

It was estimated that thawing frozen manti with dielectric processes caused less damage to the protein–starch matrix of manti. Thus, starch granules played an important role in preserving the sensory quality of manti by less allowing of dry matter into cooking water. On the other hand, other processes (hot air and running water thawing) decreased the quality of manti in terms of both shape and texture. Hence, dielectric processes are preferred for thawing of manti. A study reported that heating at high frequency (0.1–2.5 GHz) is required for thawing of meat in a microwave. Accordingly, high-frequency microwaves were used in this study.

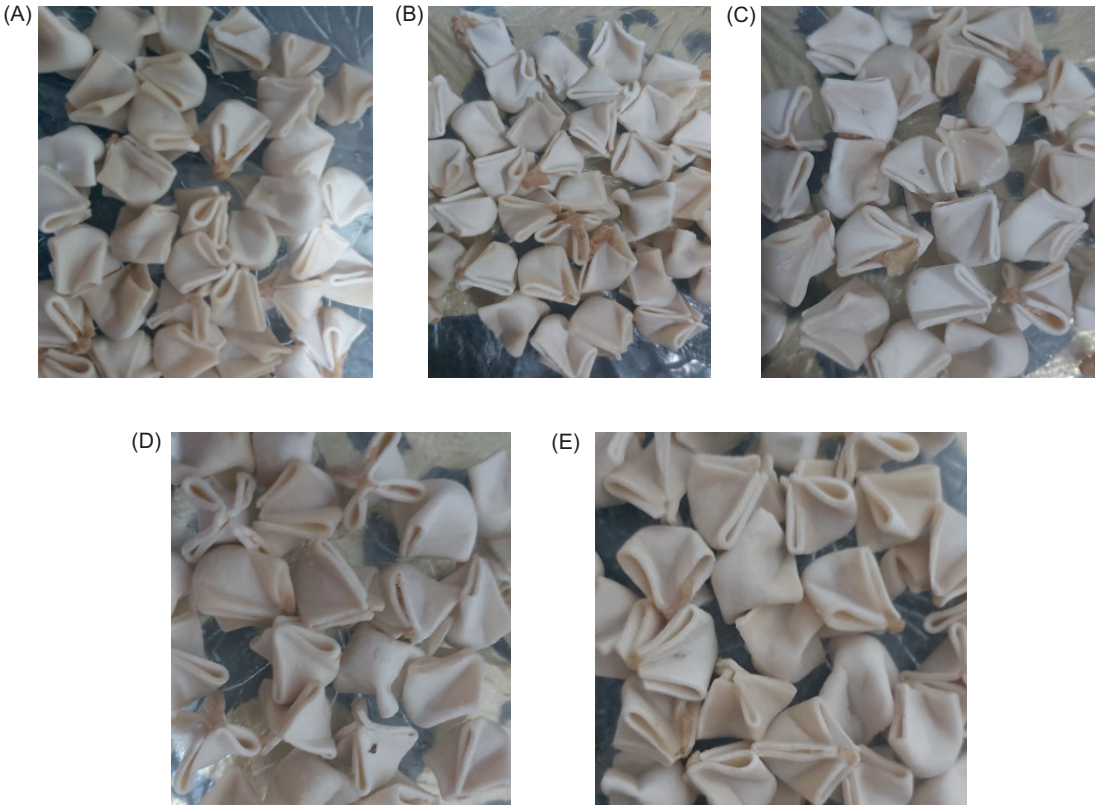


Figure 5. Mantı samples produced with (A) microwave, (B) ınfrared-assisted microwave, (C) ınfrared, (D) running water, and (E) dry hot air thawing processes.

Especially in control samples, it was estimated that the reason for the change in the amount of dry matter passing into the water was due to this. Presence of amylose and amylopectin fractions in the starch structure was another parameter that directly affected the cooking quality of manti. The starch particles embedded in flour protein were passed into water depending on the thawing process used for production. In addition, this condition caused the surface of manti grains to soften and their appearance to become worse. The passing of starch into water negatively affected the taste and aroma of manti. Likewise, while it is cooking manti, does not stick of the dough and does not come out of the mortar affects the mouth feeling parameter of manti. For this reason, it was estimated that mouth feeling values were high in tempered samples and low in control samples (Gallegos-Infante *et al.*, 2010).

Accordingly, it was determined that dissolving of frozen manti with thawing processes prior to cooking preserved the sensory quality of manti, and decreased the amount of dry matter passing into water. Besides, annealing processes had no effect on the pH and moisture content of manti. It was also determined that the thawing time of frozen manti was short and the most effective process was infrared thawing. More studies are required on this subject.

One of the parameters that determined the quality of cooking of manti was the amount of dry matter that passed into water. The higher the amount of dry matter passing into water, the more turbid would be the cooking water, thus decreasing the adhesion and visual quality of manti. There would also be a loss of mouth feeling and aroma of manti (Gökmen *et al.*, 2015). Owing to all these factors, it was estimated that the sensory quality of control samples was low compared to thawed manti samples. In order to increase the shelf life, fresh manti was stored and packed in vacuum and modified atmosphere. Although positive results were obtained in the studies conducted by Sitti *et al.* (2009) and Uzunlu and Var (2016), it was reported that the production cost of the product increased. Gökmen *et al.* (2015) reported that the sensory and chemical properties of various brands of manti available in the market were different. Ozturk *et al.* (2009) reported that the microbiological quality of manti samples available in Kayseri province was found to be quite low. One course to avoid all these negativities is to freeze manti on commercial level. Hence, it was concluded that different thawing processes must be used to

preserve the quality of manti. In addition, it was determined that the sensory quality of manti decreased with increase in dry matter passing into the water during cooking of frozen manti. It was also determined that of all the dissolving processes, microwave dissolution was the best approach for preserving sensory parameters of manti

Studies conducted by Chipley (1980) and Emam *et al.* (1995) compared ultrasound-assisted vacuum thawing (UVT) or microwave vacuum thawing (MVT) of red sea bream fillets with fresh, cold storage thawing, vacuum thawing, microwave thawing, and ultrasound thawing. Results demonstrated that UVT and MVT caused limited damage to fillets compared to other thawing processes. Similar results were found in our study with the use of microwaves.

Conclusion

In this study, physicochemical properties and sensory qualities of manti samples were investigated due to different thawing processes employed prior to consuming frozen manti. It was concluded from the results of sensory and physicochemical analyses that freezing was necessary for manti.

Future directions

In recent years, industrial-microwave systems with band have been designed for microwave application, which is one of the dielectric methods. In these systems, the product is transported by conveyor belts and subjected to microwave application in a closed environment. It is estimated that these systems could be applied in the industrial thawing of frozen manti. The most important challenges in optimizing microwave applications with conveyor belts are that all products are thawed equally and their temperature distributions are homogeneous. For this, mathematical models could be used. Hence, these problems could be solved by making separate modeling for each machine in conveyor belts microwave applications.

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