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Impact of refrigerated storage on the bioactive compounds and antioxidant capacity of two Algerian carrot varieties (*Daucus carota* L.)

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Abstract. Carrot (*Daucus carota* L.) is one of the main root vegetables rich in bioactive compounds with appreciable health-promoting properties, largely consumed in Algeria. In the current study, the storage effect (at 4 °C throughout 12 days) on bioactive compound stability and the antioxidant activity of two Algerian orange carrot varieties (Supermuscade and Touchon) were investigated. Total phenolic content of samples was determined by the Folin–Ciocâlteu method. Antioxidant capacity was determined spectrophotometrically, based on the evaluation of Free Radical Scavenging Activity (FRSA) using DPPH radical and Ferric Reducing Power (FRP). The results showed that the Touchon variety is richer in phenolics, flavonoids, and carotenoids and presents higher antioxidant activity in comparison with the Supermuscade variety. At the end of storage, the bioactive compound content and antiradical activity increased significantly ($p < 0.05$). Also, an extremely significant correlation ($p < 0.001$) was observed between the antioxidant contents and the antioxidant capacities of aqueous carrot extracts.

1 Introduction

Vegetables generally possess a good antioxidant activity, which is linked to their high contents of phenolic compounds. Numerous studies have suggested that the phytochemical content and the corresponding antioxidant activity of the vegetable contribute to their protective effect against chronic and degenerative diseases. The evaluation of the antioxidant capacity of foods commonly consumed in the diet, mainly after storage, is of great importance.

Carrot (*Daucus carota* L.) is a vegetable belonging to the family of *Umbelliferae*, also known as *Apiaceae*. *Daucus* is the largest genus in the family (*Rubatzky et al.*, 1999). Carrot is a root vegetable largely consumed on a global level, particularly in Algeria. It plays an important role in human nutrition and constitutes a rich source of health-promoting ingredients, such as carotenoids (*Kammerer et al.*, 2004; *Pace et al.*, 2020; *Shami & Naz*, 2019), in which antioxidant β-carotene acts as an anti-mutagenic and immunity booster (*Saleh et al.*, 2019; *Sharma et al.*, 2020; *Young & Lowe*, 2018). The phytonutrient content of carrots also includes phenolics, polyacetylenes, L-(+)-ascorbic acid (AA), and tocopherol, wherefore it is classified as a vitaminized food (*Encalada et al.*, 2016; *Numan*, 2019; *Vorobiev & Lebovka*, 2020).

Several epidemiological and clinical studies suggest that a high intake of carrot plays an important role in metabolism regulation, retaining a healthy skin and vision, and decreasing the risks associated to different types of cancer (*Chen et al.*, 2018; *Deding et al.*, 2020; *Jayaprakasha et al.*, 2019; *Luo et al.*,

2017; *Nkondjock & Ghadirian*, 2004; *Soares et al.*, 2018; *Su et al.*, 2002; *Surh*, 2003; *Tiwari*, 2016; *Tomita et al.*, 2020), cardiovascular diseases (*Alissa & Ferns*, 2017; *Castelletti*, 2019; *Louis et al.*, 2018; *Nicolle et al.*, 2003; *Nicolle et al.*, 2004; *Soleti et al.*, 2020), and cataract (*Braakhuis et al.*, 2017; *Chen & Chen*, 2017; *Haslam*, 2019; *Stahl & Sies*, 2020). Moreover, carrot is considered beneficial against urogenital diseases (*Aslam et al.*, 2014; *Chakraborty et al.*, 2018; *Chohra & Ferchichi*, 2019). The health-promoting effects of carrot have been attributed to the various antioxidant components present in this root vegetable (*Numan*, 2019; *Pace et al.*, 2020; *Shami & Naz*, 2019; *Soares et al.*, 2018).

Carrot is a source of various crucial macro- and micronutrients, including carbohydrates, proteins, fats, vitamins, antioxidants, minerals (potassium and sodium), folic acid, fibres, and carotenoids (*Ahimed et al.*, 2012; *Ludong et al.*, 2017; *Madu & Bello*, 2018; *Naseer et al.*, 2019; *Que et al.*, 2019; *Surbhi et al.*, 2018; *Vorobiev & Lebovka*, 2020). It contains significant quantities of thiamine, riboflavin and is also rich in sugars (*Naseer et al.*, 2019; *Surbhi et al.*, 2018). Carrot comprises several carotenoids (α- and β- carotenes) (*Ahmad et al.*, 2019; *Ludong et al.*, 2017; *Pace et al.*, 2020), which are the main pigments responsible for their colour, presenting nutritional importance due to their provitamin A and antioxidant activity (*Ellison et al.*, 2017; *Yoo et al.*, 2020). The β-carotene constituent is the major carotenoid, followed by α-carotene, lutein, and the other minor carotenoids such as cryptoxanthin, lycopene, or zeaxanthin (*Hà & Nguyễn*, 2015; *Ahmad et al.*, 2019; *Pace et al.*, 2020).

Interest in the role of antioxidants in human health has promoted research in the field of food sciences to assess fruit and vegetable antioxidants and determine how their content and activity can be maintained or improved, as the content of phytochemical substances is influenced by numerous factors such as ripening, genotype, cultivation technique, or climatic conditions during the pre-harvest period, but operations carried out during the post-harvest storage are also very important. In order to extend shelf life and maintain the quality of fresh carrot, refrigerated storage is largely used. However, storage at low temperatures may affect the composition and the activity of carrot phytonutrients. Therefore, the main objective of the present study was to evaluate the impact of refrigerated storage at 4 °C throughout 12 days on the antioxidant compounds (total phenolics, total flavonoids, and total carotenoids) and antioxidant activity of two Algerian orange carrot varieties.

2 Materials and methods

Chemicals

Folin–Ciocâlteu reagent (FCR) was purchased from Biochem, Chemopharma (Montreal, Quebec); sodium carbonate from Sigma-Aldrich (Switzerland); aluminium chloride and potassium ferricyanide from Biochem, Chemopharma (Georgia, USA); gallic acid and β-carotene from Prolabo (Montreuil, France); 1,1-diphenyl-2-picrylhydrazyl (DPPH) from Sigma-Aldrich (Germany).

Samples and preparation of extracts

Two varieties of orange carrot (Supermuscade and Touchon) were purchased from the local market. The varieties are fresh and without infection or damage. The carrots were washed with distilled water and then stored in refrigerator for 12 days at a temperature of 4 °C. The choice of this period is related to the average carrot's shelf life after purchase, stored in consumers' refrigerators and then divided into four sampling periods. The tested parameters were determined before storage of the samples and after storage, therefore sampling every three days: after 3, 6, 9, and 12 days.

The edible portions were separated from the inedible portions with manual peeler. An amount of 10 g of edible portion of fresh carrots were grated into small pieces (2.4 cm length and 1 mm width) using a manual grater and mixed with 50 mL of distilled water. After 30 min of agitation, the homogenate was centrifuged at 4,500 g for 15 min at 5 °C (Sigma 2-16 K; Germany). The supernatant was collected and the residue re-extracted with 50 mL of distilled water. The collected supernatants were combined and then concentrated under vacuum at 35 °C using a BÜCHI rotavapour (R-200, Germany) until the volume of 10 mL was reached, and the extracts were stored at -10 °C until analysis.

Sample analysis

To evaluate the effects of storage at 4 °C, the fresh-stored carrots were analysed every three days regarding their antioxidant constituents and antioxidant activity as follows:

Determination of Total Phenolic Contents (TPC)

The TPC of carrot extract was estimated following colorimetric assay of *Naithani et al.* (2006). Briefly, to 100 μL of the diluted extract (1:1, V:V), 2.2

mL of sodium carbonate water solution (2%) was added and mixed thoroughly. After 3 min, 100 μL of FCR (50%) was added under mixing. The absorbance of the mixture was measured spectrophotometrically at the wavelength of $\lambda_{abs} =$ 750 nm by using a spectrophotometer (UV-mini 1240 Shimadzu, China). The results were expressed as milligram Gallic Acid Equivalent per one hundred gram of the fresh weight (mg GAE/100 g FW) using a standard curve ($y = 1.8986\,x, R^2 = 0.9973$).

Determination of Total Flavonoid Content (TFC)

The TFC of carrot extract was evaluated following the colorimetric assay of *Djeridane et al.* (2006). To 1.5 mL of extract, an amount of 1.5 mL of 2% aluminium chloride solution (w/v) was added. After 10 min, the absorbance was measured at the wavelength of $\lambda_{abs} = 410$ nm. The total flavonoid was reported as milligram quercetin equivalent per one hundred gram of the fresh weight (mg QE/100 g FW) using standard curve ($y = 0.0095\,x, R^2 = 0.991$).

Determination of Total Carotenoid Content (TCC)

Carotenoids were extracted from the samples using the method of *Sass-Kiss et al.* (2005). In brief, 20 mL mixture of hexane-acetone-ethanol (2:1:1, V: V: V) was added to 0.5 g of homogenized fresh carrot samples. After 30 min of agitation, the supernatant was collected, and the residue was added with 10 mL hexane for a second extraction. The absorbance of the combined hexane layers was measured at the wavelength of $\lambda_{abs} = 450$ nm. The TCC in carrot samples was determined from the standard curve using β-carotene ($y = 0.1282\,x, R^2 = 0.996$), and the results were expressed as milligram β-carotene equivalent per one hundred gram of the fresh weight (mg βCE/100 g FW).

Antioxidant activities

DPPH Free Radical Scavenging Activity (DPPH-FRSA)

The antiradical activity of carrot extracts against DPPH (1,1-diphenyl-2-picryl-hydrazyl) free radical was evaluated according to the method described by *Peschel et al.* (2006). In brief, 500 μL of the extract was mixed with 2 mL methanolic solution of DPPH; the mixture was left in the dark for 90 min before measuring the absorbance at the wavelength of $\lambda_{abs} = 517$ nm. The reduction of DPPH was calculated using the following formula:

$$\text{DPPH radical scavenging activity } (\%) = [(A_c - A_e)/A_c] \cdot 100, \qquad (1)$$

where: A_c was the absorbance of the control, and A_e was the absorbance in the presence of the sample extracts.

Ferric Reducing Power (FRP)

The reducing power of carrot extracts was measured according to the method described by *Bhandari & Kawabata* (2004). Briefly, 1 mL of carrot extract, 0.5 mL of phosphate buffer (0.2 M, pH 6.6), and 2.5 mL of potassium ferricyanide solution (1% w/v) were mixed in a test tube and reacted for 20 min at 50 °C. The tubes were cooled immediately, and 0.5 mL of trichloroacetic acid (10%) was added in. After centrifugation at 3,000 g during 10 min (Sigma 2–16 K; Germany), 1 mL of supernatant was mixed with 1 mL of distilled water and 100 μL of ferric chloride (0.1% w/v) and reacted for 10 min. Then, the absorbance at the wavelength of $\lambda_{abs} = 700$ nm was measured. The FRP of carrot extracts was determined from the standard curve using Trolox standard ($y = 0.002\,x, R^2 = 0.997$), and the results were expressed as milligram Trolox equivalent per one hundred gram of the fresh weight (mg TE/100 g FW).

Statistical analysis

All data are reported as mean ± standard error of mean of three replicates. The analysis of variance (ANOVA) at $p < 0.05$ was calculated using STATISTICA 5.5 (StatSoft, Inc., USA) in order to determine the significant differences between the results. Correlations were performed using the correlation matrix at three different significance levels ($p = 0.05$, 0.01, and 0.001).

3 Results and discussion

Total Phenol Content (TPC)

Phenolic compounds are the most abundant antioxidants in the human diet and are widespread constituents of fruits and vegetables. These compounds are of considerable interest due to their antioxidant properties. Phenolic compounds in carrots are primarily found with a single aromatic ring known as phenolic acids. Major phenols found in carrots are chlorogenic, caffeic, and p-hydroxybenzoic acids along with numerous cinnamic acid derivatives. Chlorogenic acids are hydroxycinnamic acid derivatives formed by the esterification of cinnamic acids, such as caffeic, ferulic, or p-coumaric acids, with L-quinic acid (*Hà & Nguyễn*, 2015). The TPC of the aqueous carrot extracts of the

Supermuscade variety was significantly different (12.70 ± 0.65 mg/100 g FW) from that of the Touchon variety (38.81 ± 0.44 mg/100 g FW) (*Figure 1*). The two varieties present a significant difference ($p < 0.05$); the Touchon variety contains 2.5-fold more phenolics than Supermuscade. These results are in agreement with those reported by *Alasalvar et al.* (2001), who noted differences on the phenolic content of carrot varieties. In orange, yellow, and white varieties, the phenolic content varies from 7.74 to 16.2 mg/100 g; for purple carrot, it was 74 mg/100 g of fresh weight. Furthermore, the phenolic content of carrots has varied from 12.59 to 290.18 mg GAE/100 g FW (*Koley & Singh*, 2019). *Alasalvar et al.* (2005) have reported that orange and purple carrots contain 34.8 and 102 mg/100 g respectively. *Yu et al.* (2005) and *Cieslik et al.* (2006) found that the phenolic content of carrot was 198 and 15.6 mg/100 g FW respectively. Moreover, in orange carrot varieties, the phenolic content varied from 18.7 to 58.6 mg/100g FW (*Leja et al.*, 2013). These differences on the TPC may be caused by varietal differences, the geographic origin, and solvent and/or extraction method or measurement.

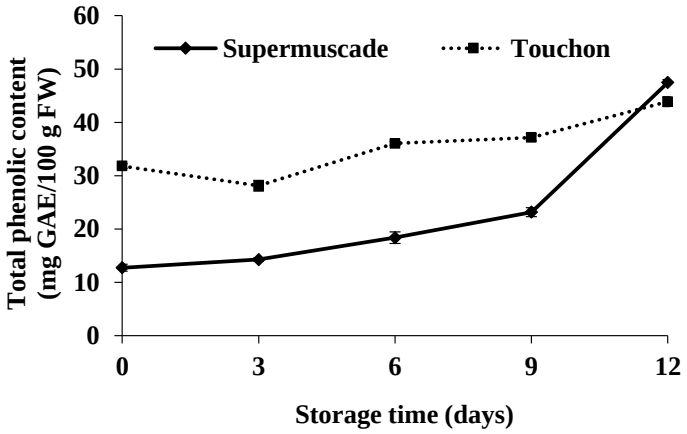

Figure 1. Effects of refrigerated storage on the phenolic content of carrot

Figure 1 shows that TPC increased significantly from the third day of storage until the 12th day. After 12 days of storage, TPC increased with 73.18% and 25.52% for Supermuscade and Touchon respectively. These results are in accordance with those obtained by *Zhang et al.* (2005), where the phenolic content of the Kend, Ricardo, and Stefano carrot varieties increased after 10 days of storage at 4 °C. Increase of TPC due to storage may be a result of the increased transcription of genes encoding the corresponding biosynthetic

enzymes (*del Rosario Cuéllar-Villarreal et al.*, 2016; *Dixon & Paiva*, 1995), i.e. changes in phenolic compound metabolism (*Alasalvar et al.*, 2005) and the synthesis of these compounds during storage (*Klimczak et al.*, 2007). According to *Tavarini et al.* (2008), the increase of total phenolic content during storage could be attributed to changes occurring in phenol metabolism as well as to the increase of phenylalanine ammonia lyase (PAL). PAL has been found to be associated with post-harvest disorders induced after prolonged storage at low temperature (*Martinez-Tellez & Lafuente*, 1997; *Zhao et al.*, 2019b).

Total Flavonoid Content (TFC)

The presence of phenolic compounds in carrots influences the organoleptic properties of fresh and processed carrots, including colour, bitterness, and aroma. Therefore, they could be used as a good quality indicator during processing and storage (*Hà & Nguyễn*, 2015). *Ahmad et al.* (2019) reported that carrots are rich in phenolic acids as well as in anthocyanins, a class of flavonoids. Flavonoids are among the most studied phytochemicals in foods of plant origin and include a large number of different molecules with various biological activities. Similarly to phenolic compounds, the TFC of Supermuscade and Touchon aqueous extracts significantly differed with rates of 3.20 ± 0.04 and 7.93 ± 0.21 mg/100 g FW respectively (*Figure 2*). These results are in accordance with those reported by *Miean & Mohamed* (2001) and *Marinova et al.* (2005), who registered carrot flavonoid contents of 3.7 and 26.7 mg/100 g respectively.

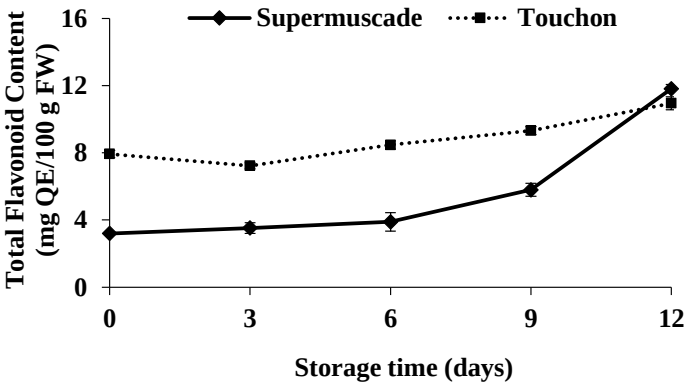

Figure 2. Effects of refrigerated storage on the flavonoid content of carrot

Furthermore, in orange carrots, a flavonoid content of 5.33 mg CE/100 g FW was indicated by *Singh et al.* (2018a). Similarly, *Leja et al.* (2013) and *Koley & Singh* (2019) estimated the highest amount of flavonoids in carrots. On the contrary, *Al-Dabbas et al.* (2015) recorded low flavonoid content in carrots with a value of 0.029 μg/g. The published data may vary according to the extraction methods, sample preparations, and other factors such as cultivars, post-harvest handling, and processing conditions (*Hà & Nguyễn*, 2015). In addition, *Dixon & Paiva* (1995) stated that the flavonoid composition of plants depends on the temperature, the solar exposition, the cellular damage, and the available quantities of phosphorus and nitrogen. Furthermore, *Ahmad et al.* (2019) reported that phenolic compounds are affected by multiple factors such as the cultivar, storage conditions and temperature, fertilizer application, processing procedures, and various biotic and abiotic stress factors.

Similarly to TPC, TFC increases significantly ($p < 0.05$) from the third day of storage at 4 °C to the 12th day. In fact, TFC increases with 72.92% (Supermuscade) and 27.58% (Touchon) after 12 days of storage. These results are in agreement with those reported by *Lafuente et al.* (2011), *Gorrepati & Bhagat* (2018), and *Youryon & Supapvanich* (2019). In addition, *Ahmad et al.* (2019) reported that in a recent study conducted by *Kamiloglu et al.* (2015) with black carrots it was found that after 20 weeks of storage the preserved amount of main flavonoids (anthocyanins) in samples stored at 4 °C (53.4%–81.0%) was higher than in samples stored at 25 °C (7.8%–69.3%). Moreover, *Del Caro et al.* (2004) noted an increase in the flavonoid content of lemon after 12 days of storage at 4 °C, explained by the stimulation of phenylalanine ammonia lyase (PAL) activity and consequently a synthesis of these compounds. According to *Gorrepati & Bhagat* (2018), the increase of flavonoid content after refrigerated storage may be attributed to stress due to low temperature. On the other hand, further studies have shown a decrease in flavonoid content in carrot (*Al-Dabbas et al.*, 2015), lettuce (*DuPont et al.*, 2000), and fresh-cut onion during storage (*Berno et al.*, 2014). These differences may be explained by the differences in the time and/or temperature of the storage.

Total Carotenoid Content (TCC)

Carotenoids are compounds very sensitive to light, heat, air, and other variables; consequently, their determination, involving the steps of extracting, can be accompanied by degradations and/or loss. For this reason, it is important to make a careful evaluation of the analytical procedure to avoid causes of variation and inaccuracies (*Chiosa et al.*, 2005). *Figure 3* shows the effects

of storage at 4 °C throughout 12 days on the TCC of the two orange carrot varieties. The varieties analysed present significant differences ($p < 0.05$); the TCC of Touchon variety (19.09 ± 0.06 mg/100 g FW) was initially 2.2-fold higher than that of the Supermuscade variety (8.9 ± 0.1 mg/100 g FW). This is in agreement with values reported by *Alasalvar et al.* (2005), who found that the total carotenoid of purple carrots (19.5 ± 0.05 mg/100 g) was 2.3-fold higher than that of orange carrots (8.6 ± 0.2 mg/100 g). *Edwards et al.* (2002) reported that the total carotenoid content of the Apache variety was 11.45 mg/100 g, while *Sun & Temelli* (2006) recorded carrot carotenoids content of 15 mg/100 g. In addition, *Scarano et al.* (2018), *Singh et al.* (2018a), and *Hasan et al.* (2019) estimated the highest amount of carotenoids in carrot compared to the results found in the present study. *Koley & Singh* (2019) noted that the β-carotene content in various coloured carrot genotypes ranged between 0 and 4.62 mg/100 g and high β-carotene content was observed in the orange-coloured genotype. These differences in TCC are probably due to the extraction method and/or the sensibility of the measurement method, the varietal differences and geographic origin of the sample analysed. According to *Sun & Temelli* (2006), total carotenoid content can be influenced by carrot genotype, development stage, and growing conditions such as temperature and use of fertilizers.

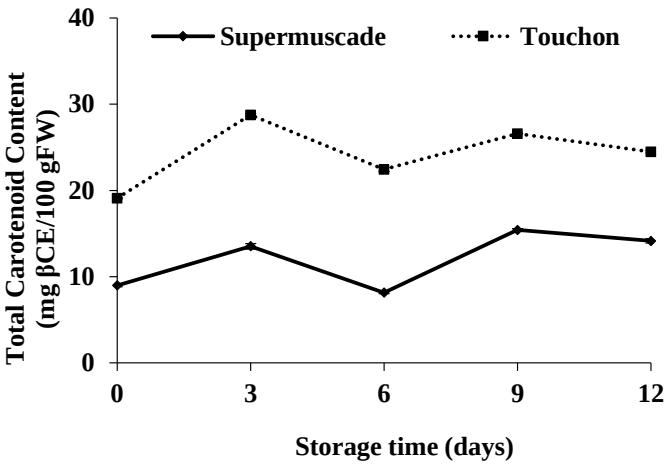

Figure 3. Effects of refrigerated storage on the carotenoid content of carrot

Figure 3 shows that the evolution of TCC during storage varies significantly. An increase of TCC has been noted after 3 and 9 days of storage, which is not

the case after 6 days of storage, when a slight decrease was recorded in TCC. This effect may be attributed to the cis-trans isomerization of carotenoids during storage. According to *Vieira et al.* (2018), this behaviour may be related to the matrix disruption and the polyene chain instability of carotenoids, promoting their isomerization or oxidation. Overall, the storage of carrot at 4 °C for 12 days causes a significant increase in TCC, with a percentage of 36.46% in Supermuscade and 21.98% in Touchon varieties. Similar results were obtained by *Murcia et al.* (2009), *Preethi et al.* (2018), and *Nunes et al.* (2019). *Berger et al.* (2008) stated that the storage of carrot for 14 days at 4 °C causes a significant increase in carotenoid content: 8% and 23% for Nevis and Kingston varieties respectively. These results were explained by the carotenoid biosynthesis during storage and the good extractability of these pigments after enzymatic decomposition, i.e. the fibrous structure of the carrot matrix could be disaggregated by cellulases and hemicellulases, and further quantities of carotene could be released during the extraction process. According to *Ahmad et al.* (2019), carotenoids are influenced by two main factors – namely, inherited characteristics and environmental conditions (during growth and packaging and/or storage conditions and temperature).

Many authors have reported a decrease in total carotenoid content during storage of fruits and vegetables. The Kintoki variety lost 30% of total carotenoid after 9 weeks of storage at 1 °C (*Mayer-Miebach & Spieß*, 2003). Similarly, a decrease of TCC was obtained by *Alasalvar et al.* (2005) in purple and orange carrots after 13 days of storage at 5 ± 2 °C and by *Macura et al.* (2019) in purple carrots. These differences registered in relation to TCC as effects of storage may be explained by the differences in storage temperature and duration and/or the varietal differences.

Antioxidant activity

In order to assess the antioxidant capacity of orange carrots during storage, two methods based on different reaction mechanisms were applied. The first is radical scavenging activity, which is based on the extract's ability to neutralize DPPH radical, and the second is ferric reducing power (FRP), which is based on the ability of the extract to reduce Fe^{3+}.

DPPH Free Radical Scavenging Activity (DPPH-FRSA)

The results obtained (*Figure 4*) indicate that the antiradical activity of aqueous carrot extracts presents significant differences at $p < 0.05$. The inhibition percentages of the DPPH radical were initially 10.90% and 20.60% for

Supermuscade and Touchon respectively. These results indicate that the antiradical activity of the Touchon variety was roughly 2-fold higher than that of the Supermuscade variety. These results are comparable with those reported by *Singh et al.* (2018b). *Singh et al.* (2018a) claimed that the antioxidant activity of coloured tropical carrots ranged from 1.22 to 43.98 μmol TE/g FW. *Koley & Singh* (2019) reported that the antioxidant activity varied from 0.58 to 29.72 μmol TE/g in the carrots' genotype. Also, *Leja et al.* (2013) noted a high antioxidant capacity in orange, white, and yellow roots, showing radical scavenging activity with a rate of 6%, and only the red roots had a high activity with a percentage of 9.3%. *Gajewski et al.* (2007) found higher antioxidant capacity in methanolic extracts from purple carrots as compared to extracts from orange and yellow carrots. *Yen et al.* (2008) observed very high, reaching even 80–98%, DPPH neutralization activity in red carrot roots; however, these authors used very high (2–20 mg DM/cm^3 of extract) tissue concentration. Furthermore, in their study on some selected fruits in Ekiti State, Nigeria, *Ogunlade et al.* (2019) noted that carrots presented a good antioxidant activity comparatively to other fruits as tangerine, lime, or watermelon. These differences may be related to differences in varieties and/or geographic origin. According to *Smeriglio et al.* (2018), antioxidant activity is not only related to the main constituents, but it may be modulated by several other compounds, wherefore the concepts of synergism and antagonism can be highly relevant.

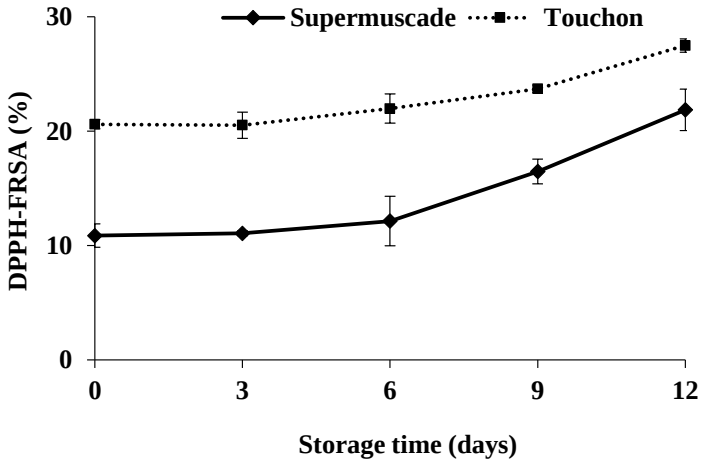

Figure 4. Effects of refrigerated storage on the antiradical activity of carrot

Figure 4 shows that the antiradical activity of carrot varieties increases progressively from the 6^{th} day until the 12^{th} day of storage at 4 °C. Indeed, the antiradical activity increased by 50.22% in Supermuscade and 25.06% in Touchon aqueous extracts. This effect is probably due to the increase of bioactive compounds (TPC, TFC, and TCC) during storage. Similar results were reported by *da Silva et al.* (2018), *Magalhães et al.* (2019), and *Zahoor & Khan* (2019). According to *Shivashankara et al.* (2004), an increase in antioxidant activity during the storage of vegetables was generally attributed to the phenolic content. *Lattanzio et al.* (1994) reported that during the storage of vegetables the cellular walls lost their integrity, which led to browning due to the enzymatic oxidation of phenolic compounds. *Zahoor & Khan* (2019) explained this increase in antioxidant activity by the occurrence of Maillard reactions during storage. Moreover, *Pinelo et al.* (2004) reported that these oxidations were coupled with the formation of highly polymerized phenolic compounds, possessing higher antioxidant activity in comparison with their natural precursors. However, many other authors have registered that storage decreases antioxidant activity in different foods (*Ang & Deocampo*, 2019; *Corleto et al.*, 2018; *Louaileche & Djaoudene*, 2016; *Murcia et al.*, 2009; *Nayik & Gull*, 2018; *Vieira et al.*, 2018). In fact, these differences in the effects of storage may be explained by the variety in duration and/or the different storage temperatures.

Ferric Reducing Power (FRP)

The presence of reductants causes the reduction of Fe^{3+} ferricyanide complex to the ferrous form; Fe^{2+} is monitored by measuring the formation of Perl's Prussian blue at the wavelength of $\lambda_{abs} = 700$ nm. Ferric reducing ability may serve as an indicator of the antioxidant potential. Prior to storage, the analysed carrots exhibited a ferric reducing ability of 25.46 mg TE/100 g FW for Supermuscade and 94.20 mg TE/100 g FW for Touchon (*Figure 5*). These results indicate that the FRP of Touchon variety was 3.7-fold higher than that of Supermuscade variety due to the highest content of bioactive phytochemicals (TPC, TFC, and TCC) in the Touchon variety. Our results approximate the value reported by *Allane & Benamara* (2019). Moreover, *Šeregelj et al.* (2017) noted a high reducing ability of carrot extracts obtained with ethanol-acetone solvent mixture with a rate of 7368.07 and 3167.91 μmol TE/100 g DW. However, a low reducing power was registered for the ethyl acetate extract with a level of 71.19 μmol TE/100 g DW. The observed differences may be explained by the differences in the polarity of solvents used for

carrot extraction, yielding different compositions of the extracts. The ethanol, acetone, and ethyl acetate extracts had the highest total bioactive compound content (i.e. carotenoids and polyphenols) resulting in superior reducing power (*Šeregelj et al.*, 2017). In addition, the modifications in the extraction procedures, in particular the homogenized sample weight: solvent volume ratio, extraction solvent type and technical extraction, and the varietal differences cannot be excluded in this case.

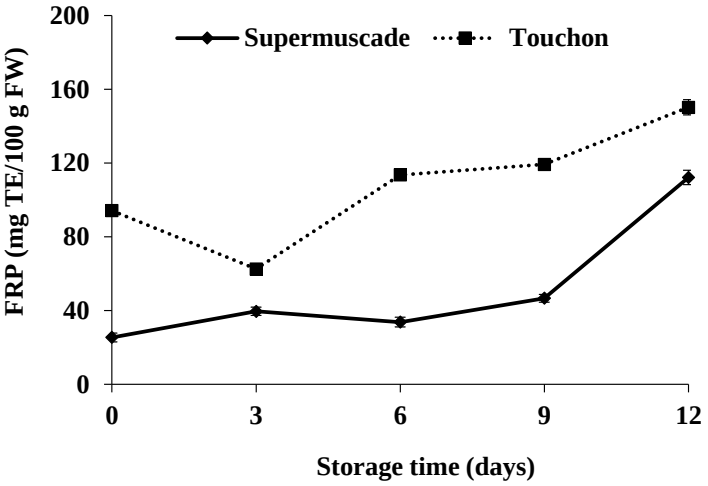

Figure 5. Effects of refrigerated storage on the reducing power of carrot

As shown in *Figure 5*, FRP increases gradually from the third day of the storage at 4 °C until the 12th day. The initial values of the ferric-reducing potential of orange carrot extracts are 25.46 mg TE/100 g FW (Supermuscade) and 94.20 mg TE/100 g FW (Touchon). It rises with a proportion of 77.30% for Supermuscade and 37.28% for Touchon. This effect could be attributed to the increase of the antioxidant compounds (TPC, TFC, and TCC) during storage, which is responsible for the antioxidant power. These results are in concordance with those reported by *Kallithraka et al.* (2009), *Zhao et al.* (2018), and *Zhao et al.* (2019a).

Nevertheless, several other authors have registered a decrease of reducing power during storage: *Saci et al.* (2015), *Louaileche & Djaoudene* (2016), *Panigrahi et al.* (2018), and *Ang & Deocampo* (2019). These differences observed regarding the impact of storage conditions on reducing power may be attributed to the differences in storage conditions (time and temperature) and/or the food matrices.

Correlations

Correlation analysis was used to explore the relationship between the different measured variables. The correlation matrix presented in *Table 1* revealed a correlation between bioactive compound content and antioxidant activity. A strong positive correlation was observed between total phenolic, total flavonoid, and total carotenoid content ($r = 0.99$ and $r = 0.85$). The antioxidant capacity of aqueous extracts was influenced by the content of bioactive compounds; FRSA and FRP activities were highly and significantly correlated ($p < 0.001$) with TPC ($r = 0.83$ and $r = 0.82$ respectively), TFC ($r = 0.85$ and $r = 0.83$ respectively), and TCC ($r = 0.75$ and $r = 0.63$ respectively). This indicates that these compounds are most responsible for the free radical scavenging ability and ferric-reducing power of the investigated carrots. A similar trend was observed in carrot extracts by other researchers, who observed a direct significant association between the total phenolics, total flavonoids, total carotenoids, and antioxidant ability (*Carrillo et al.*, 2017; *Koley & Singh*, 2019; *Koley et al.*, 2014; *Leja et al.*, 2013; *Singh et al.*, 2017) of root vegetables. Furthermore, a strong positive significant correlation was also observed between both antioxidant assays of FRSA and FRP ($r = 0.93$); this may be due to the presence of molecules displaying simultaneously antiradical and reducing properties. Similar results were reported by *Louaileche & Djaoudene* (2016) in orange jam and *Koley & Singh* (2019) in various carrot genotypes.

Table 1. Correlation matrix between the phytochemical content and antioxidant activity of orange carrots

	TPC	**TFC**	**TCC**	**FRSA**	**FRP**
TPC	1.00				
TFC	0.99***	1.00			
TCC	0.85***	0.85***	1.00		
FRSA	0.83***	0.85***	0.75***	1.00	
FRP	0.82***	0.83***	0.63***	0.93***	1.00

Notes: **TPC**: Total Phenolic Content; **TFC**: Total Flavonoid Content; **TCC**: Total Carotenoid Content; **FRSA**: Free Radical Scavenging Activity; **FRP**: Ferric-Reducing Power *** $p < 0.001$: extremely significant correlations)

4 Conclusions

In conclusion, the result of this investigation confirmed that Algerian orange carrots are a good source of bioactive molecules. The Touchon variety is richer in TPC (38.81 ± 0.44 mg/100 g FW), TFC (7.93 ± 0.21 mg/100 g FW), and TCC (19.09 ± 0.06 mg/100 g FW) and presents higher antioxidant activity (20.60% and 94.20 mg TE/100 g FW for FRSA-DPPH and FRP respectively) in comparison with the Supermuscade variety. Refrigerated (4 °C) storage for 12 days caused a significant increase of the bioactive compounds (phenolics, flavonoids, and carotenoids) and antioxidant activity of orange carrots as well as an increase of PAL, which led to an increase in antioxidant activity. Moreover, there was a strong linear correlation between the antioxidant compounds and antioxidant activity, which confirmed that these substances are the main compounds responsible for the carrots' antioxidant activity. Therefore, the refrigerated storage of carrots is a promising method that not only extends shelf life but also improves the bioactive compound content and antioxidant activity of carrots.

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Physico-chemical and sensory properties of *pupuru* and *pupuru* analogues from co-fermented cassava (*Manihot esculenta Crantz*) and breadfruit (*Artocarpus altilis*) blends

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Abstract. The physico-chemical and sensory qualities of *pupuru* analogues produced from co-fermented cassava and breadfruit blends were investigated. Cassava and breadfruit were processed separately and co-fermented at different proportions to produce *pupuru* and *pupuru* analogues. Seven different samples were produced with the ratios of 100:0, 90:10, 80:20, 50:50, 20:80, 10:90, and 0:100 cassava:breadfruit respectively. The proximate composition, bulk density, hydrogen cyanide, pH, TTA, and sensory properties of the sample were determined using standard methods. The results showed that the protein (2.86–6.41%), fat (0.43–2.05%), ash (0.36–1.17%), crude fibre (0.68–2.83%), and energy values (393.84 to 399.38 kcal/100 g) increased together with breadfruit substitution. The bulk density, pH, total titratable acidity, and hydrogen cyanide content of the sample was in the ranges of 0.47–0.60 g/ml,

Keywords and phrases: bulk density, co-fermented, hydrogen cyanide, proximate composition, sensory properties

4.30–5.30, 0.18–0.31%, and 0.56–1.68 mg/100 g respectively. The *pupuru* analogues had lower hydrogen cyanide content than *pupuru*. The *pupuru* analogues up to 50% breadfruit substitutions had acceptable sensory attributes, comparable to *pupuru*. The study concluded that *pupuru* analogues of acceptable quality can be produced from co-fermented cassava and breadfruit; this entails increasing the utilization of breadfruit.

1 Introduction

Breadfruit (*Altocarpus altilis*) is a crop native to Malaysia and countries of the South Pacific and Caribbean (*Ajani et al.*, 2012). Other botanical names by which the plant is known include *Artocarpus communis* and *Artocarpus incise*. It is widely cultivated to an appreciable extent in the south-western states of Nigeria (*Adejuyitan et al.*, 2018). The present level of breadfruit production in south-western Nigeria has been estimated at about 10 million tonnes of dry weight per year, with potential to exceed 100 million tonnes every year (NTBG, 2009; *Ajani et al.*, 2016). This starchy fruit is sometimes round or oval in shape, with rough green skin, having pale yellow or white flesh. The fruit is high in carbohydrate, low in fat, protein, and is a good source of minerals (iron), vitamins, especially niacin, riboflavin, and pro-vitamin A (*Ajatta et al.*, 2016). However, the traditional use of breadfruit is limited to boiled and pounded breadfruit among the "Ifes", but its use can be expanded by exploring other value-added products in this regard.

Fermentation is one method of processing cassava into another food form, which not only improves the flavour and taste of the product but extends its shelf life *(Falade & Akingbala*, 2010). Acid production during cassava fermentation has been attributed to the activities of lactic acid bacteria on the carbohydrate content of cassava tuber (*Oyewole & Afolami*, 2001). Fermentation enhances the reduction of the cyanide level and detoxification of the root (*Kostinek et al.*, 2005). One of the notable products from fermented cassava is *pupuru*.

Pupuru, a fermented cassava product, is usually consumed by the people living in the riverine areas of the southern and middle belts of Nigeria, where it is also known as "Ikwurikwu" (*Shittu et al.*, 2003; *Daramola et al.*, 2010). Pupuru and other cassava products are widely accepted and consumed in Nigeria (*Adejuyitan et al.*, 2018). It is moulded into the shape of a smoke ball, which is usually made into dough in boiling water before consumption with any desired soup (*Ikujenlola & Lawson*, 2005). Breadfruit is nutritious, cheap, and available in high abundance during its season, while it also helps

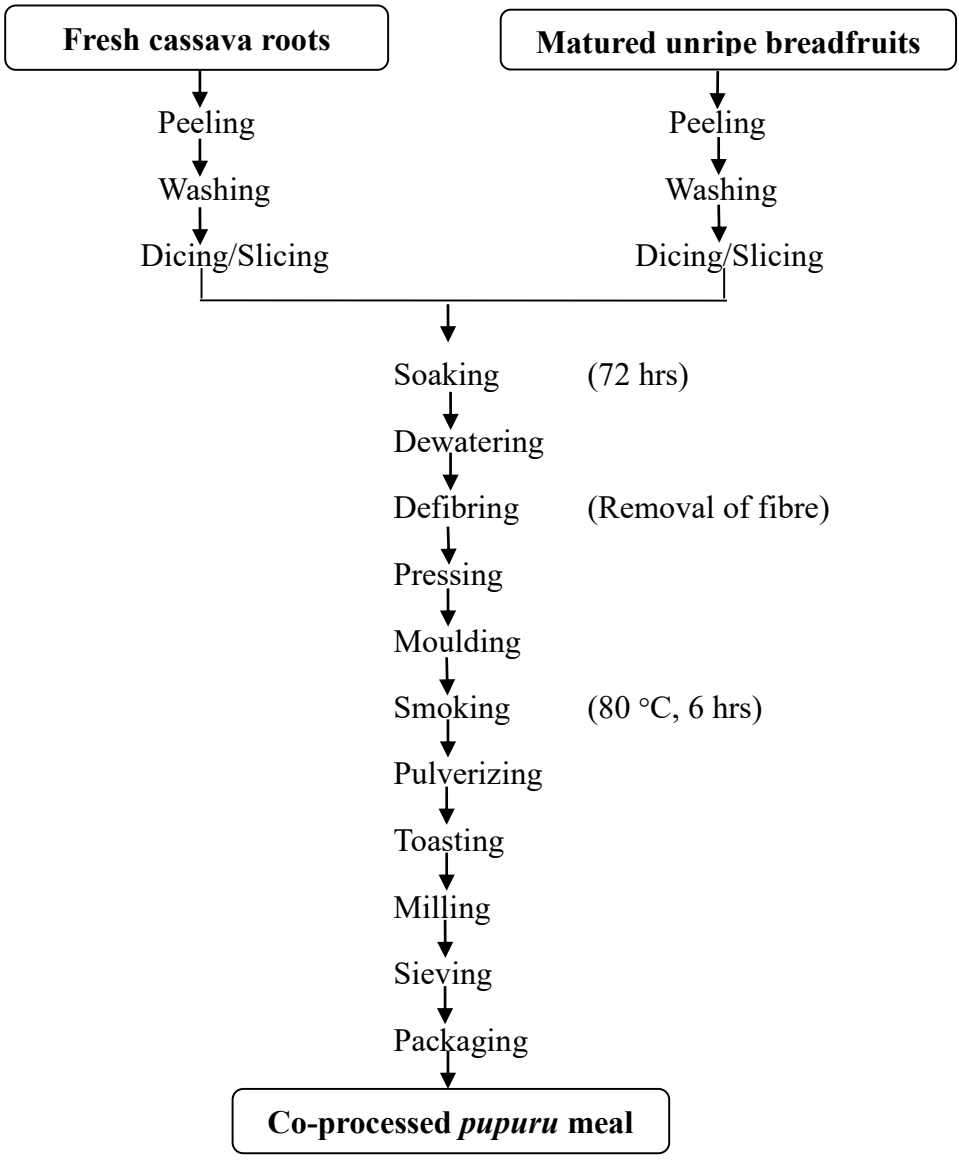
out the poor people in rural areas, providing them with an extra layer of food security (*Omobuwajo*, 2003). The expansion of breadfruit utilization has not been extended to the production of *pupuru analogue*. However, the putrid smell has limited its sensory acceptability, and there is also need for a further investigation into the physico-chemical properties of *pupuru* analogues to increase the utilization of breadfruit and make it a food of first choice, especially in several food deficit regions and countries. Hence, the objectives of this study were to produce *pupuru* analogue from co-fermented breadfruit and cassava blends and to evaluate the physico-chemical and sensory properties in a culturally familiar form, analogous to *pupuru* from cassava.

2 Materials and methods

Matured unripe breadfruits (*Artocarpus altilis*) and matured cassava root (*Manihot esculenta*) were purchased at Ilode and Tonkere markets in Osun State, Nigeria.

Production of *pupuru* and *pupuru* analogue meals

Cassava tuber and matured unripe breadfruits were washed and peeled. The cassava and breadfruit were mixed at different ratios of 100:0, 90:10, 80:20, 50:50, 20:80, 10:90, and 0:100 (wt/wt). It was sliced into easy-to-manage pieces using dicing machine. This is to ensure regular shape and size; this will guarantee the uniform fermentation of the diced pieces. The diced/sliced breadfruit and cassava roots were co-fermented (this is to allow for synergy in the fermentation process of the two biomaterials) in water (1:3 solid:water) inside a plastic container for 72 hrs at ambient temperature (28–32 °C) to allow the inherent fermenting microorganisms to act on it and soften the pieces. The fermented mash was drained of excess water and the fibres were removed manually. Thereafter, the mash was packed inside bags and pressed using hydraulic press for 30 minutes to further reduce the water. The dewatered mash was moulded into balls of 5–10 cm in diameter. The moulded balls were smoked in the kiln dryer at 80 °C for 6 hrs. In order to produce meal from the smoked balls, they were scraped of the dark outer portion of the balls, pulverized, sieved, and toasted (> 90 °C) for 10 min in a traditional toaster. The toasted mass was cooled, re-milled, sieved (630 micron sieve), and packaged to obtain *pupuru* and *pupuru* analogue meals (*Figure 1*) (*Ikujenlola & Lawson*, 2005).



Source: *Ikujenlola & Lawson* (2005)

Figure 1. Production of *pupuru* and *pupuru* analogue meals

Formulation of samples

Table 1 shows the various *pupuru* and *pupuru* analogues produced from cassava and co-fermented cassava and breadfruit respectively.

Chemical analysis

The proximate compositions of the samples were determined using standard methods of AOAC (2010). The samples were analysed for moisture, ash, crude fibre, crude protein, crude fat, and carbohydrate. Calories was calculated using Atwater factors; the sum of 4 × percentage of Protein, 4 × percentage of carbohydrate, and 9 × percentage of fat (*Onoja et al.*, 2014).

Table 1. Formulation of *pupuru* and *pupuru* analogues from co-processed cassava and breadfruit

Samples	Cassava	Breadfruit
100% PF	100	–
100% BP	–	100
90:10 PF/BP	90	10
80:20 PF/BP	80	20
50:50 PF/BP	50	50
20:80 PF/BP	20	80
10:90 PF/BP	10	90

Source: *Ikujenlola & Lawson (2005)*

Keys: **100% PF** – 100% cassava; **100% BP** – 100% breadfruits; **90:10 PF/BP** – 90% cassava co-processed with 10% breadfruits; **80:20 PF/BP** – 80% cassava co-processed with 20% breadfruits; **50:50 PF/BP** – 50% cassava co-processed with 50% breadfruits; **20:80 PF/BP** – 20% cassava co-processed with 80% breadfruits; **10:90 PF/BP** – 10% cassava co-processed with 90% breadfruits

Physico-chemical properties

Bulk density

The bulk density was determined by the method of *Okezie & Bello* (1988). A 10 ml graduated cylinder, previously tared, was gently filled with the sample. The bottom of the cylinder was gently tapped on a laboratory bench several times until there was no further diminution of the sample level after filling to the 10 ml mark. Bulk density was calculated as weight of sample per unit volume of sample (g/ml).

pH

The pH was measured by making a 10% w/v suspension of the sample in distilled water. The suspension was mixed thoroughly in a Sorex blender and the pH was measured with a Hanna checker pH meter (Model HI1270).

Total titratable acidity

The total titratable acidity of the sample was determined using the method described by AOAC (2010). Five grams of the sample was weighed in a clean beaker and 50 ml of distilled water was added and homogenized, from which 25 ml of the solution was taken into another conical flask, and three drops of 2% phenolphthalein indicator was added. The mixture was titrated against 0.1 N sodium hydroxide (NaOH) until a permanent pink-coloured end-product was obtained. Total titratable acidity was calculated as follows and expressed as percentage lactic acid.

$$\%\ \text{lacid acid (wt/vol)} = N \cdot V \cdot Eq.wt \cdot W \cdot 1000 \cdot 100, \tag{1}$$

where: N = normality of titrant, usually NaOH (mEq/ml); V = volume of titrant (ml); $Eq.wt$ = equivalent weight of predominant acid (mg/mEq); W = mass of sample (g); 1000 = factor relating mg to gram (mg/g) (1/1000).

Hydrogen cyanide determination

The cyanogenic potentials of *pupuru* meals were determined using the picrate paper kits method as described by *Bradburg et al.* (1999). One gram sample of *pupuru* meal was homogenized in a 250 ml conical flask containing 25 ml of water. A strip of spot paper soaked in an alkaline sodium picrate solution was fixed in the solution with the cork; the flask was kept for 18 hrs at 27 °C (room temperature), the strip was removed and later eluted in 60 ml, and the absorbance was read at 540 nm using a spectrophotometer. The hydrogen cyanide content was extrapolated using a cyanide standard curve.

Sensory evaluation

A voluntary panel of 15 judges made up of both males and females were selected from Obafemi Awolowo University, Ile-Ife. The selection was based on the fact that they were familiar with *pupuru*. The samples were placed on white plates coded with alphabetic letters under normal lighting condition at

room temperature. Panellists were instructed in assessment terminology and requested to evaluate the various *pupuru* and *pupuru analogue* samples for taste, colour, aroma, texture, mouldability, and overall acceptability using a 9-point Hedonic scale as follows: 1 = dislike extremely, 2 = dislike very much, 3 = dislike moderately, 4 = dislike slightly, 5 = neither like nor dislike, 6 = like slightly, 7 = like moderately, 8 = like very much, and 9 = like extremely (*Iwe*, 2002).

Statistical analysis

The data obtained were expressed as mean ± standard deviation of nine experiments and were subjected to statistical analysis using one-way analysis of variance to determine the significant differences between means with significance level taken at $\alpha = 0.05$. Tukey's least significant difference test was used to compare the means. All statistical procedures were carried out using SPSS version 17.0 (SPSS, Chicago, IL, USA).

3 Results and discussion

Physical appearance and proximate composition of *pupuru* and *pupuru* analogues

The *pupuru* and *pupuru* analogue meals produced from cassava and breadfruit, respectively, are presented in *Figure 2*. It was observed that *pupuru* had a brighter white colour compared to *pupuru* analogues, which are creamy in colour. The higher the proportion of breadfruit is, the darker the colour. The locally produced *pupuru* and industrial *pupuru* were of brighter colour.

The results of the proximate compositions of *pupuru* and *pupuru* analogues from cassava and cassava co-processed with breadfruit, respectively, are presented in *Table 2*. The protein content of all the *pupuru* analogues ranged between 2.86 and 6.41%. It was observed that the protein content of *pupuru* analogues from 100% breadfruit was 6.41%, which was the highest of all the samples. The protein content of *pupuru* from 100% cassava was higher than 0.55% as reported by *Ojo et al.* (2017) for 100% cassava starch obtained from cassava starch-mushroom flour blends. It was observed that the protein content increased with increase in the level of substitution of breadfruit. The protein obtained was comparable with the range of 1.52–7.22% reported by *Alozie et al.* (2017) for gari fortified with soybeans, melon seed, and moringa seed flours. However, it was higher than the range (1.70–3.75%) reported by

Adejuyitan et al. (2018) for *pupuru* from breadfruit and tigernut flour. There was no significant difference ($p > 0.05$) in the protein content of 100% BP and 10:90 PF/BP. *Padmaja & Jisha* (2005) reported that the protein content of cassava-based composite flours increased with the incorporation of legume flours.

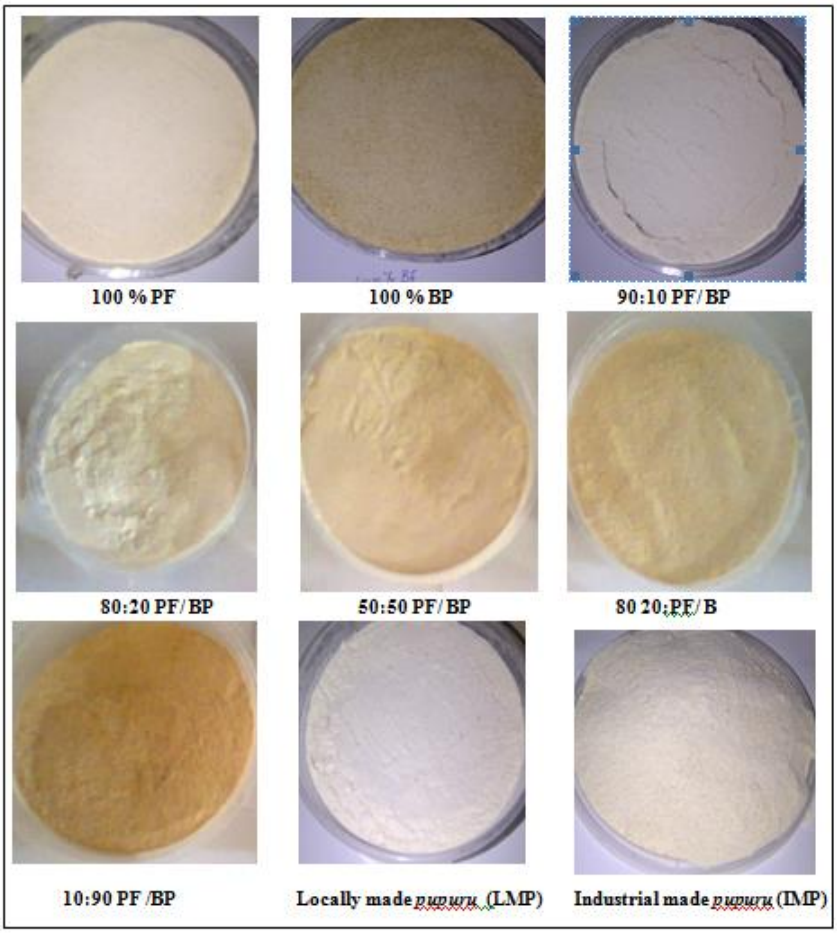


Figure 2. Finished products of *pupuru* and *pupuru* analogue meals

Keys: **100% PF** – 100% cassava; **100% BP** – 100% breadfruits; **90:10 PF/BP** – 90% cassava co-processed with 10% breadfruits; **80:20 PF/B**P – 80% cassava co-processed with 20% breadfruits; **50:50 PF/BP** – 50% cassava co-processed with 50% breadfruits; **20:80 PF/BP** – 20% cassava co-processed with 80% breadfruits; **10:90 PF/BP** – 10% cassava co-processed with 90% breadfruits

Table 2. Proximate compositions of *pupuru* and *pupuru* analogue meals (% dry basis)

Samples	Moisture	Ash	Fat	Crude fibre	Protein	Carbohydrate	Energy value (kcal/100 g)
100% PF	11.80 ± 0.35^{cd}	1.17 ± 0.22^{a}	0.43 ± 0.07^{g}	0.90 ± 0.00^{c}	2.86 ± 0.19^{e}	94.64 ± 2.08^{ab}	393.84 ± 1.99^{b}
100% BP	12.98 ± 0.02^{a}	1.07 ± 0.07^{a}	2.05 ± 0.17^{a}	2.83 ± 0.23^{a}	6.41 ± 0.18^{a}	87.64 ± 3.77^{c}	394.67 ± 0.85^{c}
90:10 PF/BP	11.47 ± 0.42^{d}	0.36 ± 0.14^{c}	0.70 ± 0.02^{f}	0.68 ± 0.11^{c}	3.33 ± 0.16^{de}	94.93 ± 1.82^{a}	399.38 ± 0.65^{a}
80:20 PF/BP	12.97 ± 0.67^{a}	0.54 ± 0.23^{bc}	0.85 ± 0.02^{e}	0.88 ± 0.06^{c}	3.77 ± 0.41^{cd}	93.96 ± 2.30^{ab}	398.58 ± 1.73^{b}
50:50 PF/BP	12.72 ± 0.14^{ab}	0.59 ± 0.03^{bc}	1.40 ± 0.01^{d}	2.32 ± 0.17^{b}	4.26 ± 0.37^{c}	91.43 ± 2.37^{abc}	395.33 ± 0.56^{bc}
20:80 PF/BP	12.73 ± 0.18^{ab}	0.69 ± 0.17^{b}	1.62 ± 0.02^{c}	2.40 ± 0.35^{b}	5.52 ± 0.35^{b}	89.77 ± 3.10^{bc}	395.79 ± 1.30^{bc}
10:90 PF/BP	12.21 ± 0.30^{bc}	1.06 ± 0.13^{a}	1.88 ± 0.01^{b}	2.48 ± 0.17^{b}	5.87 ± 0.19^{ab}	88.71 ± 3.64^{bc}	395.24 ± 3.41^{b}

Mean ± standard deviation of triplicate determinations.

Means with the same superscripts in the same column are not significantly different at 5% probability level.

Keys: **100% PF** – 100% cassava; **100% BP** – 100% breadfruits; **90:10 PF/BP** – 90% cassava co-processed with 10% breadfruits; **80:20 PF/BP** – 80% cassava co-processed with 20% breadfruits; **50:50 PF/BP** – 50% cassava co-processed with 50% breadfruits; **20:80 PF/BP** – 20% cassava co-processed with 80% breadfruits; **10:90 PF/BP** – 10% cassava co-processed with 90% breadfruits

The ash content of the *pupuru* and *pupuru* analogues ranged between 0.36% and 1.17%. The ash content of 100% PF was the highest but was not significantly different ($p < 0.05$) from that of 100% BP and 10:90 PF/BP. The value obtained was lower than the range (1.55–2.47%) recorded by *Alozie et al.* (2017) for gari fortified with soybean, melon seed, and moringa seed flours. The ash content of the *pupuru* analogue from 10% breadfruit (0.36%) was comparable with 0.33% as reported by *Monayajo & Nupo* (2011) for *pupuru* fortified with soy flour. However, ash values obtained in this study were lower than the maximum 3% recommended by the Codex Alimentarius Commission (1995) for edible cassava flour.

The fat content of the *pupuru* and *pupuru* analogues ranged from 0.43 to 2.05%. The *pupuru analogue* from 100% breadfruit had the highest fat content (2.05%). 100% PF had the lowest value (0.43%). These values were higher than the range of 0.26–0.56% reported for cassava-African yam bean fufu blends by *Nwokeke et al.* (2013). It was observed that the fat content of *pupuru analogue* from 100% breadfruit was lower than 100% breadfruit *pupuru analogue* (3.25%) as reported by *Adejuyitan et al.* (2018) but higher than the fat content of breadfruit flour (1.09%) reported by *Adepeju et al.* (2011). The increase in the fat content of the products could be attributed to the increase in the substitution level of breadfruit.

Crude fibre contents increased with increase in the level of substitution of breadfruit. The value ranged between 0.68 and 2.83%, with the highest in 100% BP and the lowest value in 90:10 PF/BP. There was no significant difference ($p > 0.05$) between the crude fibre of 100% PF, 90:10 PF/BP and 80:20 PF/BP. However, they were significantly different ($p < 0.05$) from those of 50:50 PF/BP and 20:80 PF/BP. The values were comparable to the crude fibre 1.34–2.01% from cassava-breadfruit fufu by *Agbon et a*l. (2010) but lower than the values (1.38–5.11%) reported by *Adejuyitan et al.* (2018) for *pupuru* flour from breadfruit and tigernut flour. The values in this study were higher than the 2% upper limit specified for edible cassava flour by the Codex Alimentarius Commission (1995). Crude fibre helps in maintaining the normal peristaltic movement of the intestinal tracts, thereby preventing colon diseases such as piles, cancer, or appendicitis (*Famurewa & Oluwalana*, 2007).

The moisture content of the *pupuru* and *pupuru* analogues ranged between 11.47 and 12.98%. The moisture content range in this study was lower than the range (12.10–14.00%) reported for three traditional fermented cassava products by *Shittu & Adedokun* (2010) but higher than the range (8.79–9.35%) reported by *Ojo et al.* (2017) for cassava starch and mushroom blends. The moisture content of *pupuru* analogues from 50% and 80% breadfruit substi-

tutions were significantly different ($p < 0.05$) from 100% PF. The moisture content of flour products is a function of drying temperature, time, and loading depth (*Ikujenlola & Lawson*, 2005). The higher the moisture content of food materials, the lower the shelf stability (*Aluge et al.*, 2016). Generally, the moisture content of the products was within the acceptable levels (10–14%) for flours (*Butt et al.*, 2004).

The values of carbohydrate decreased from 94.93 to 87.64% with increase in the level of substitution of breadfruit. The value of the carbohydrate for 90:10 PF/BP was the highest as compared to other samples. There was no significant difference ($p > 0.05$) between the values of the carbohydrate content for *pupuru* analogues substituted with 80% and 90% breadfruit. However, it was significantly different ($p < 0.05$) from the values obtained for 100% PF and 90:20 PF/BP. The decrease in the carbohydrate content of the analogues could be explained based on the lower level of carbohydrate present in breadfruit (87.64%) compared to cassava (94.93%). This observation agrees with the report of *Agbon et al.* (2010).

The energy value of *pupuru* and *pupuru* analogues ranged between 393.84 and 399.38 kcal/100 g. It was observed that the energy value of *pupuru analogue* substituted with 10% breadfruit had the highest value of 399.38 kcal/100 g. There was no significant difference ($p < 0.05$) between the energy value of *pupuru* analogues from 80% and 90% breadfruit substitution. The energy value of *pupuru* from 100% cassava was higher than the value 363.73 kcal/100 g (100% cassava starch) for cassava starch-mushroom blends (*Ojo et al.*, 2017). However, *Alaba et al.* (2013) reported reduced energy levels (358.03–359.32 kcal/100 g) for cassava flour.

Physico-chemical properties

The bulk density (*Figure 3*) ranged from 0.47 to 0.64 g/ml with a significant difference ($p < 0.05$). 100% BP had the highest value (0.64 g/ml) compared to other samples. There was no significant difference ($p < 0.05$) between the bulk density of 50:50 PF/BP and 20:80 PF/BP. However, those of 10:90 PF/BP and 90:10 PF/BP were the same. The range was comparable to the bulk density (0.40–0.62) reported by *Alaba et al.* (2013) for cassava flour (*pupuru*) but lower than 0.82–0.85 g/ml for composite flours made from wheat, breadfruit, and cassava starch, as reported by *Ajatta et al.* (2016). Bulk density is a measure of heaviness of flour (*Adejuyitan et al.*, 2009), and low bulk density is desired in flour blends as it contributes to lower dietary bulk, ease of packaging and transportation (*Aluge et al.*, 2016).

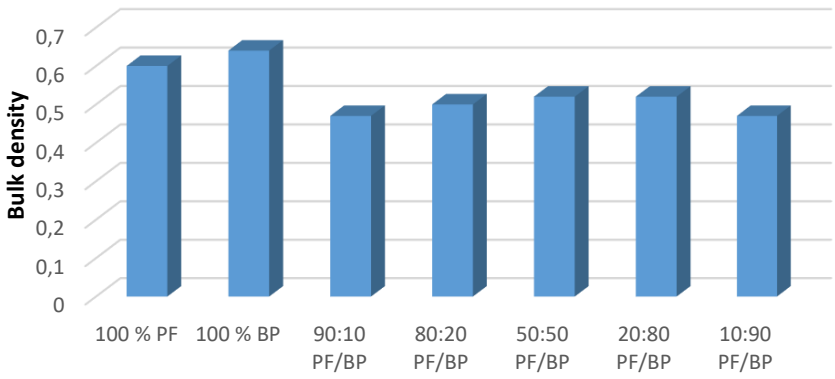


Figure 3. Bulk density (g/ml) of the *pupuru* and *pupuru* analogues

pH values (*Figure 4*) ranged between 4.37 and 5.30. pH value gives a measure of the acidity or alkalinity of the flour. The substitution of cassava in the *pupuru* analogues of breadfruit showed a gradual increase in the pH of the products. This was due to the pH of breadfruit, which was higher than that of cassava. There was no significant difference ($p < 0.05$) between the pH of the *pupuru* 100% PF and of *pupuru* analogues 20:80 PF/BP.

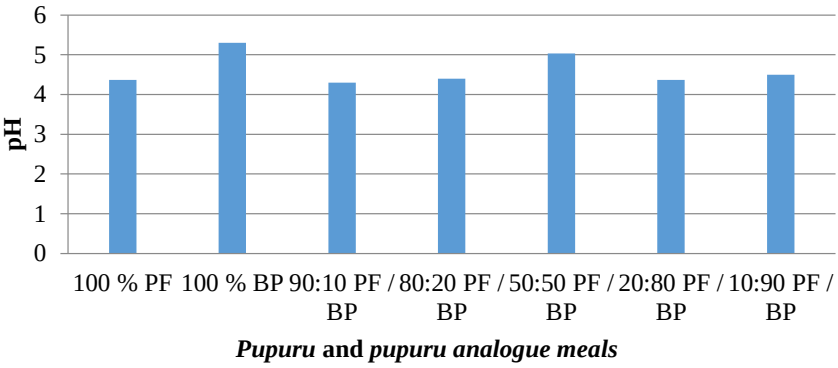


Figure 4. The pH of *pupuru* and *pupuru analogue meals*

The pH value of *pupuru* from 100% cassava (4.37) was lower than the value reported by *Adejuyitan et al.* (2018) for similar products. The pH decreased as a result of secretion of lactic acid, which implies that the more the cassava stays in the water during fermentation, the more there is reduction in the pH

by the action of fermenting organisms. Acidic products are more shelf-stable than their non-acidic counterparts (*Caballero et al.*, 2015).

The total titratable acidity (TTA) (*Figure 5*) expressed as percentage lactic acid of *pupuru* samples ranged between 0.18 and 0.31%. There was no significant difference ($p < 0.05$) in the total titratable acidity of all the samples. The value was higher than 0.13–0.16% for cassava flour (*pupuru*) as reported by *Alaba et al.* (2013). TTA values obtained for 100% BP are comparable to the value (0.25%) for 100% breadfruit *pupuru* flour reported by *Adejuyitan et al.* (2018). Titratable acidity gives a measure of the amount of acid present in the food. The level of this index is used to estimate the quality of the flour. These values were in agreement with the Nigerian Industrial Standard recommendation of less than 10 g/100 ml total titratable acidity for gari samples. This shows that the period of fermentation of the various samples was adequate.

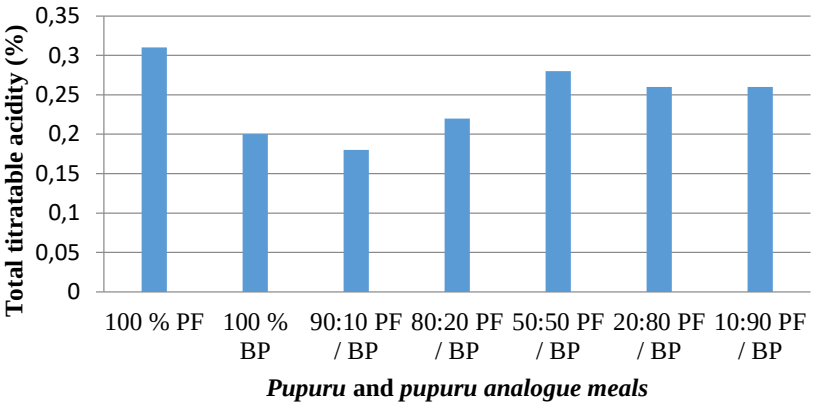


Figure 5. The total titratable acidity of *pupuru* and *pupuru analogue meals*

The cyanide concentration (*Figure 6*) of the *pupuru* samples ranged between 0.56 and 1.68 mg/100 g. The *pupuru* analogues produced from 100% breadfruit had the least cyanide value (0.56 mg/100 g) while those from 100% cassava had the highest (1.68 mg/100 g). The cyanide content decreased as the level of substitution of breadfruit increased. Hydrogen cyanide (HCN) is the predominant antinutrient/toxic substance in cassava tubers and cassava products. The knowledge of cyanogenic glycoside content of food is vital because cyanide, being an effective cytochrome oxidase inhibitor, interferes with the aerobic respiratory system (*Onwuka*, 2005). The level of cyanide (0.42–0.47 mg/100 g) reported by *Alaba et al.* (2013) for cassava flour (*pupuru*) is lower than the values obtained. The reduction in cyanide could be attributed to the

synergistic effect of loss by hydrolysis into the steep water during fermentation and toasting (*Irtwange & Achimba*, 2009).

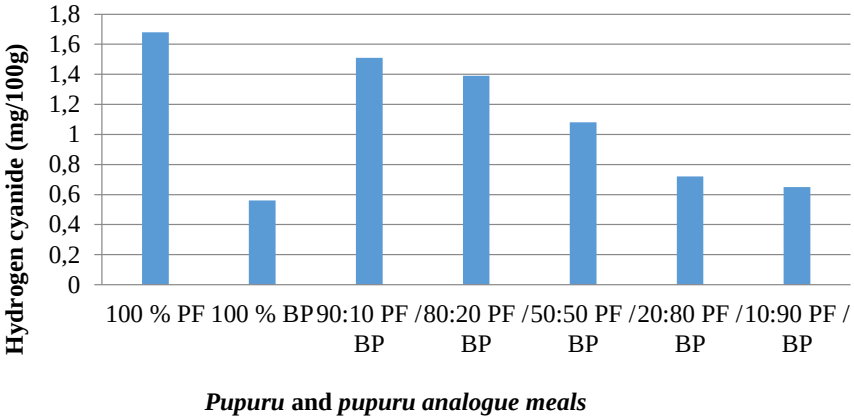


Figure 6. The hydrogen cyanide of *pupuru* and *pupuru analogue meals*

Sensory evaluation

Table 3 shows the results of the sensory evaluation of *pupuru* and *pupuru* analogues. The scores obtained for the colour of *pupuru* and *pupuru* analogues were significantly different ($p < 0.05$) from each other, with 90:10 PF/BP having the most preferred colour (8.6). The scores for colour of locally (LMP) and industrially made *pupuru* (IMP) (7.47 and 7.07 respectively) were significantly different ($p < 0.05$) from the colour of 100% PF (5.40). There was the least preference for 10:90 PF/BP in terms of colour, texture, mouldability, and taste. Pupuru and *pupuru* analogues from 100% PF, 50:50 PF/BF, locally-made *pupuru* (LMP), and 20:80 PF/BP did not differ significantly ($p < 0.05$) in terms of aroma and texture. However, the other samples were significantly different ($p < 0.05$) from industrially-made *pupuru* (IMP). Usually, during smoking, there is a deposition of organic components, such as phenols, alcohols, aldehyde, or ketones, which influences flavour and the antimicrobial effects on the products (*Tewe*, 2004). In the case of mouldability, there was no significant difference ($p > 0.05$) between 100% PF, 100% BP, 50:50 PF/BP, 20:80 PF/BP, and LMP respectively. Of all the samples, the 10% breadfruit substitution was the most acceptable meal. Meanwhile, *pupuru* analogues up to 50% breadfruit substitution had sensory attributes comparable to those of *pupuru* produced locally and industrially.

Table 3. Sensory evaluation of *pupuru* and *pupuru* analogues

Samples	Colour	Aroma	Texture	Mouldability	Taste	Overall acceptability
100% PF	5.40 ± 1.55^{de}	5.73 ± 1.39^{bc}	5.53 ± 0.99^{bc}	5.87 ± 1.36^{bc}	5.60 ± 1.80^{bcd}	6.00 ± 1.36^{bc}
100% BP	4.60 ± 1.92^{ef}	4.47 ± 1.77^{d}	4.67 ± 1.63^{cd}	5.27 ± 2.37^{bc}	4.67 ± 2.19^{de}	5.80 ± 2.24^{cd}
90:10 PF/BP	8.60 ± 0.63^{a}	7.47 ± 1.06^{a}	7.87 ± 0.74^{a}	7.67 ± 0.82^{a}	7.53 ± 1.19^{a}	8.13 ± 0.83^{a}
80:20 PF/BP	6.73 ± 1.10^{cb}	6.60 ± 1.06^{ab}	6.40 ± 1.12^{b}	6.27 ± 1.53^{b}	5.93 ± 2.09^{bcd}	6.53 ± 1.46^{b}
50:50 PF/BP	5.53 ± 1.55^{de}	5.93 ± 1.16^{bc}	5.40 ± 1.18^{bc}	5.53 ± 1.30^{bc}	5.67 ± 1.45^{bcd}	6.00 ± 1.41^{bc}
20:80 PF/BP	6.00 ± 1.25^{cd}	6.20 ± 1.01^{bc}	5.73 ± 1.79^{bc}	5.27 ± 1.75^{bc}	5.20 ± 1.82^{cd}	5.73 ± 1.53^{bc}
10:90 PF/BP	3.67 ± 1.80^{f}	4.67 ± 1.76^{d}	4.13 ± 1.96^{d}	4.53 ± 2.23^{c}	3.60 ± 2.10^{e}	4.20 ± 2.11^{d}
LMP	7.47 ± 1.06^{b}	6.27 ± 1.22^{bc}	5.80 ± 2.37^{bc}	5.60 ± 1.96^{bc}	5.80 ± 2.37^{ab}	6.60 ± 1.80^{b}
IMP	7.07 ± 0.96^{b}	5.40 ± 1.84^{cd}	6.07 ± 1.67^{b}	6.40 ± 1.84^{b}	6.07 ± 1.67^{ab}	6.33 ± 1.63^{b}

Mean ± standard deviation.

Mean with the same superscripts in the same column are not significantly different at 5% probability level.

Keys: **100% PF** – 100% cassava; **100% BP** – 100% breadfruits; **90:10 PF/BP** – 90% cassava co-processed with 10% breadfruits; **80:20 PF/BP** – 80% cassava co-processed with 20% breadfruits; **50:50 PF/BP** – 50% cassava co-processed with 50% breadfruits; **20:80 PF/BP** – 20% cassava co-processed with 80% breadfruits; **10:90 PF/BP** – 10% cassava co-processed with 90% breadfruits; **LMP** – Locally made *pupuru*; **IMP** – Industrially made *pupuru*

Results from this study suggest that the co-processing of cassava with breadfruit up to 50% breadfruit substitution will produce a meal that is acceptable, having a functional quality index. The abundance experienced during the breadfruit season in the south-western part of Nigeria can be exploited by utilizing breadfruit in the production of *pupuru* analogues. The value-added product can also boost the foreign earning of the country if the exportable product is exported to neighbouring countries. The local production of *pupuru* analogues is expected to be cheaper than the price per unit of *pupuru* made from cassava because breadfruit is abundant and cheaper.

4 Conclusions

The study concluded that *pupuru* analogues of acceptable sensory and physicochemical properties could be produced from cassava co-processed with breadfruit. The study provides valuable information regarding the utilization of breadfruit in food material, thereby preventing wastage of the crop during its season as well as expanding the use of breadfruit in food deficit regions.

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Functional and pasting characteristics of *pupuru* and *pupuru* analogues from cassava (*Manihot esculenta*) and breadfruit (*Artocarpus altilis*) blends

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Abstract. *Pupuru* and *pupuru* analogues are fermented, smoked food products usually produced from cassava or cassava substituted with a varying ratio of breadfruit. This study aims at determining and comparing the functional and pasting characteristics of *pupuru* and *pupuru* analogues with a view to expanding the utilization of breadfruit as *pupuru* analogue. The functional properties (water absorption capacity (%), swelling power (g/g), solubility (%)) and pasting characteristics were determined using standard methods. The results showed that the yield of the products ranged between 24.66 and 29.65%, and it was not affected by the amount of breadfruit substituted. The water absorption capacities of the *pupuru* and *pupuru* analogues ranged between 216.0 and 449.0%; this parameter increased with temperature increase. Both swelling power and solubility had a rapid increase from 80 °C to 90 °C. Pasting temperature ranged between 73.15 and 83.66 °C, with peak time between 4.58

Keywords and phrases: breadfruit, cassava, *pupuru* analogue, swelling power, pasting, viscosity, water absorption capacity

and 5.33 min. The final viscosity ranged between 94.08 and 391.83 RVU, and it decreased with increase in breadfruit substitution. The study concluded that adding breadfruit to cassava in *pupuru* analogue production improved some of the functional and pasting properties of the product.

1 Introduction

Pupuru is a fermented cassava-based food product (*Daramola et al.*, 2010). *Pupuru* and other cassava products are widely accepted and consumed in Nigeria, the consumption of which is steady and increasing in this country and beyond its borders too (*Adejuyitan et al.*, 2018). *Pupuru* is traditionally prepared by soaking cassava in water for about 3–5 days to become soft. After fermentation, the wet mash is packed into sacks and dewatered using a mechanical press. The fibres are handpicked from the mash and the mash is moulded into ball or circular shape and placed over fire to be smoke-dried. The resulting products are spherical materials with brown, appealing appearance (*Alaba et al.*, 2013). The outer covering is then scraped off with knife, and the inner white component is milled and sieved into *pupuru* flour.

Previous studies (*Ikujenlola & Lawson*, 2005; *Osundahunsi & Oluwatoyin*, 2005; *Ayodeji et al.*, 2005; *Sanni et al.*, 2003; *Osunsami et al.*, 1989) were focused on the various aspects of *pupuru* processing and rheological properties. However, there is no information on the substitution of cassava with breadfruit for the production of *pupuru* analogues. Breadfruit (*Artocarpus altilis*) is a widely cultivated crop in south-western Nigeria. It is grown mainly as a subsistence crop and is a popular staple food in Polynesia, Jamaica, and the Caribbean (*Ajatta et al.*, 2016). Breadfruit is nutritious, cheap, and highly available during its season, but it has found limited applications in the food industries (*Omobuwajo*, 2003). It has been processed to starches (*Akanbi et al.*, 2009) and flour (*Adepeju et al.*, 2011). The quality and nutritional properties of starch-based foods are largely determined by the changes that starch undergoes during processing/cooking and subsequent storage (*Ojo et al.*, 2017). However, its utilization in *pupuru* analogue production has not been exploited. Therefore, the aim of this study was to produce *pupuru* analogues by blending cassava with breadfruit in different proportions and to determine the functional and pasting characteristics of the products.

2 Materials and methods

Matured unripe breadfruits (*Artocarpus altilis*) were purchased at Ilode market, Ile-Ife, Osun State, and matured cassava roots (*Manihot esculenta*) were bought from Tonkere, Osun State, Nigeria.

Preparation of *pupuru* meals

Cassava tubers and matured unripe breadfruits were washed, peeled, and sliced. The sliced breadfruit and cassava roots were steeped in water at ambient temperature (30 ± 2 °C) inside a plastic container for 72 hrs; after fermentation, the fermented and softened mash was dewatered, and the fibres were sorted out. Thereafter, the mash was packed inside bags, pressed using hydraulic press for 30 min, and moulded into balls of 5–10 cm in diameter. The moulded balls were smoked in the kiln dryer at 80 °C for 6 hrs, pulverized and toasted for 10 min. It was milled and sieved (d = 630 μm) to obtain *pupuru* meal. This method was repeated for blends of cassava and breadfruit in the production of *pupuru* analogues at different proportions (90:10, 80:20, 50:50, 20:80, and 10:90).

Yield of *pupuru* and *pupuru* analogue meal from breadfruit and cassava

The yield of the *pupuru* meals produced from cassava and breadfruit was determined using the method proposed by *Apea-Bah et al.* (2011). The total yield of *pupuru* meals was determined by recording/monitoring the material balance of each unit operation until the final product (*pupuru*) was obtained.

$$\text{Yield of } \textit{pupuru} \text{ meal} = \frac{\text{Weight of } \textit{pupuru} \text{ meal}}{\text{Weight of whole roots}} \cdot 100 \tag{1}$$

Functional properties of the products

Determination of least gelling concentration

The method of *Sathe & Salunkhe* (1981) was employed for the determination of the gelling concentration. Sample suspensions of 1–17% (step 2%) and 20% (w/v) were prepared in 5 ml of distilled water, and the test tubes were heated in a boiling water bath for 1 hr followed by rapid cooling under cold tap water flow. The test tubes were further cooled for 2 hrs at 4 °C. Least gelling

concentration was determined as that concentration when the sample from the inverted test tube did not fall down or slip.

Determination of water absorption capacity (WAC)

The WAC was determined at room temperature and at temperatures ranging between 60 to 90 °C using a combination of the AACC (1995) method and those of *Sosulski* (1962) and *Rutkowski & Kozlowska* (1981). A 2 g sample was dispersed in 20 ml of distilled water. The contents were mixed for 30 s every 10 min using a glass rod; after mixing it five times, it was centrifuged at 4,000 g for 20 min. The supernatant was carefully decanted, and then the contents of the tube were allowed to drain at a 45 ° angle for 10 min and then weighed. Water absorption capacity was expressed as the percentage increase of the sample weight.

Determination of swelling power and solubility

Swelling power and solubility were determined using the modified methods of *Takashi & Sieb* (1988) and *Sathe & Salunke* (1981). Exactly 3 to 5 g sample was weighed into a tared 50 ml centrifuge tube. About 30 ml of distilled water was added and mixed gently. The slurry was heated at a constant temperature (60, 70, 80, and 90 °C) in a water bath for 15 min. During heating, the slurry was stirred gently to prevent clumping of the starch. Upon completion of the 15 min, the tube containing the paste was centrifuged at 3000 × g for 10 min. The supernatant was decanted immediately after centrifugation. The tubes were dried at 50 °C for 30 min, cooled, and then weighed (W_2). Centrifuge tubes containing sample alone were weighed prior to adding distilled water (W_1). From the supernatant, 10 ml was dried in the air oven at 120 °C for 4 hrs in a crucible to constant weight, and swelling power was calculated as follows:

$$\text{Swelling power} = \frac{W_2(\text{g}) - W_1(\text{g})}{\text{Weight of sample(g)}} \cdot 100 \tag{2}$$

$$\text{Solubility (\%)} = \frac{\text{Dry weight at 120 °C}}{\text{Weight of sample(g)}} \cdot 100 \tag{3}$$

Determination of pasting properties

Pasting properties of *pupuru* and *pupuru* analogue meals were determined using the Rapid Visco Analyser (RVA) (model 3D, Newport Scientific, Warriewood, Australia). *Pupuru* meal (3 g, 14% moisture basis) was mixed with 25 g of accurately weighed water in the aluminium canister. During the programmed heating and cooling cycle, the mixture was held at 50 °C for 1 min, heated to 95 °C for 7.5 min at 6 °C/min, held at 95 °C for 5 min before cooling to 50 °C for 7.5 min and holding at 50 °C for 1 min. Peak viscosity, temperature at peak viscosity, temperature at initial viscosity rise, time from initial to peak viscosity, hot-paste viscosity, cold-paste viscosity, trough, breakdown, and setback were recorded (*Bhattacharya et al.*, 1997).

Statistical analysis

The data obtained were expressed as mean ± standard deviation and were characterized by one-way analysis of variance (at the significance level of $\alpha = 0.05$). For mean value comparison, Tukey's least significant difference test was used. All statistical procedures were carried out using SPSS 17.0 (SPSS, Chicago, IL, USA) software.

3 Results and discussion

Yield of the *pupuru* and *pupuru* analogues

The yield (24.66–29.65%) of the *pupuru* and *pupuru* analogues produced from cassava and breadfruit blends is presented in *Table 1.* The peels of both cassava and breadfruit accounted for the bulk of the waste. The values of the peels ranged between 12.46% and 17.59%. The peel loss is lower than the 22% peel loss reported by *Ikujenlola* & *Opawale* (2007) for cassava products. According to *Opara* (1999), hand peeling losses and mechanized peeling losses are on average between 25 and 30% and 30 and 40% respectively.

The percentages of chaff, water, and other waste materials accounted for losses between 54.53 and 61.26%. Water losses entailed the removal of hydrogen cyanide and starch from the product, while the losses of other materials included the removal of chaff, fibre, and the dark surface covering of smoked balls. *Hahn* (1992) reported that the dry matter content of cassava roots is affected by season, type, and variety.

The yields (24.66–29.65%) of the *pupuru* and *pupuru* analogues obtained were comparable to the range of 12.8–32.3% reported by *Oyewole* & *Ogundele* (2001) for *fufu*. To obtain a higher yield, increasing the monitoring of all production operations is indicated. For waste reduction, peeling must be carried out with care.

Table 1. Yields of the *pupuru* and *pupuru* analogues (%)

Sample	**Starting material**	**Peeled material**	**Peeled material losses**	**Water, chaff, and other**	**Yield of *pupuru***
100% PF	100	85.56	14.44	58.70	26.86
100% BP	100	86.72	13.28	61.26	25.46
90:10 PF/BP	100	82.41	17.59	54.53	27.88
80:20 PF/BP	100	87.54	12.46	57.89	29.65
50:50 PF/BP	100	84.35	15.65	57.74	26.61
20:80 PF/BP	100	84.40	15.60	57.88	26.52
10:90 PF/BP	100	85.68	14.32	61.02	24.66

Keys: **100% PF** – 100% cassava; **100% BP** – 100% breadfruits; **90:10 PF/BP** – 90% cassava co-processed with 10% breadfruits; **80:20 PF/BP** – 80% cassava co-processed with 20% breadfruits; **50:50 PF/BP** – 50% cassava co-processed with 50% breadfruits; **20:80 PF/BP** – 20% cassava co-processed with 80% breadfruits; **10:90 PF/BP** – 10% cassava co-processed with 90% breadfruits

Functional properties of *pupuru* and *pupuru* analogues

The least gelation concentration of the products increased (7–11%) with increase in the level of substitution with breadfruit (*Table 2*). This result compared favourably with the least gelation concentration (10–13%) of composite flour reported by *Ajatta et al.* (2016) but was lower than (30–50%) the least gelation concentration for *Dioscorea alata* reported by *Udensi et al.* (2008). The ability of protein to form gels and provide a structural matrix for holding water, flavours, sugars, and food ingredients is useful in food application and in new product development (*Aremu et al.*, 2006). The differences observed in the gelling concentration may be a result of the relative proportion of different flour constituents such as carbohydrates, proteins, lipids, and fibres and the interactions between the components (*Sathe et al.*, 1982).

The effect of temperature on the water absorption capacity, swelling power, and solubility of *pupuru* and *pupuru* analogues are presented in *Figures 1*, *2*, and *3* respectively. The water absorption capacity represents the ability of a product to associate with water under conditions where water is limited.

Table 2. Least gelation concentration of *pupuru* and *pupuru* analogues (%)

Samples	**Partial gelation (%)**	**LGC** (%)
100% PF	7.00	9.00
100% BP	9.00	11.00
90:10 PF/BP	9.00	11.00
80:20 PF/BP	9.00	11.00
50:50 PF/BP	7.00	9.00
20:80 PF/BP	11.00	13.00
10:90 PF/BP	9.00	11.00

Keys: **100% PF** – 100% cassava; **100% BP** – 100% breadfruits; **90:10 PF/BP** – 90% cassava co-processed with 10% breadfruits; **80:20 PF/BP** – 80% cassava co-processed with 20% breadfruits; **50:50 PF/BP** – 50% cassava co-processed with 50% breadfruits; **20:80 PF/BP** – 20% cassava co-processed with 80% breadfruits; **10:90 PF/BP** – 10% cassava co-processed with 90% breadfruits. **LGC (%)** – least gelation concentration

The water absorption capacity of the meal ranged from 216% to 449% and was observed to increase with increase in temperature. The *pupuru* analogues containing 20:80 PF/BP, 50:50 PF/BP, 100% BP, and 10:90 PF/BP showed marginal increase at varying temperatures. This range was higher than the one (240.0–275%) reported by *Ajatta et al.* (2016) for composite flours but comparable to 330–367% for *Altocarpus altilis* pulp flour reported by *Appiah et al.* (2011). According to *Adetuyi et al.* (2009), the increase in water absorption capacity could be attributed to the increase in the protein content of the co-processed flour; hence the flour could be used as thickener in liquid and semi-liquid foods since the flour has the ability to absorb water and swell for improved consistency in food. Increase in the water absorption capacity would be advantageous to food processors as little dry matter could produce reasonable volume of the reconstituted meal.

Figures 2 and *3* present the influence of temperature on swelling power and solubility. Generally, the swelling power increased with temperature increase. The *pupuru* (100% cassava) and *pupuru* analogues 10% and 90% breadfruit exhibited significant ability to swell more than 100% BP, 20:80 PF/BP, 80:20 PF/BP, and 50:50 PF/BP over a range of temperatures between 80 °C and 90 °C. This result is similar to the findings of *Adepeju et al.* (2011). The swelling power obtained ranged between 3.12 g/g and 8.5 g/g, and this is within the range of 8.70 g/g–15.00 g/g for corn starch flours reported by *Makanjuola & Makanjuola* (2018). Meanwhile, the swelling power was comparable with the one (7.84–9.25) reported by *Osungbaro et al.* (2010).

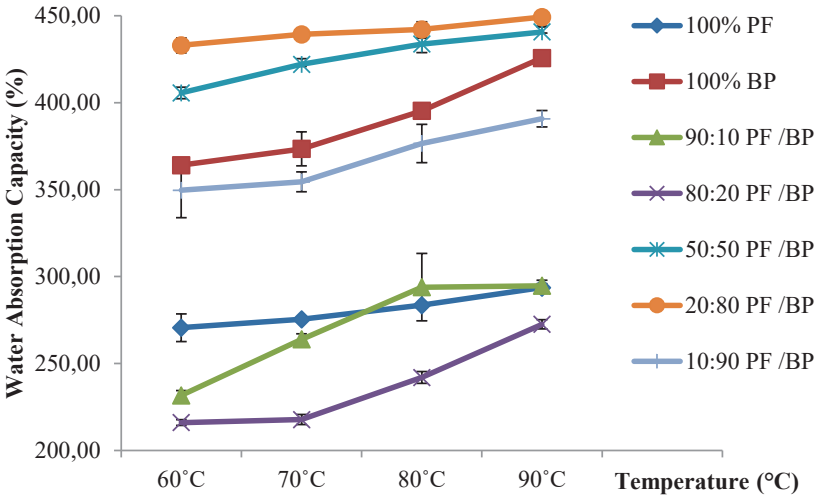

Figure 1. Effect of temperature on the water absorption capacity of *pupuru* and *pupuru* analogue meals

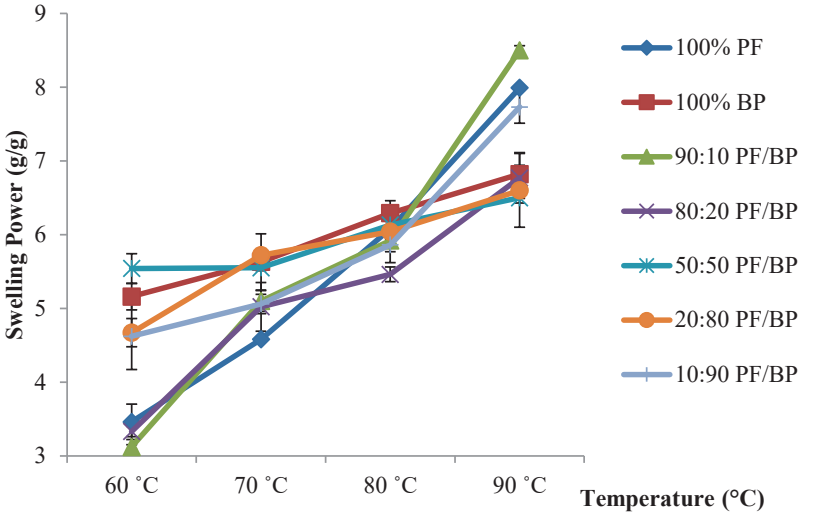

Figure 2. Effect of temperature on the swelling power of *pupuru* and *pupuru* analogue meals

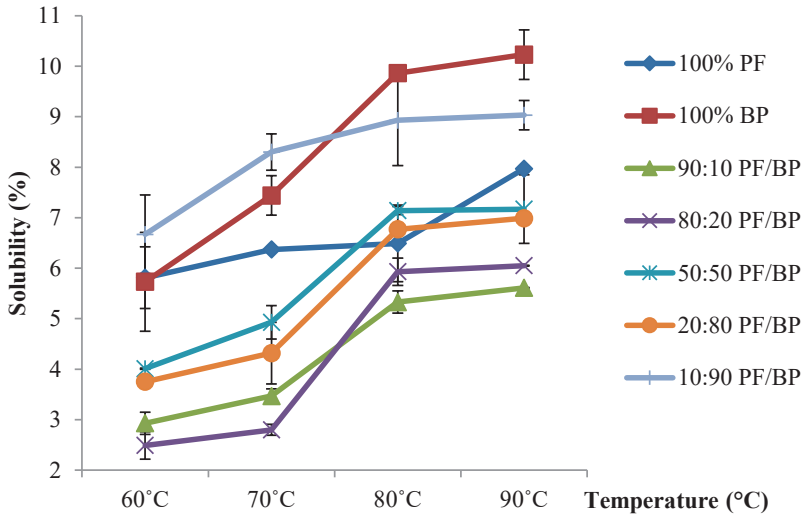

Figure 3. Effect of temperature on the solubility of *pupuru* and *pupuru* analogue meals

According to *Hoover* & *Maunal* (1996), the temperature increase allows the amylose (water-soluble fraction) molecules located in the bulk amorphous regions to interact with the branched segment of amylopectin (water-insoluble fraction) in the crystalline regions. This implies that high temperature weakens the starch granules of flour, thus leading to improved solubility. As a result of swelling, there is an increase in the solubility, showing the highest value at 70 °C and 80 °C, with 100% BP having the highest solubility. The solubility of the starch is believed to be affected by factors such as inter-associative forces, swelling power, presence of surfactants, and other associative compounds (*Sibanda* & *Sychawska*, 2000).

Pasting properties of *pupuru* and *pupuru* analogues

Figure 4 (*a–g*) shows the pasting characteristics of *pupuru* and *pupuru* analogues. The peak viscosity is the maximum viscosity developed by a starch-water suspension during heating (*Adebowale et al.*, 2005). The peak viscosity of the 100% PF (276.38 RVU) was the highest, while 100% BF (38.75 RVU) recorded the lowest value of peak viscosity. Higher peak viscosity may be attributed to differences in protein content (*Sandhu* & *Singh*, 2007). The peak viscosity of co-processed meal decreased as the proportion of breadfruit in-

creased; this agrees with the study of *Oluwamukomi & Jolayemi* (2012), who reported a significant decrease in the peak viscosity of soy-melon-enriched gari semolina.

The peak viscosity of the samples ranged between 38.75 and 276.33 RVU; this range was within the range of 203.34–340.22 RVU reported by *Nwokeke et al.* (2013) for cassava–African yam bean fufu flours. Two factors interact to determine the peak viscosity of a cooked starch paste: the extent of granule swelling (swelling power) and solubility. Higher swelling capacity is indicative of higher peak viscosity, while higher solubility due to starch degradation or dextrinization results in reduced paste viscosity (*Shittu et al.*, 2001; *Zobel et al.*, 1984). These were corroborated by results of swelling power and solubility reported in *Figures 2* and *3*. This suggests that the presence and interaction of components, such as fats and protein, from breadfruit with cassava starch lowers the peak viscosity of the blends (*Egounlety et al.*, 2002). According to *Iwe et al.* (2017), values for peak viscosity for the five cassava varieties blended with wheat ranged from 66.08 to 358.08 RVU. Peak viscosity increased with increase in the ratio of cassava flour to wheat flour, and this could be attributed to the high degree of swelling of cassava starch granules.

Trough is sometimes called shear thinning, hot-paste viscosity, or holding strength due to the accompanied breakdown in viscosity. It measures the strength of the paste to withstand breakdown during cooling. This ranged between 39.58 and 240.33 RVU, which is comparable with the range (63.08–202.33 RVU) obtained for fermented cassava-sorghum blend reported by *Osungbaro et al.* (2010).

The breakdown viscosity, which is a measure of cooked starch disintegration, ranged from 1.75 to 60.34 and was observed to be lower than the range of 692.50–924.00 for the corn starch flour sample reported by *Makanjuola & Makanjuola* (2018). Higher values of breakdown are associated with higher peak viscosities, which in turn are related to the degree of swelling of starch granules during heating (*Ragaee & Abdel-Aal*, 2006). The breakdown was the highest in 80:20 PF/BP (60.34 RVU) and the lowest in 100% BP (1.75 RVU). This implies that 100% BP is more stable to heat and mechanical shear than 80:20 PF/BF. Breakdown viscosity decreased with the increasing level of breadfruit flour substitution; therefore, breakdown viscosity is indicative of paste stability (*Akanbi et al.*, 2009).

The final viscosity ranged between 94.08 and 391.83 RVU, with 100% PF having the highest 391.83 RVU and 100% BP having the lowest 94.08 RVU. The value is comparable with 180.33–332.24 RVU for cassava-African yam bean fufu flour reported by *Nwokeke et al.* (2013). The final viscosity, ac-

cording to *Iwe et al.* (2017), is a parameter commonly used to determine a sample's ability to form a gel after cooking and cooling. The difference between final viscosity and trough gives rise to a pasting property known as setback viscosity. Setback value is the tendency of starch to associate and retrograde upon cooling (*Peroni et al.*, 2006). It is the phase of the pasting curve after cooling the starches to 50 °C. This stage involves re-association, retrogradation, or re-ordering of starch molecules. A higher setback value is synonymous to reduced dough digestibility (*Shittu et al.*, 2001), while a lower setback of the starch granule during the cooling indicates lower tendency for retrogradation (*Sanni et al.*, 2004; *Sandhu et al.*, 2007) and lower rate of staling of the product from starch (*Adeyemi & Idowu*, 1990). Among the studied *pupuru* and *pupuru* analogues, 100% PF had the highest retrogradation tendency, yielding 151.50 RVU for setback viscosity, while the 50% inclusion of breadfruit reduced it to 55.58 RVU.

The peak time, a measure of the cooking time, ranged between 4.58 and 5.33 min for the *pupuru* samples. The time to attain peak viscosity is considerably higher than the range (3.93–4.07 min) reported by *Oluwamukomi & Jolayemi* (2012) for soy-melon-enriched gari semolina but comparable to the 5.33–5.53 min obtained for corn starch flours reported by *Makanjuola & Makanjuola* (2018). However, it fell within the range (5.02–9.00 min) reported by *Osungbaro et al.* (2010), who worked on fermented cassava–sorghum flour. The result obtained might be due to the fact that *pupuru* and *pupuru* analogues were partially gelatinized during smoking and toasting.

Pasting temperature is a measure of the minimum temperature required to cook a given food sample (*Sandhu et al.*, 2005), and it is related to paste stability – gives an indication of the strength of associative forces within the granules of the biomaterials (*Iwe et al.*, 2017). The pasting temperature of the *pupuru* samples ranged between 73.15 and 83.66 °C. The pasting temperature of *pupuru* from 100% cassava was the highest, while the *pupuru* analogue from 50% breadfruit substitution had the lowest pasting temperature. This may be due to the buffering effect of fat (from breadfruit) on starch, which interferes with the gelatinization process (*Egouletey et al.*, 2002). The pasting temperatures (61.41–61.80 °C) were higher than those of the composite flours reported by *Ajatta et al.* (2016).

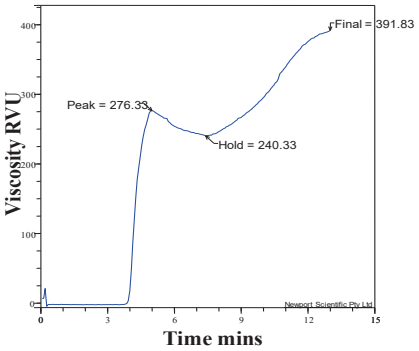

Figure 4a. Pasting property of 100% *pupuru* flour

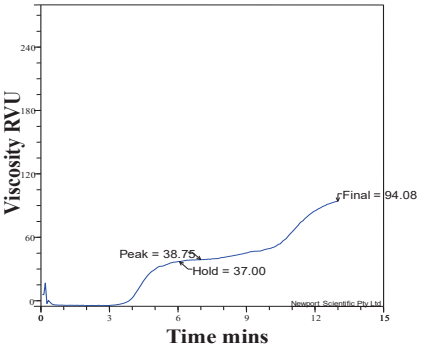

Figure 4b. Pasting property of 100% Breadfruit *pupuru* flour

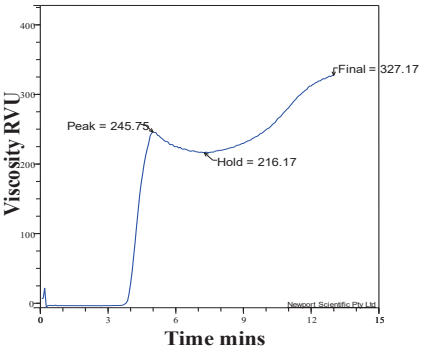

Figure 4c. Pasting property of 90:10 Cassava: Breadfruit *pupuru* flour

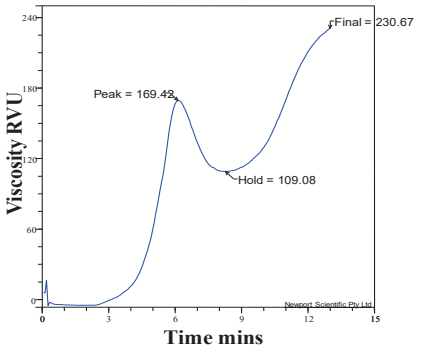

Figure 4d. Pasting property of 80:20 Cassava: Breadfruit *pupuru* flour

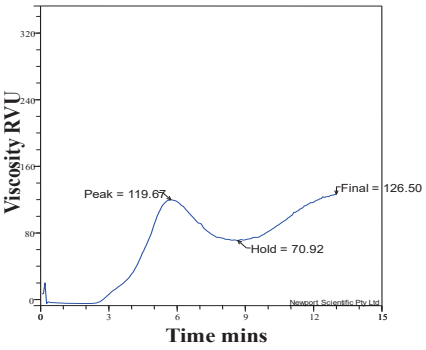

Figure 4e. Pasting property of 50:50 Cassava: Breadfruit *pupuru* flour

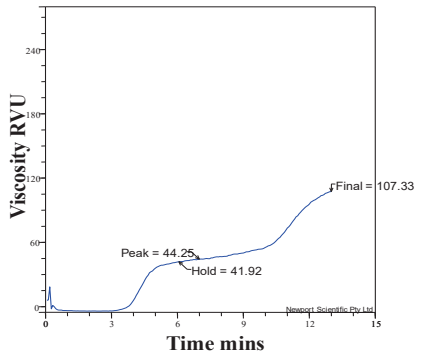

Figure 4f. Pasting property of 20:80 Cassava: Breadfruit *pupuru* flour

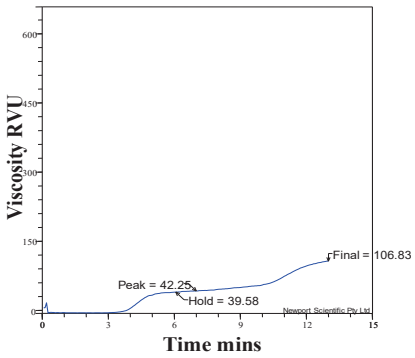

Figure 4g. Pasting property of 10:90 Cassava: Breadfruit *pupuru* flour

4 Conclusions

The study concluded that the functional and pasting characteristics of *pupuru* and *pupuru* analogues from cassava and cassava substituted with breadfruit improved with increase in the proportion of breadfruit. This study has shown another avenue to increase the utilization of breadfruit.

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Biotic and abiotic risks of soil biochar treatment for food safety and human health

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Abstract. Pyrolysis technology facilitates the heating of organic waste biomass in a very low oxygen environment to temperatures over 400 °C. The high carbon content and surface area of the char produced via slow pyrolysis makes it suitable for a range of purposes that would sequester the carbon it contains. For example, there is a growing interest in its use as a soil amendment, which enhances plant growth and nutrient use efficiency.

Keywords and phrases: biochar, adsorption, PAHs, pathogens, *Escherichia coli*

Biochar application to soils is being considered as a means to improve fertility while concurrently improving soil functions. Wider issues, including environmental conditions, applicational health, and safety associated with biochar production and handling, are put into context. Biochar also might contain organic and inorganic contaminants, which developed during the pyrolysis processes. The aim of this study is to measure both a biochar product's Polycyclic Aromatic Hydrocarbons (PAHs) content to get scientific basis for policy development and the potential changes in the microbial community relating to biochar soil application, with special attention to soil-borne pathogens. Based on our results, we found that biochar increased the microbial biomass values even before the incubation. In single and combined biochar–alginite treatments, more bacterial biomass was adsorbed due to the higher adhesion capability and the increased surface area. The volume of the microbial adsorption is different from species to species and even strains.

1 Introduction

In line with ever-changing consumer needs, the production of healthy and safe food poses increasing challenges to agriculture, food industry, and, last but not least, soil (micro-)biology professionals. The constantly degrading soils or the effects of climate change further reinforce these challenges and highlight their significance (*Lajtha et al.*, 2018; *Fekete et al.*, 2014; *Kotroczó et al.*, 2020). Numerous studies report that these processes need to be mitigated. There have also been a number of studies finding the use of biochar a good solution, highlighting its positive properties (*Ding et al.*, 2016), but only a few publications present the critical aspects of using biochar (*Hardy et al.*, 2019).

Biochar-charcoal is an organic-related biomass material which could be produced by reductive pyrolysis (*Di Blasi*, 2008; *Bridgwater*, 2007). There is a growing interest in its use as a soil amendment, which enhances plant growth and nutrient use efficiency (*Van Zwieten et al.*, 2010a; *Shomana et al.*, 2020). Beneficial effects of biochar in terms of increased crop yield and improved soil quality have been reported. Its application into soil is a well-accepted process in sustainable agricultural systems, even though there are large discrepancies about its positive and negative effects. Biochar might improve the physical-chemical-biological properties of soil (*Brady & Weil*, 2008) and its water retention (*Shomana et al.*, 2020), the clay and organic matter content (*Glaser et al.*, 1998; *Lehmann et al.*, 2003), the pH levels (*Van Zwieten et al.*, 2010b), and the availability of macro- and micronutrients due to its adsorption capacity (*Brown et al.*, 2006; *Chan et al.*, 2008).

Data in the literature suggest that biochar products could be applied on a wide scale to influence soil-plant-microbe interactions. Biochar has a highly porous structure with a surface that can reach an area of 1,000 m^2/g (*Downie et al.*, 2009). In addition to the adsorption of various organic and inorganic substances, it provides habitats for bacteria, actinomycete, and fungi (*Thies & Rillig*, 2009). The observed actions of biochar on soil microbiological activity result from at least three main effects: alteration of physico-chemical interactions, such as increased water and nutrient retention; electron donor provision; provision of habitat (*Ennis et al.*, 2012; *Chan et al.*, 2008). The soil microbiota need an efficient surface protection by the large absorptive capacity of biochar products and an improved water/nutrient supply. Although the combined and enhanced role of biochar and soil microbial populations in ecosystem amelioration are recognized (*Fischer & Glaser*, 2012; *Cocozza et al.*, 2017), limited research has been reported on microbial diversity/functional response to the approach. Publications on the integration of biochar into crop production technologies report yield increases, at least in the short term (*Gorovtsov et al.*, 2019). *Matsubara et al.* (2002) have shown that biochar inoculated with mycorrhizal fungi is effective in reducing *Fusarium* root disease in an *Asparagus* species. In an experiment with tomato plant, *Nerome et al.* (2005) found that biochar from municipal organic waste reduced contamination in soil by the pathogenic bacterial wilt (*Ralstonia solanacearum*).

Besides the already known benefits, however, some environmental risk of biochar application was also published. Numerous studies have supported the effects of biochar on various herbicides and pesticides. *Zheng et al.* (2010) found that biochar efficiently adsorbed them, thereby reducing their efficiency (*Yang et al.*, 2006). On the other hand, during the pyrolysis process, some contaminants might be created in the biochar products, which might reduce its agricultural applicability. Such contaminants are the polycyclic aromatic hydrocarbon (PAH) compounds, which might create some environmental threat (*Wang et al.*, 2017). PAH compounds have been detected both in pyrolysis products and also during forest fires in nature (*Ré-Poppi*, 2002; *Kim et al.*, 2003; *Kocsis et al.*, 2018). Determination of the PAH content of any biochar products is of utmost importance to assess the human/environmental risk. Some authors stated (*Kaal et al.*, 2008) that PAHs are the result of the pyrolysis process, being formed when biomass undergoes a variety of physical, chemical, and molecular changes. The PAHs' content might exceed the permissible limits of biochar products very frequently (*Rajapaksha*, 2016; *Kocsis et al.*, 2018). This fact can reduce the soil applicability of biochar products when considering the environmental and food safety aspects.

The aim of our work was to find out how biochar as a potential abiotic contaminant (PAH) affects the soil, what its biotic risk is, as it can also support the growth of microbes and opportunistic pathogens that are harmful from the point of view of food safety, and study the adhesion factors of microorganisms on species and strains level. We also aimed to provide a biochar product's PAH content measurement to get scientific basis for policy development and to measure the potential changes in the microbial biomass relating to biochar soil application, with special attention to soil-borne pathogens.

2 Materials and methods

Pollution parameters

PAH content in the applied biochar product was investigated by HPLC (CEN/TS 16181:2013), as suggested by *Beni et al.* (2014) and *Włóka et al.* (2015). In order to provide a wide range of statistically correct results, 6 subsamples were measured for PAHs content. 30 ml of acetonitrile was used as sample preparation for the accurately measured 1.00-gram samples. The samples were then treated for 30 minutes in an ultrasonic bath. The extracts were shaken for 24 hours. After that, extracts were purified by centrifugation and filtration through a 0.45-μm pore-size PP membrane filter. The final phase of sample preparation was the concentration of extracts by using Solid-Phase Extraction Technique. For this purpose, ChromaBond C_{18} 6 ml/500 mg columns were used as follows: flow rate: 1.5 ml/min, temperature: 30 °C, detector: UV 254 nm, and injector volume: 20 μl.

Testing of soil-borne microorganisms by biochar contaminants

The aim was to investigate the biochar effect on soil biota. Biochar-treated slightly humus sandy soil's microbial abundance was determined by the pour-plate method. 50 grams of dried and sieved (2 mm) soil samples were prepared in Petri dishes. The samples were subjected to the following treatments (in 4-4 replicates): A) control, no amendment, B) 5 g biochar, C) 5 g biochar + 3 g alginite as a slow-releasing nutrient source. Water-holding capacity was set to 60%, while incubation temperature was adjusted to mesophilic (30 ± 1 °C) conditions for 48 hours. After incubation, the samples were decimally diluted until $1/10^{\text{th}}$ of the original concentration, and then 100 μl of all dilution was pipetted onto Nutrient-agar media (Oxoid Ltd.) surface and spread around using a sterile glass rod. The CFU values were counted after 24 hours, 30 ± 1 °C incubation.

Testing of the microbial adsorption capacity

To investigate the microbial adhesion ability on different surfaces, bacteria strains from the collection of the Department of Microbiology and Biotechnology, Szent István University (*Table 1*) were separately incubated in a liquid medium (pH 6.6) containing glucose (20 g/l), peptone (10 g/l), and yeast extract (2 g/l) until 10^8 CFU/cm^3 concentration. All of these species are common in the soil, and if they contaminated the raw materials, they would cause food spoilage or illness.

Table 1. Experimental strains with their incubating temperature

Strain	**Collection no.**	**Incubation temperature**	**Properties**
Pseudomonas aeruginosa	ATCC 27853	37 °C	Opportunist pathogen
Pseudomonas lundensis	ATCC 49968	30 °C	Causes spoilage of milk, cheese, meat, and fish
Bacillus cereus	ATCC 14579	30 °C	Causes foodborne illness
Micrococcus luteus	ATCC 10240	30 °C	Opportunist pathogen
Escherichia coli (four strains)	ATCC 8724 ATCC 8739 ATCC 25992 ATCC 43895	37 °C	Opportunist pathogen

In the measurement, sterilized soil column was prepared in three different treatments. The soil was pre-treated by γ-irradiation with 20 kGy doses (1600 TBq activity of 60Co source). The assay followed OECD Test No. 312: "Leaching in Soil Columns" protocol. The following treatments were set in 4-4 replicates: A) control, 50 g soil; B) 45 g soil + 5 g biochar; C) 40 g soil + 5 g biochar + 5 g alginite. Two pieces of filter paper were placed on the plastic plate to avoid the outflow of soil particles from the soil column. A sterilized (autoclave 121 °C, 21 min) 15 mm thick quartz sand layer was also added on the top and bottom of the soil to facilitate a uniform distribution of the eluent. After the preparation, 100 ml sterile deionized water was added to the column to restore moisture content. After flowing down, 100 ml separately prepared liquid bacteria culture was also added. The leachate was later collected by a 250 ml flask under the soil column, and its volume was recorded. A total of 12 samples of leachate (each sample contained approximately 200 ml of leachate in volume) for each soil column were collected. Finally, the microbial

concentration of the leachate was also determined by pour-plate method.

Data analysis

For evaluation of the results, one-way ANOVA test was applied. Normality assumption was proven by Kolmogorov-Smirnov test ($p > 0.05$, $p = 0.200$) or Shapiro–Wilk test ($p > 0.05$), and the homogeneity of variances was checked by Levene's test ($p > 0.05$). Where data had homogeneity of variance, Tukey's honestly significant difference (HSD) post-hoc test was used, and where the data were 131 heteroscedastic, Games-Howell's post-hoc analysis was applied. The differences are presented with the letters a, b, c, and d over the corresponding column of the graph. As above, the significantly highest group is denoted with the letter a, the next highest with b, c, and this pattern continues up to letter d, if needed.

3 Results and discussion

Risk assessment of biochar samples

Even though soil properties can be improved by biochar application, concern should be given to proper biochar quality. As it was reviewed by *Kocsis et al.* (2016), the biochar might contain chemicals of persistent organic pollutants, which may reduce its general agricultural applicability. The levels of various PAH compounds were assessed from several biochar samples of agricultural origin. Results are shown in *Table 2*.

As we found beforehand (*Kocsis et al.*, 2018), the PAH concentration of the biochar sample exceeded the permissible limit value of the 1 $mg.kg^{-1}$ product (*Table 2*). There is an International Biochar Initiative, which recommends classification tools regarding the nutrient and PAH content of these pyrolysed products, but it is not a widespread norm. In Hungary, there is a standard and a decision of the Hungarian Agricultural and Land Management Ministry (36/2006.V. 18. FvM) on yield-enhancing materials. Furthermore, the Hungarian soil conservation and protection law (129/2007) also stated that caution is needed with any products with a potential of soil application. The PAH concentration in biochar-treated soils cannot exceed the level of 1 mg/kg on a dry soil basis. Neither of the adjusted biochar-soil treatments exceeded the statutory requirement.

Compared to the control after 48 hours at 30 °C temperature, both the biochar and biochar + alginite treatments showed a one-order increase in log

CFU values after the start of the incubation (*Figure 1*) – these increased values were significant based on the ANOVA test result. The sterile biochar did not contain microorganisms (due to incineration and lack of water), wherefore the explanation might be that biochar provided additional nutrients and space (niche) for microbial growth. Numerous studies report that due to its porous structure, biochar is not only able to bind certain substances, but the large surface area also promotes the adhesion of microorganisms, providing habitat for them (*Lehmann et al.*, 2011; *Abujabhah et al.*, 2016).

Table 2. Characteristics and levels of various Polycyclic Aromatic Hydrocarbon (PAH) compounds of the biochar product

Characteristics	**Biochar**
Raw material	Separated cow manure/wood chips (80:20%)
Obtaining temperature	(°C) 650–750
pH (water)	9.66
Total dissolved solids (mg/kg)	2125
PAH compounds (μ**g/g**)	
Anthracene	0.1209
Benzo[a]anthracene	0.3276
Benzo[b]fluoranthene	n.d.
Benzo[a]pyrene	n.d.
Chrysene	7.3454
Fluoranthene	2.4044
Fluorene	0.4437
Phenanthrene	n.d.
Pyrene	n.d.
SUM	**10.6419**

The content of some polycyclic aromatic hydrocarbon (PAH) compounds was measured by the HPLC method.

The short time between mixing the biochar in the soil and the measurement was sufficient for this increase. The same results can be observed for biochar + additions, with slightly higher values compared to the single biochar treatment and a higher rate of increase after incubation, which can be explained by the slower exploration of alginite. As biochar, alginite has a number of beneficial properties. It improves soil structure, has a significant content of minerals and organic matter, and contributes to improving soil biological activity and thus fertility (*Borowik & Wyszkowska*, 2018; *Strachel et al.*, 2018). In this case, the alginite could not be revealed due to the short measurement period, which could be the reason why no statistically substantiated differences between the biochar and the biochar + alginite treatments were found.

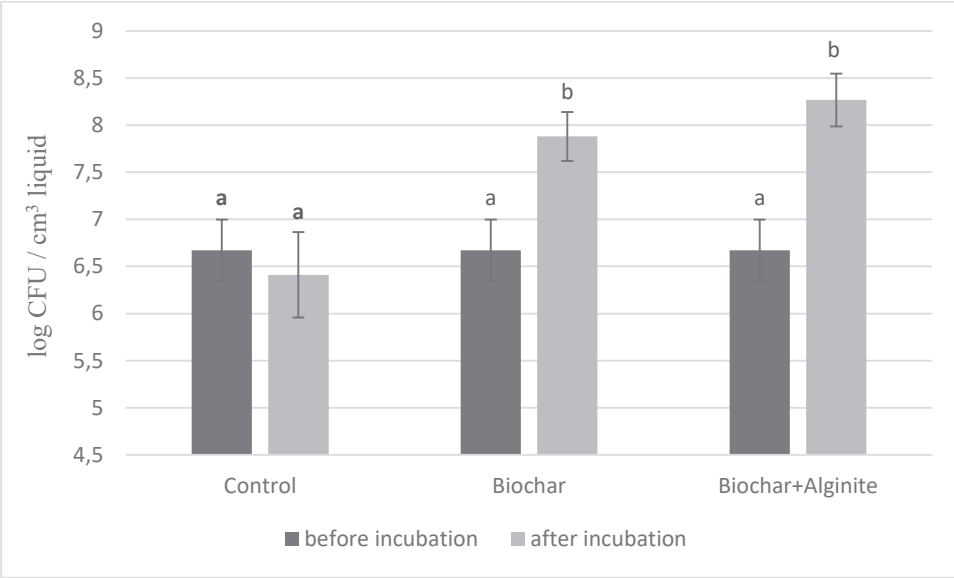

Figure 1. Development of CFU cultivable germ count values under the influence of biochar and biochar + alginite compared to the untreated control

The log CFU values of pure cultures filtered through soil columns were significantly lower in the biochar and biochar + alginite treatments compared to the control. This means that the biochar and the combined biochar-alginite treatments adsorbed more bacteria, which is due to the higher adhesion capability and the larger surface area. *Elmer et al.* (2010) reported a similar result in their work with *Asparagus*. In their experiment, they observed a decrease in the number of *Fusarium* fungi in biochar-treated soils. Likewise, *Ogawa* (2010) describes the use of biochar and biochar-amended composts in reducing bacterial and fungal soil-borne diseases.

There was no significant difference between biochar and biochar + alginite treatments, except for one *Escherichia coli* strain ATCC 8739 (*Figure 3*), where the biochar-alginite combination produced a synergistic effect compared to the single biochar treatment.

The microbial adsorption capacity rate of the cultures also varied with species and strain levels (figures 2 and 3). The measured *Pseudomonas aeruginosa* strain leached in greater values than *Pseudomonas lundensis*, *Bacillus cereus*, and *Micrococcus luteus* (*Figure 2*).

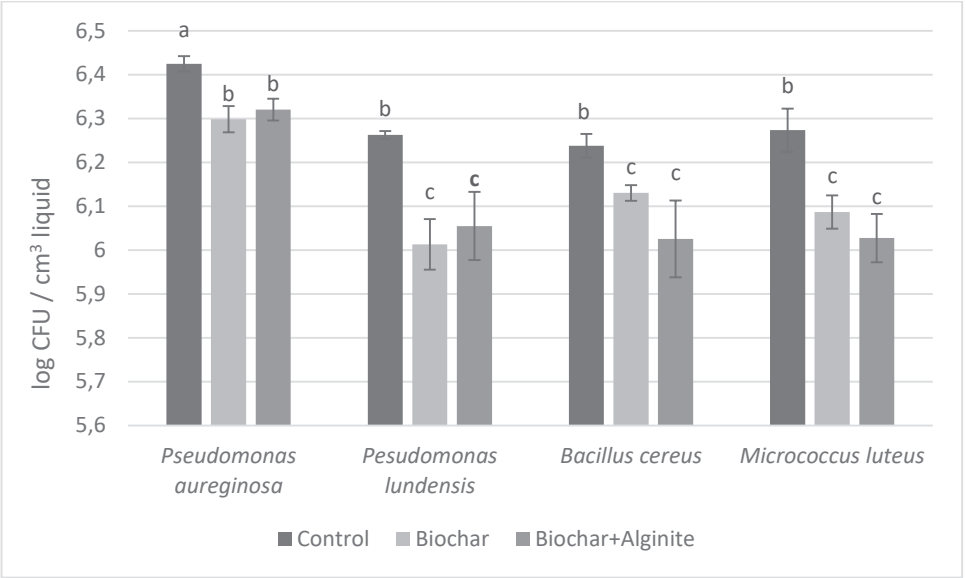

Figure 2. The number of the different bacteria under the influence of biochar + alginite, compared after leaching through a soil column

In the case of *E. coli*, the leaching properties show also diverse results, suggesting differences in the microbial adhesion factors (*Figure 3*). The ATCC 43895 (O157:H7) strain produced the largest binding compared to the control, while ATCC 8739 uniquely shows a significant difference between the combined biochar-alginite and the single biochar treatments. The CFU concentration of the starting liquid was "log 8". The soil columns reduced the number of bacteria in the liquid by orders of magnitudes of 1.4–2.1. Based on the reduction, more bacteria remained in the leached column; thus, the biochar-treated soil may potentially pose a greater food safety risk of pathogenic microbes.

There is a huge variability in biochar structures depending on the parent material and the conditions present at their formation. This determines many properties of biochar, including how many, if any, microorganisms are able to adhere to its surface (*Czimczik* & *Masiello*, 2007). Several studies reported that different groups of microbes are able to bind to biochar to varying degrees.

The reasons for changes in microbial abundance may differ for the different groups of microorganisms (*Warnock et al.*, 2007; *Lehmann et al.*, 2011).

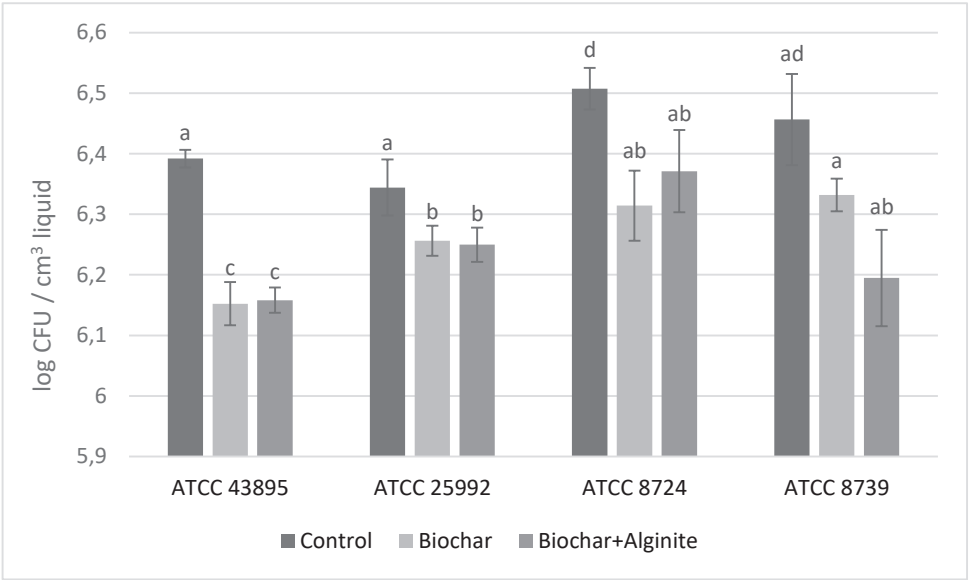

Figure 3. The number of different *Escherichia coli* strains under the influence of biochar + alginite, compared after leaching through a soil column

Differences in the adsorption of microbe species or strains onto biochar are explained by phenomena such as sorption of signalling compounds, detoxification of allelochemicals, soil physico-chemical properties, or indirect effects through alterations of other soil microbial processes (*Warnock et al.*, 2007; *Elmer & Pignatello*, 2011; *Lehmann et al.*, 2011).

4 Conclusions

Based on the results of this study, the main risks of the biochar products of various industrial technologies cover two main directions. One of them is the risk of PAH content, which might diminish the proper nutrient availability of crops in arable soils. The other direction is the microbiological contamination of the changed soil niche. Increased countable microorganism number can be adsorbed by biochar application, which helps soil life by providing additional nutrients and ecological space in the treated soil, which also supports the survival of pathogens. In this case, the added alginite did not yield a significantly different result compared to biochar treatment. The measurement of micro-

bial adsorption capacity revealed that biochar and biochar-alginite treatments adsorbed microbes in higher amount, and so they can be found in higher numbers, which is also a food safety issue. The magnitude of these changes is different from species to species and even strains. Thus, it is difficult to determine why there might be such a difference between individual microbial strains in their binding to biochar. However, it supports our hypothesis that potentially pathogenic microbial strains need to be tested separately based on their adsorption affinity to biochar. Based on our results, we can state that their different binding determines the amount of microbes in biochar-enriched soils, and thus they can pose a food safety risk even if they are too enriched.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Characterization of some bottled Romanian mineral waters on the basis of the total mineral content

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Abstract. Romania has many mineral water sources due to its geological features. In the present study, bottles of 26 Romanian mineral water brands were purchased from the market to make a characterization based on the pH, conductivity, and fixed residue content. Focusing on the total fixed residue, the distribution of low, medium, and highly mineralized water was 43.9%, 41.46%, and 14.63% respectively. The mean of fixed residue concentration was 763.3 mg/L, ranging from 40.37 mg/L to 2,603 mg/L. The pH values of the still mineral waters varied between 6.86 and

Keywords and phrases: mineral water, pH, conductivity, fixed residue, cluster analysis

7.91, while the pH values of the sparkling mineral waters were the lowest (4.7). The conductivity was strongly related to the concentration of the ions, so the maximum measured conductivity for the still waters was 573 μS/cm, for the partially sparkling waters 2,133 μS/cm, and for the sparkling mineral waters 3,079 μS/cm. The chemical composition of the mineral waters was highly dependent on the rock types. Using the hierarchical cluster analysis, two different clusters were detected according to the main characteristics of mineral waters.

1 Introduction

Romania owns 60% of the hydro-mine sources in Europe, but only one-fifth of these resources are exploited (*FRD Center Market*, 2016). According to the EU legislation (80/777/EEC): "mineral water is microbiologically wholesome water from an underground aquifer tapped via one or more natural or drilled wells" (*The Council of the European Communities*, 1980). According to the definition of the Food and Drug Administration, mineral water contains at least 250 mg/L of dissolved solids originated from a biologically and physically protected underground water source (*Sharma*, 2017).

According to the literature, the most significant mineral water source with high CO_2 content is found in the Eastern Carpathians due to the Oaş-Gutâi-Călimani-Harghita volcanic chain (*Ionete et al.*, 2015; *Vaselli et al.*, 2002).

The World Health Organization's (WHO) recommendations for the average daily water requirements for women, men, and children are 2.2 L, 2.9 L, and 1.0 L respectively. In the case of hard physical work at elevated temperature, this requirement may be increased to 4.5 L. For a woman in pregnancy and lactation period, the daily water intake should be 4.8 L and 3.3 L respectively (WHO, 2005). Besides body hydration, mineral water consumption supports essential macro-nutrients (Ca^{2+}, Cl^{-}, PO_4^{3-}, Mg^{2+}, K^{+}, Na^{+}, SO_4^{2-}) and micro-nutrients at trace levels (Co^{2+}, Co^{3+}, Cr^{3+}, Fe^{2+}, Fe^{3+}, F^{-}) (*Ingegerd*, 2014; *Quattrini et al.*, 2016; *Whelton et al.*, 2007).

The mineral water bottling process consists of the following operation series: extraction of water from the well, drilling, water filtration, water treatment (iron and manganese removal), carbon dioxide enrichment, and, finally, bottling (*Galanakis*, 2020). The total soluble mineral content of the mineral waters is strongly dependent on CO_2 concentration because the acidulated water dissolves more components from the rocks (*Misund et al.*, 1999). The chemical composition and the mineral variety of water are strongly dependent on the original geological state, the rock types, and some other parameters:

temperature, CO_2 concentration, redox conditions, and adsorption complex type (*Kis & Baciu*, 2014; *van der Aa*, 2003).

The consumption of mineral water types is recommended to different types of meat; therefore, the consumption of CO_2-saturated mineral water is recommended with fatty foods, and the consumption of still mineral water is suitable for fish (*Feru*, 2012). Based on the dissolved mineral content, mineral water with low mineral content is more proper for newborns, and rich mineral water is suitable for sportsmen to compensate the minerals lost in transpiration (*Feru*, 2012).

The chemical composition of natural mineral waters in Romania collected from the original springs was analysed by many researchers (*Kis et al.*, 2013; *Papp & Niţoi*, 2006; *Szakács & Krézsek*, 2006); however, bottled mineral waters were analysed by only a few (*Levei et al.*, 2016).

The main objective of the present research is to investigate the fixed residue, pH, and electrical conductivity of some commercially available bottled mineral waters from the Romanian market and to group them according to the abovementioned properties in order to provide supplementary information for the customers.

2 Materials and methods

In order to determine the pH and the conductivity of the mineral waters, a portable laboratory equipment was used (HI 9828 multimeter, Hanna Instruments). The measurements were carried out at room temperature (20 °C).

For the determination of the fixed residue, the water was evaporated entirely at 180 °C, and the fixed residue was measured using an analytical balance with four decimal precision. For the calculated fixed residue, the following equation was used:

$$\mathrm{Rez}_{fix} = [(m_{sp-dry} - m_{wa-em})/(m_{sp-weet} - m_{wa-em})] \cdot \mathrm{CF}, \qquad (1)$$

where: Rez_{fix} – fixed residue at 180 °C (mg/L); m_{sp-dry} – the weight of the ampoule with samples (solid) after the evaporation (g); m_{wa-em} – the weight of the empty ampoule (g); $m_{sp-weet}$ – the weight of the ampoule with the sample (liquid) (g); CF – conversion factor (10^6).

In order to add the confidence interval to the results, all samples were measured in triplicate. After the determination of the fixed residues, the mineral water brands were categorized into four groups, based on the European legislation: 1 – very low mineral content (< 50 mg/L), 2 – low mineral content

(50–500 mg/L), 3 – medium mineral content (500–1500 mg/L), and 4 – rich mineral content (> 1500 mg/L) waters (*EU Commission Directive*, 2003). According to the CO_2 content indicated on the label of the bottles, the waters were marked with st – still, spp – partially sparkling, and sp – sparkling.

In the local market (Miercurea Ciuc), the following 26 brands were available: *Apa Craiului sp*, *AQUA Carpatica Forte sp*, *AQUA Carpatica st*, *Aquatique st*, *Artesia st*, *Azuga sp*, *Azuga st*, *Borsec sp*, *Bucovina st*, *Cezara light spp*, *Dorna st*, *Harghita Tiva sp*, *Izvorul Zăganului sp*, *K-classic sp*, *K-classic spp*, *Perla Harghitei sp*, *Perla Harghitei spp*, *Poiana Negri cump. sp*, *Siculaqua sp*, *Spring Harghita sp*, *Stânceni sp*, *Tuşnad sp*, *Tuşnad spp*, *Vâlcele sp*, *Wonder Spring st*, and *Zizin st.*

Using the IBM SPSS Statistics 22 version, the hierarchical cluster analysis was used to classify the mineral water brands based on their similarities (centroid clustering method and Euclidean distance), and the results were presented in a dendrogram.

3 Results and discussion

The statistical description of mineral water characteristics based on CO_2 content

The main characteristics of all studied brands are presented in *Table 1.* According to the CO_2 content, the mineral waters were classified into three categories: still mineral waters, partially sparkling mineral waters, and sparkling mineral waters. Still mineral waters had higher pH values, ranging between 6.86 and 7.91. It is well-known that there is a strong correlation between electrical conductivity and mineral concentration – namely, high conductivity indicates a high ion and mineral concentration. The highest fixed residue was measured in the case of highly carbonated mineral water brands (Vâlcele sp – 2604 mg/L, K-classic sp – 2384 mg/L, Borsec sp – 1553 mg/L). Based on the pH value, the partially sparkling mineral waters were situated between the still and sparkling mineral waters, with values between 5.72 and 6.19, while the lowest pH value (Azuga sp – 4.7) was detected in the case of the sparkling mineral water brands. On average, the mineral content of the sparkling and partially sparkling mineral waters was quite similar, exhibiting 1008 mg/L and 987 mg/L respectively.

Table 1. Statistical description of the studied mineral waters

		N	Min.	P(25)	Mean	Med.	P(75)	Max.
Still mineral water	pH	8	6.86	7.49	7.59	7.73	7.83	7.91
	Cond. μS/cm		93	182	298	296	365	573
	Fix res. mg/L		40	68	148	137	179	345
Partially sparkling	pH	4	5.72	5.74	5.91	5.88	6.06	6.19
	Cond. μS/cm		1003	1148	1507	1447	1807	2133
	Fix res. mg/L		756	788	1008	939	1160	1402
Sparkling	ph	14	4.7	5.27	5.45	5.39	5.88	6.33
	Cond. μS/cm		254	421	1216	1024	1813	3079
	Fix res. mg/L		104	480	987	711	1360	2604

Abbreviations: **N** – number of samples; **Min.** – minimum; **P(25)** – 25^{th} percentile; **Mean** – average; **Med.** – median; **P(75)** – 75^{th} percentile; **Max.** – maximum; **Cond.** – conductivity; **Fix res.** – fixed residue.

Characterization of mineral waters based on the pH value

As it can be observed in *Figure 1*, the dissolved CO_2 content highly influences mineral waters' pH. Three sparkling mineral waters, Azuga, Izvorul Zăganului, and Apa Craiului, had pH values lower than 5. For 12 types of mineral waters (46.15%), pH values varied between 5 and 6, while 4 and 7 types of mineral waters exhibited pH values in the range of 6–7 and 7–8 respectively.

Characterization of mineral waters based on electrical conductivity

The relationship between the fixed residue and the conductivity of the mineral waters was strong (Pearson correlation: r = 0.96). The conductivity varied between 92 and 3078 μS/cm (*Figure 2*).

For 14 mineral water brands, conductivity was lower than 600 μS/cm; from these brands, 8 were still mineral waters and 6 were sparkling. The conductivity of the other 12 mineral waters was higher than 1000 μS/cm, including eight sparkling and four partially sparkling mineral waters.

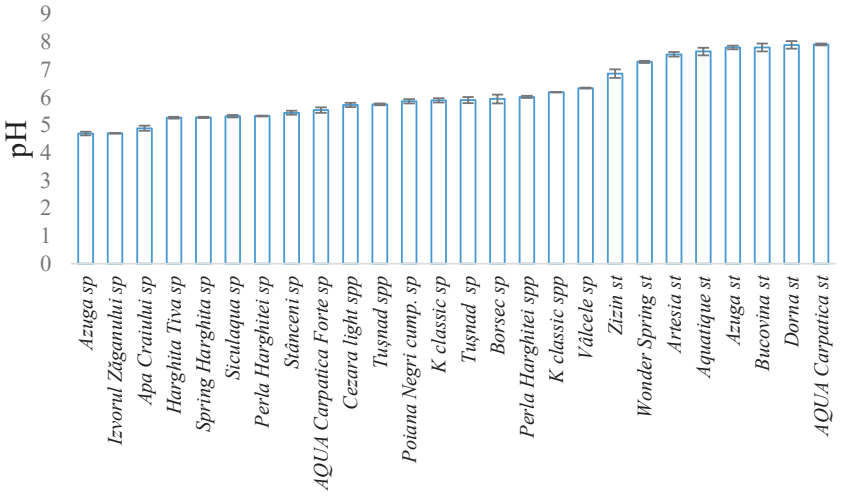

Figure 1. The pH values of the studied mineral waters

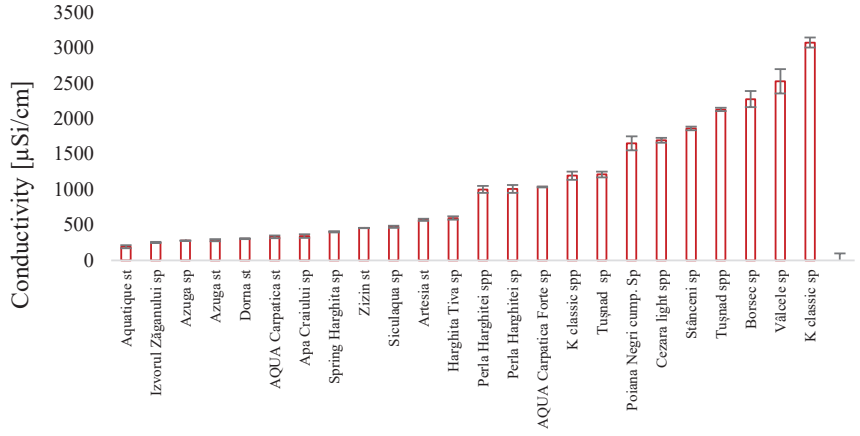

Figure 2. Electrical conductivity of the mineral waters

Classification of mineral waters based on the fixed residue

According to the results obtained for the 26 studied brands for the fixed residue, we can emphasize that in the case of 12 brands (46.2%) the fixed residue was between 50 and 500 mg/L, which corresponds to a low mineral

content, followed by 10 brands (38.5%) with medium mineral content. However, one brand was detected as having a very low mineral content, while brands with rich mineral content represented 11.5% of the samples, namely Borsec sp, K-classic sp, and Vâlcele sp (*Table 2*).

Table 2. Mineral water classification based on the fixed residue

Fix res. at 180 °C	No.	%	Brand description
Very low (< 50 mg/L)	1	3.85	Wonder Spring st
Low (50–500 mg/L)	12	46.2	Bucovina st, Aquatique st, Azuga sp, Dorna st, Izvorul Zăganului sp, Azuga st, AQUA Carpatica st, Apa Craiului sp, Zizin st, Artesia st, Siculaqua sp, Spring Harghita sp
Medium (500–1500 mg/L)	10	38.5	Harghita Tiva sp, Perla Harghitei sp, AQUA Carpatica Forte sp, Perla Harghitei spp, K-classic spp, Cezara light spp, Poiana Negri cump. sp, Stânceni sp, Tuşnad spp, Tuşnad sp
Rich (> 1500 mg/L)	3	11.5	Borsec sp, K-classic sp, Vâlcele sp

Two categories were identified based on the fixed residue values as follows: very low and low (*Figure 3*) and medium and rich mineral content (*Figure 4*). The lowest fixed residue was detected in the case of Wonder Spring and the highest in Vâlcele mineral waters.

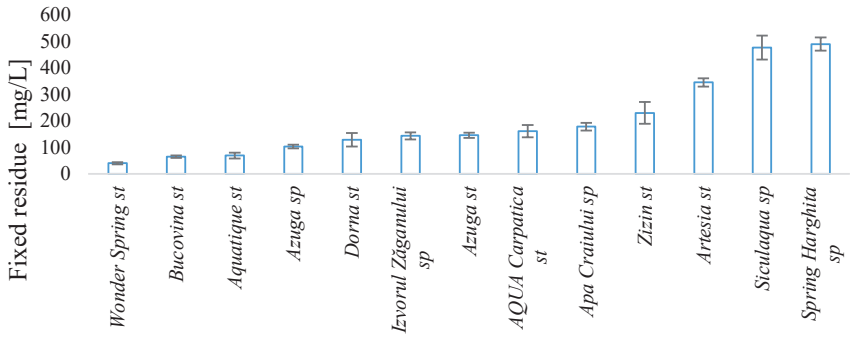

Figure 3. Very low and low fixed residue mineral water brands

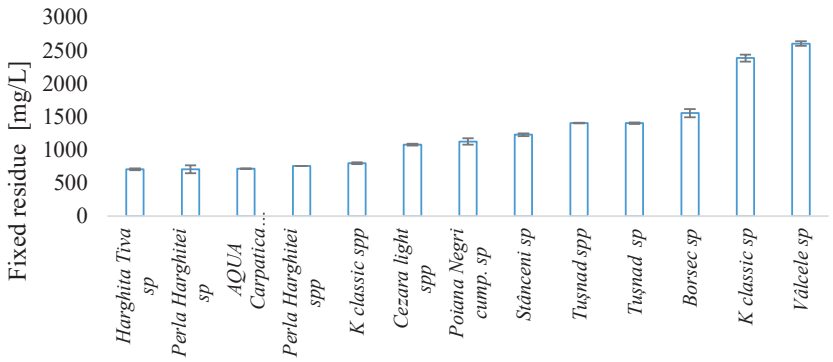

Figure 4. Medium and rich fixed residue mineral water brands

Comparison of the studied waters with some European waters

The measured parameters were compared to other European mineral waters (*Table 3*). The lowest and highest pH values were registered in Italy (4.1, 8.8), while the measured conductivity varied on a large scale from 18 to 26000 μS/cm. Based on pH values, the Romanian mineral waters' variability (4.7–7.91) was similar to that of Estonian mineral waters (4.7–7.76). The maximum value of measured electrical conductivity in Romanian waters (3079 μS/cm) was almost identical to the one measured in Italian mineral waters (3020 μS/cm).

Table 3. The pH and the electrical conductivity of mineral waters from Europe

	pH	**Cond, μS/cm**	**Reference**
Romania	4.7–7.91	92–3079	
Serbia	5.6–7.5	340–4560	(*Petrović et al.*, 2010)
Italy	4.1–8.8	18–3020	(*Dinelli et al.*, 2010)
Estonia	4.7–7.76	175–4370	(*Bityukova & Petersell*, 2010)
Poland	4.7–8.3	188–6510	(*Astel et al.*, 2014)
Hungary	5.3–8.3	250–26000	(*Fugedi et al.*, 2010)
Spain	6–8.1	30–1257	(*Devesa et al.*, 2012)
Slovakia	6–6.75		(*Dušan et al.*, 2010)
Croatia	6–7.9	340–3680	(*Peh et al.*, 2010)
Germany	3.8–8.10	38.1–6340	(*Birke et al.*, 2010)

Comparison of the studied parameters with the reported ones on the labels

The comparative analysis between the determined and the reported values on the labels is presented in *Table 4*. We would like to mention that not all studied brands displayed the pH values and fixed residues on the labels.

Table 4. The comparison of the measured ^{m} and the reported ^{r} values

	pH^{m}	**pH^{r}**	**Fix res^{m}**	**Fix res^{r}**
Apa Craiului sp	4.89	5.00	178	194
Aquatique st	7.66	7.8	70	90
Artesia st	7.55		345	465.5
Azuga sp	4.70		104	191
Azuga st	7.80		146	191
Borsec sp	5.94	5.64	1554	1655
Bucovina st	7.80	7.27	65	78
Cezara light spp	5.72	6.11	1079	
Dorna st	7.89	7.71	129	192
Izvorul Zăganului sp	4.70		144	147
K-classic sp	5.89		2384	2453
K-classic spp	6.19		798	920
Poiana Negri cump. Sp	5.86	5.65	1125	1173
Spring Harghita sp	5.28	5.3	490	510
Stânceni sp	5.45	5.37	1229	1375
Tuşnad sp	5.90		1403	1674
Tuşnad spp	5.75		1402	1674
Vâlcele sp	6.33	6.55	2604	2440
Wonder Spring st	7.28		40	81.4
Zizin st	6.86		230	202

Hierarchical Cluster Analysis

In order to find similarities and differences among the studied samples, the hierarchical cluster analysis was carried out, taking into consideration the pH, the electrical conductivity, and the fixed residue. The results showed two main clusters with sub-clusters (*Figure 5*). Cluster 1 contained two sub-clusters. The sub-cluster 1.1 contained 8 still mineral waters, characterized by average high pH (7.59) and low electrical conductivity (298 μS/cm) and fixed residue (148 mg/L). The 1.2 sub-cluster covers the majority of the studied brands (16), represented by partially sparkling and sparkling mineral waters. In comparison with cluster 1.1, lower pH (5.49), higher electrical conductivity (1090 μS/cm) and fixed residue (804 mg/L) were observed. Two brands, K-classic st and

Vâlcele sp, appeared in cluster 2, which formed a group of waters with very high fixed residue and electrical conductivity (2494 mg/L, 2805 μS/cm).

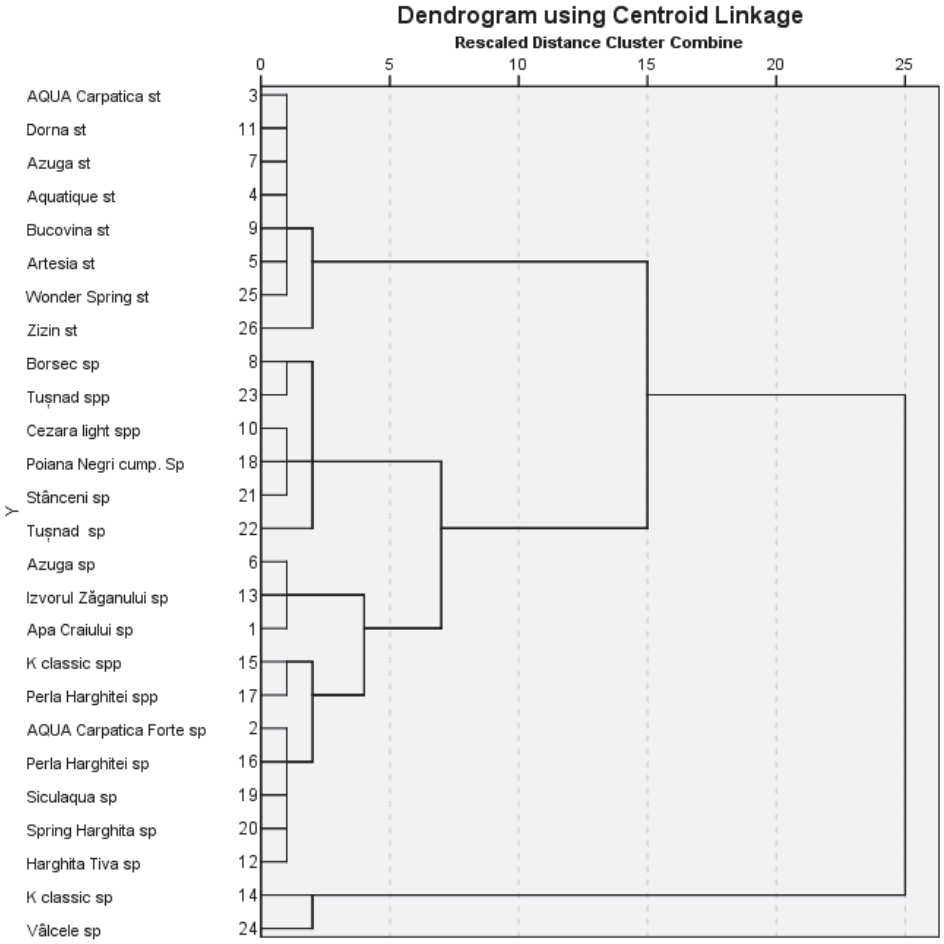

Figure 5. Classification of the mineral waters by hierarchical cluster analysis

4 Conclusions

In this study, three parameters (pH, electrical conductivity, and fixed residue) were determined for selected commercialized mineral waters and analysed in more detail using descriptive statistics. The results revealed that the mineral

content was very low for 3.84%, low for 46.2%, medium for 38.5%, and rich for 11.5% of the selected waters. The total mineral content of sparkling and partially sparkling mineral waters was remarkably close, as we found close values for the electrical conductivity. According to the hierarchical cluster analysis, cluster 1.1 covers the still mineral waters with high pH and low fixed residue. Cluster 1.2 was represented by the medium mineral content, and in cluster 2 two brands were observed with very high fixed residue. There were no considerable differences between the reported values on the labels of the bottles and the values determined by our team.

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The aflatoxin content of milk and dairy products as well as breast milk and the possibilities of detoxification

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Abstract. Aflatoxins are fungal toxins produced by *Aspergillus* species, which, due to increasing temperature and climate change in the temperate zone, appeared in the most important feeding plant and food ingredients. The most toxic of them is aflatoxin B1 (AFB1), which hydroxylates to aflatoxin M1 in the body of dairy animals and humans, and excretes in the milk. With the development of analytical methods, researchers are now able to detect toxins with a concentration of ng/kg. It was found that in most countries in Europe both breast milk and cow's milk may contain AFM1, and therefore increased attention should be paid to the toxin content of milk, and that those above the limit should be excluded from consumption. In addition to cow's milk, the AFM1 toxin content

Keywords and phrases: mycotoxins, aflatoxins, aflatoxin M1, milk, breast milk, detoxification of mycotoxins, determination of aflatoxins

of breast milk can also be significant, the precursors of which are introduced into the mother's body with food. Aflatoxins are highly resistant to physical, chemical, and microbiological effects, so the detoxification of foods, especially milk, is almost impossible. The best solution appears to be feeding the animals with toxin-free feeds or feeds containing toxins below the permitted limit, without giving opportunity to the toxins to enter the milk from the feed and from there into the human body.

1 Introduction

Aflatoxins are one of the main classes of mycotoxins, being secondary products of metabolism of microscopic fungi such as *Aspergillus flavus*, *Aspergillus parasiticus*, or *Aspergillus nomius* (*Creppy*, 2002). The main classes of aflatoxins are aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2). Conditions such as drought, high temperatures, substrate composition, and storage time play a very important role both in the growth of fungi and in the production of toxins. Of the toxins listed, AFB1 has the most toxic, carcinogenic, teratogenic, and mutagenic effects (*Sweeney & Dobson*, 1999).

Aflatoxins are colourless, pale yellow crystalline compounds that fluoresce in ultraviolet (UV) light. They dissolve only slightly in water (10–20 μg/mL) but are well dissolved in slightly polar solvents such as chloroform or dimethyl sulphoxide. UV light breaks it down in the presence of oxygen and is unstable even below pH = 3 or above 10. The lactone ring splits in a reversible reaction under alkaline conditions but may re-form under the influence of acid. At high temperatures in an irreversible reaction and after decarboxylation, ammonia also splits the lactone ring (*Kumar*, 2018).

AFB1 shows absorption maximum at 223, 265, and 362, AFB2 at 265 and 363, AFG1 at 243, 257, 264, and 362, and AFG2 at 265 and 363 nm. AFB1 occurs in almost all foods, and its hydroxylated derivative, aflatoxin M1 (AFM1), is also found in milk and dairy products as well as in breast milk. In addition to milk, it has also been shown in cheeses, ice cream, and yoghurt. In particular, a lot of AF may be contained in fat-rich milk and dairy products, and especially a lot of AF is accumulated in butter thanks to the high volume of apolar triglyceride content (*Kumar*, 2018).

The AFM1 is the hydroxylated metabolite of AFB1, which is excreted through milk in humans and dairy animals (*Fallah et al.*, 2009). Approximately 0.3–6.2% of AFB1 is transformed to AFM1 by the genetics of the animals, season, type of milk production, and environmental factors. The toxic effect of

AFM1 is much less than that of AFG1; however, it causes health problems because in many countries the milk and dairy products are a significant part of daily food, and so the toxin can enter the body of many people not only with milk but also with dairy products (*Unusan*, 2006; *Fallah et al.*, 2009).

Symptoms of acute toxicity in mammals include the development of lethargy, loss of appetite, rough or halo hairs, ataxia, and fatty liver. In animals, jaundice may develop, feed is rejected, and milk production is significantly reduced. Aflatoxins reduce disease resistance and inhibit immunity developed by vaccines (*Diekman & Green*, 1992). In beef cattle, the 700 μg/kg toxin volume did not reduce mass gain, but, since the liver has increased significantly, it is considered that the toxin content of the feed should not exceed 100 μg/kg. In dairy cows, this amount has already significantly increased the concentration of aflatoxin in the milk. It has been reported that feed containing 120 μg/kg toxins decreased significantly the reproduction, and when the cows were fed with a feed that did not contain toxins, milk production increased by 25%. Milk production decreased more when cows took the toxin with contaminated feed than when it was added to feed in synthetic form (*Guthrie*, 1979; *Patterson & Anderson*, 1982).

2 How to prevent toxins from entering the milk?

Today, *Aspergillus* species, which are capable of producing aflatoxins due to the rising global average temperature and climate change, have also appeared in temperate zone environments, such as in Hungary, and the toxins can be found in the different food and basic feed materials such as maize or wheat. The most toxic of them is aflatoxin B1, which, absorbed from feed, is able to form an aflatoxin M1 in the bovine body, which reaches the milk gland during milk production and is excreted with the milk.

Milk is one of the most important basic foods, whose production is estimated to double by 2050 to provide the growing population with the necessary nutrients. It is very important that milk does not contain AFM1 at all or only to an acceptable degree because otherwise this important food material can become a source of toxic materials (*Iqbal et al.*, 2015).

According to the Food and Feed Safety Administration of the National Food Chain Safety Office of Hungary (NÉBIH), the most important is to ensure that cattle are treated with feeding stuffs with the lowest possible contamination and low aflatoxin content because the toxin is introduced through the feed into the blood through the cattle's digestive tract and from there into the

milk. In Hungary, due to the changed climatic conditions, aflatoxins have also appeared in maize, which are the main source of contamination (*Szeitzné Szabó & Frecskané Csáki*, 2013).

When feeding with contaminated feed, the toxin appears in the milk within up to a day, and for the depletion of the toxin, when feeding toxin-free or low-toxin-content feed, it is necessary to wait two to three days. In the case of basic feed materials, the maximum permissible limit for aflatoxin B1 is 20 μg/kg, but this is lower, 5 μg/kg, for feed mixtures of dairy cows. In the case of milk, the maximum amount of toxin is 50 ng/kg, and milk with a higher toxin content cannot be released in Hungary (*Szeitzné Szabó & Frecskané Csáki*, 2013).

In Hungary, the sources of pollution are feed mixtures made from by-products of grain maize and maize processing. The most important means of preventing the AFB1 contamination of milk is the feeding of toxin-free or low-toxin-content feed to dairy cows. If the toxin content of the feed is known, feeding feed with a toxin content exceeding the limit value can be prevented, or the feed formula can be assembled in such a way that its toxin content falls below the limit value.

If the feed of dairy cows is contaminated with AFB1, the toxin is hydroxylated in the bovine body and enters into the milk in the form of aflatoxin M1. Toxin absorption from feed varies per person, and the lactation status of the individual and the milk yield also influence it. In cows with low milk yields, 1–2% of the toxin and with high milk yields 5–6% of the toxin enters into the milk, and more toxins are excreted at the beginning of lactation since in fresh-milk cows, under the same toxin load, the toxin content of the milk may be three to four times higher than at a later stage of lactation. As a result, the toxin content of milk may be above the limit value even if cows still consume feed with the permitted concentration of toxins. In general, however, it can be stated that the toxin content of milk remains at the permitted level if cows do not take more than 40 μg of toxins per day (*Szeitzné Szabó & Frecskáné Csáki*, 2013).

The toxin content of milk may also be affected by the physiological state of the animal since if the pH of the rumen is reduced (rumen acidosis), the effectiveness of the absorption of the toxin can also increase several times. If the toxin content of the milk is nevertheless above the permitted limit, an immediate replacement of the feeding stuffs may be required, or toxin bindings may be used, which may reduce the toxin content of the milk by 20–30%. The use of toxin binders is only an emergency solution; the final result can only be obtained by feeding toxin-free or low-toxin feeds. Attention is drawn to

the analysis of the toxin content of feed carried out in accredited laboratories, because the toxin content of milk can be estimated from the concentration of the toxin and the amount of the fed feed (*Szeitzné Szabó & Frecskáné Csáki*, 2013).

The AFM1 in milk, especially coupled to casein, can be found in the aqueous phase, and so the AFM1 content of cream and butter is relatively low. During cottage cheese and cheese making, most of the toxin is transferred to the curd, and only a smaller part of it is left in the whey; so, the AFM1 concentration of soft cheeses is about three times and of hard cheeses five times higher than the toxin concentration of the milk used as a raw material (*Szeitzné Szabó & Frecskáné Csáki*, 2013).

3 Stability of AFM1 and the possibility of reduction in milk and milk products

AFM1 is very stable at high temperatures, so, during normal heat treatment methods, it does not suffer significant decomposition in milk. Many researchers have also studied the stability and the changes in the composition of dairy products and how much AFM1 of milk passes into the dairy product. AFM1 was stable in Kashar cheese even after the 60-day maturation period and had an unchanged concentration in traditional cheeses during 90 days of maturation (*Oruc et al.*, 2006).

When assessing the stability of AFM1 in artificially contaminated yoghurt, it was found that the volume of either at concentrations of 0.05 or 0.10 μg/ L during four-week storage, pH = 4.6 did not change, but if pH was reduced to 4.0, the volume of both concentrations decreased significantly by the end of the third and fourth week. In a similar experiment, the AFM1 amount of yoghurt decreased significantly during the production and storage of yoghurt. The decrease is attributed to low pH, to the effect of organic acids and fermentation by-products, and to the presence of lactic acid bacteria (*Govaris et al.*, 2002). In another experiment, 13% more AFM1 was found in yoghurt than in the starting milk, but the difference was not significant (*Bakirci*, 2001).

When making Ricotta cheese, 94% of AFM1 remained in the liquid phase, and only 6% were included in the cheese. When ultrafiltration was used, almost 90% of AFM1 remained in the liquid phase, and only a minimum quantity was transferred to the cheese. Spray drying also decreased the amount of AFM1 in significant quantities (40–60%) (*Cattaneo et al.*, 2013).

During UHT heat treatment, different technologies use temperatures of 130–

150 °C and different heat maintenance periods, so the results of the degradation of AFM1 to heat are also contradictory. Some (*Purchase*, 1967; *Kabak & Ozbey*, 2012) reported of about 32% decrease, while others (*Galvano et al.*, 1996) claimed that at this temperature the AFM1 is heat stable, and its concentration is not affected by UHT treatment. Looking at the effect of different technologies in UHT treatment, it was found that decomposition was 12–35% depending on the circumstances, but in most cases it was also found that heat treatment had no effect on the concentration of AFM1 (*Prandini et al.*, 2009).

Several researchers experimented with reducing the AFM1 content of cow's milk and dairy products by the use of clay and clay minerals (*Carraro et al.*, 2014). These studies have shown that benthonic is very effective in reducing the AFM1 content of cow's milk if its concentration has not exceeded 80 ng/L. The concentration was reduced to 50 ng/L for adults and to 25 ng/L for children without significantly altering the organoleptic properties of milk.

Among the cultures used in the production of yoghurt, *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, and *Lactobacillus plantarum* were the most effective at reducing AFM1 levels during the storage of yoghurt (*Elsanhoty et al.*, 2014). Using probiotic strains, the concentration of AFM1 was reduced by 19.9–25.4%, and in *in vitro* trials with probiotic strains, depending on its type, they were able to achieve a decrease of 23–45% (*Serrano-Nino et al.*, 2013).

In rare cases, the concentration of AFM1 in dairy products exceeds 1 μg per litre. The AFM1 content of cheeses is not produced during fermentation, but it comes from milk, and the milk is obtained through the feed consumed by the animal. AFB1 absorbed from feed is converted into AFM1 in the liver and is found in almost all dairy products.

Since cows also consume a lot of imported nutrients, the toxin can also be found in milk even if the basic foodstuffs are toxin-free. AFM1 is stable both during heat treatment and other cheese-making processes, and its concentration does not change when stored. The detoxification of AFM1, without damaging the valuable components of food, is almost impossible (*Tabata*, 1998).

Milk and dairy products can be contaminated with mycotoxins in an indirect and direct manner. The easiest way to indirect contamination is to eat feed infected with microscopic fungi. AFM1 has the greatest importance, which is the main metabolite of AFB1. The effect of AFM1 on the human body is virtually the same as that of AFB1. Direct contamination occurs when, for example, moulds proliferate in the inside or on the surface of cheeses, which are capable of producing toxins. These can even be components of the

starter cultures used to make different cheeses. *Aspergillus* strains can infect even milk or dairy products, after which they produce a toxin AFB1, whose concentration is generally much smaller than that of AFM1 (*Fisher et al.*, 2011).

AFB1, which is added to the body with food, is converted there to AFM1, which is either excreted in urine or reaches the foetus through the placenta during pregnancy, and so the new generation already encounters the toxin in intrauterine life or is excreted with breast milk and with it enters the newborn's body. Aflatoxins are found in a lot of basic food and feed materials and are thus introduced directly or through the animal, e.g. with milk, and get into the human body.

During 2013–2014, 80 milk samples and 21 infant formulas have been examined with the AFM1 content. It was found that its concentration varied from 0.02 to 0.32 μg/kg, and the mean value was 0.13 μg/kg. In 75% of the samples, the AFM1 concentration exceeded the 0.05 μg/kg, which is considered by the EU as a limit. On the basis of the results, the levels of toxins that may be consumed by each animal species as well as the types of exploitation have been determined. The lowest value, 0.05 μg/kg AFM1, was determined for dairy animals (*Spanjer*, 2018).

4 Aflatoxin content of breast milk

In recent times, sensitive methods for determination of the concentration of aflatoxins and their metabolites have been developed, with LOD (limit of detection) valued at 3–6 ng/kg, with the help of which the small toxin content of breast milk can also be measured. During the experiments conducted in Germany, they were unable to detect AFM1 neither in the 120 samples collected from Kiel nor in the 75 samples from Munich. By contrast, for Sudanese samples from the same time, 75% were tested positive, and 11% of the Zimbabwean samples showed positive results, with a maximum value of 51 ng/L (*Coulter et al.*, 1984). All the 42 French samples produced negative results, and in Italy 1–5% of the samples were positive, but the maximum value reached nowhere the 200 ng/L level (*Galvano et al.*, 2008; *Turconi et al.*, 2004).

The situation is much worse in Africa, where mainly AFB1 and AFM1 toxins have been detected in breast milk. In Ghana 32%, in Kenya 28%, in Nigeria 12%, and in Sudan 37% of the investigated samples were positive, where the maximum amount of toxins reached the level of a few μg per litre. These results show that babies in African countries have access to aflatoxins in significant

quantities with breast milk, and some consume very serious amounts. In the town of Bishoftu, Ethiopia, of the 108 milk samples analysed for AFM1, all samples were found to be contaminated (100%) with a mean value of 0.835 μg/l. The highest AFM1 content was 2,159 μg/l and the lowest was 0.029 μg/l; both were obtained from the local milk producers (*Tadesse et al.*, 2020). The situation in some Arab countries is not much better either. In Egypt, approximately 50% of the tested samples were positive, and the amount of the toxin varied from 4 to 120 ng/L, but in some cases it reached a level of 19 μg/L (*Tomerak et al.*, 2011).

Various studies have shown that the concentration of aflatoxin in milk shows seasonal fluctuations, which for AFM1 may be associated with the life function and production of toxins of moulds in foods consumed by mothers. According to one study, the milk of Egyptian mothers contained 13.5 ng/L AFM1, which is far less than those measured in the neighbouring countries. In Abu-Dhabi, 99.5% of the tested samples were positive, the results varied from 2 to 3,000 ng/L, and the average was 68 ng/L. In the United Arab Emirates, 92% of the tested samples were positive, the results varied from 5 to 3,400 ng/L, and the average was 560 ng/L (*Abdulrazzag et al.*, 2003).

The situation is further complicated by the fact that different regions within some countries (Turkey, Iran) also show different data. Summing up, in most European countries, the load of AFM1 on infants from breast milk is negligible, but the situation may be critical for some tropical African and Middle Eastern countries. In West Africa, in the United Arab Emirates, and in Egypt, the toxin content of breast milk's AFM1 may significantly exceed the level of 25 ng/L indicated as a top level in Europe and the United States, and, in addition, the resistance and efficiency of deactivation of newborns and infants to toxins is much lower than in adults. As a result, the growth and development of infants is reduced, which justifies the development of a strategy for a significant reduction in the toxin content of breast milk in countries at risk (*Polychronaki et al.*, 2017; *Galvano et al.*, 2008; *Degen et al.*, 2013).

5 Ways to detoxify foods contaminated with aflatoxin

Several physical, chemical, microbiological, and biological methods have been developed to detoxify and remove aflatoxins. It is essential for these methods not to reduce the nutritious value of the food during detoxification, that the by-products causing carcinogenesis and mutagenesis are not produced during the

procedure, and that the method should destroy *Aspergillus* spores and mycelia so that they cannot produce new toxins (*Santini* & *Ritieni*, 2013; *Sowley*, 2016). The first step of physical methods is the separation and removal of the contaminated food fraction, followed by detoxification with heat, cooking, roasting, or irradiation. Aflatoxins are poorly soluble in water and cannot be removed from food by washing with water, but it has been reported that washing with water has been able to extract 40% of aflatoxin from food (*Hwang* & *Lee*, 2006).

The decomposition temperature of the various aflatoxins is between 273 and 306 °C; nevertheless, various heat treatment procedures have been developed to reduce its volume. In doing so, with cooking, roasting, frying, or hot steam, the content of aflatoxin has been reduced with a significant degree (50–70%) (*Jalili*, 2015). Only minor results have been achieved with gamma irradiation, while radiolysis in aqueous medium, generating free radicals, was not successful either. The destruction of microorganisms depends on the strength of gamma radiation as microorganisms did not die at a low dose (0.1 MRad), but the dose of 0.3–0.4 MRad prevented the reproduction and toxin production of moulds (*Samarajeewa et al.*, 1990).

The absorption of aflatoxin on a solid adsorbent surface is another opportunity for detoxification. Such adsorbents may include activated charcoal, aluminium oxide, diatomic earth, clay, bentonite and montmorillonite, zeolite and hydrated calcium aluminium silicate, polysaccharides, such as cellulose, or derivatives, such as glucomannans or peptido glycans, or synthetic polymers or their derivatives (*Huwing et al.*, 2001). A greater effect could be achieved when these adsorbents were used in combination instead of applying them separately (*Khadem et al.*, 2012).

Several of the chemical methods used sodium hypochlorite, chlorine dioxide or chlorine gas itself, hydrogen peroxide, ozone and sodium hydrogen sulphate for detoxification, and many used acidic or alkaline hydrolysis for the oxidation of the double bonding of the furan ring or for the oxidation or the hydrolysis of the lactone ring (*Doyle et al.*, 1982; *Samarajeeva et al.*, 1990; *Jalili*, 2015). Peroxide treatment can reduce 100% of AFM1 in milk but requires a high dose of hydrogen peroxide that may leave a residue, which is a concern for human health (*Nguyen et al.*, 2020).

Chemicals such as 75% methanol, 5% dimethylamine hydrochloride, aldehydes, benzoyl peroxide, osmium tetroxide, iodine, copper ammonium sulphate, potassium permanganate, kinons, sodium borate, or formaldehyde were used for detoxification; however, these methods were not used in practice due to the difficult removability of residual substances (*Samarajeewa et al.*, 1990).

Many bacteria and fungi are also able to detoxify aflatoxins from solutions. A strain of flavobacteria was able to detoxify and completely remove AFB1 from milk, oil, peanut butter, peanuts, and wheat and was effective in soy (*Ciegler et al.*, 1966). It has also been reported that from naturally contaminated milk, with the help of the flavobacteria, 9.9 μg/mL of aflatoxin could be completely removed at 30 °C in four hours. In addition to flavobacteria, other microbes are able to degrade aflatoxin as well, and even the *Tetrahymena pyriformis* protozoa was able to do it (*Doyle et al.*, 1982).

In addition, with the help of their peroxidase enzyme, the moulds themselves are able to dismantle the toxin, which creates free radicals by dismantling hydroperoxides, which can react with aflatoxins. Out of the peroxidases in the presence of hydrogen peroxide and chloride ions, the myeloperoxidase creates hypochlorite and nascent oxygen, which effectively react with aflatoxins (*Wogan*, 1966).

Cold plasma has been used for the degradation one of aflatoxin. Although it has never been applied for degrading AFM1 in milk, it has been used to reduce AFB1 in other food samples. The physical and chemical quality of milk following treatment with cold plasma for controlling microorganisms has shown no noticeable changes in pH, colour, and fatty acids (*Nguyen et al.*, 2020).

AFB1 is the most toxic one of aflatoxins and is the most common in different foods; therefore, its metabolite, AFM1, has also received a lot of attention in research studies (*Iqbal et al.*, 2015; *Van Egmond*, 1989). The transformation of AFB1 into AFM1 can also be considered as a detoxification process because the carcinogenic and mutagenic effect of AFM1 is only approximately 10% of AFB1 (*Wogan & Paglialunga*, 1974).

Most of the physicochemical and biological detoxification methods could lessen the concentrations of AFM1 in milk, with the apparent reduction rate ranging from 1.9 to 90.0%. It is worth noting that these detoxification methods are still substandard relative to the EU limit (50 ng/L) (*Min et al.*, 2020).

6 Methods for determining aflatoxin M1 from milk and dairy products

The first step in most methods is the extraction of AFM1 from milk and dairy products using a mixture of polar organic solvents such as acetonitrile, methanol, or acetone. The use of chlorinated hydrocarbons during extraction has been reduced for environmental reasons (*Shephard*, 2008). Most use chro-

matographic methods to separate AFM1 from other components, preceded by steps such as sample preparation and extraction, cleaning, and perhaps derivatization. In doing so, all components that may interact with the component we are looking for during weaning must be removed from the sample. Cleaning methods include column chromatography, the use of solid-phase extraction columns, fluid-fluid extraction, the use of immunoaffinity columns and multifunctional columns applied in one step (*Krska et al.*, 2005).

These procedures are simple, increase efficiency and speed, minimize solvent use, increase toxin release, and reduce costs. Solid-phase adsorption or the use of immunoaffinity columns reduces preparation time to a few minutes but requires preparatory steps such as conditioning the column, withholding the desirable components on the column, and then removing desirable components from the column. However, minimal interference may occur between the materials to be retained and the column load, but this preparatory operation allows small concentrations to be determined by the following chromatography methods (*Fuchs et al.*, 2002).

After the appropriate sample preparation, most researchers used liquid chromatography methods and ELISA to determine the AFM1 content of milk and dairy products. Many of them used thin-layer chromatography, fluorimetrics, ultra-performance liquid chromatography, linked to tandem mass spectrometry, lateral flow, and gel-based immunodeterminations. High-performance liquid chromatography is also used by fluorescent detection for the AFM1 analysis of milk and dairy products (*Fallah*, 2010; *Huang et al.*, 2014; *Anfossi et al.*, 2013).

Although thin-layer chromatography is perhaps one of the oldest chromatography procedures, it has been used widely to determine AFM1, and even in 1990 the AOAC adopted it as an official method also in cases where the concentration of the toxin developed around 1 μg/g. However, its application was reduced to HPLC fluorescent detection, especially when the latter method began to be combined with mass spectrometry or gas chromatography. In recent years, almost exclusively, both of the modifications of HPLC have been used to determine AFM1. Depending on the polarity of toxins, both normal-phase and reverse-phase chromatography were used (*Iqbal et al.*, 2013).

In the case of milk and dairy products, depending on the type of sample, HPLC procedures are practically the same, the difference being only in the composition of the mobile phase and in the stationary phase of the column. Fluorescent detectors are also excellent for use since each toxin has its own emission and excitation maximums. Moreover, the advantage of HPLC methods is the very low detection level (LOD) and the ability to detect several

components from a sample (*Valenta*, 1998).

Due to its ease of applicability, lately, the ELISA method has also become very popular for detecting AFM1. The advantage of ELISA is the ease of use, high specificity, and portability; so, it is not necessary to have a well-equipped laboratory for analysis, and a large number of samples can be analysed with it in a short period of time (*Anfossi et al.*, 2013).

A lot of methods with biosensors have also been developed to detect the AFM content of milk. One of them is such a membrane-based flow system where the antibody against AFM1 is linked to the horseradish peroxidase enzyme. This indirect, visually assessable mobile biosensor can measure AFM1 in 18 minutes with a concentration of 0.05 ng/mL. The LOD value of an impedimetric biosensor developed for AFB1 for milk samples is 0.1 ng/mL. A manual biosensor, based on immunoaffinity and linked to fluorometric detection, can measure AFM1 in less than two minutes with a concentration of 0.1 ng/mL (*Rasooly & Harold*, 2011).

7 Conclusions

With the rise of global mean temperature and climate change, conditions in the temperate zone have become optimal for the spread of Aspergillus species, which under certain circumstances are capable for the production of mutagenic, teratogenic, and carcinogenic toxins such as AFB1, AFB2, AFG1, and AFG2. AFB1 is incorporated into the body of the mother or dairy animals, where it is hydroxylated during a process considered as a detoxification step, and then it is excreted in the milk from where it enters the body of the infants or adults. AFB1 is the most toxic of aflatoxins and, despite the fact that the carcinogenic and mutagenic effect of AFM1 is only 10% of AFB1 as it can enter the body in significant quantities with milk and dairy products, this derivative has also received a lot of attention lately.

If we are to prevent the AFM1 content of milk from being more than 50 ng/kg, which is already unsuitable for human consumption, the most important step is prevention, i.e. dairy animals must be fed with a high-quality feed, preferably free of mycotoxins. In Hungary, dairy cattle feed may contain a maximum of 5 μg/kg of AFB1, but the production of milk with a toxin content lower than allowed is still in doubt even in high-milk cows. It complicates the situation that the absorption of the toxin from feeding stuffs, in addition to the concentration of the toxin, is affected by the lactation status and microbial processes in the rumen as well as by the fact whether or not the

toxin binder materials have been used in feeding.

AFM1 is extremely stable in milk, heat treatment and other technological processes have little effect on it, and it does not suffer significant decomposition during the production of dairy products. Although various physical, chemical, and microbiological methods have been tried to reduce the toxin content of milk, a major breakthrough in this area has not been achieved. Perhaps the most useful detoxification procedures appear to be when the reduction of the toxin content of dairy products has been achieved with microorganisms with a quality very similar to that of the cultures used in the production of dairy products. In summary, there is no procedure other than microbiological methods which can significantly reduce the toxin content of milk without significantly altering its nutritious value.

In addition to cow's milk, a significant amount of AFM1 can get into breast milk, such as in the previous cases, during the conversion of AFB1 to AFM1 in the mother's diet. In most European countries, one can either not detect toxins from breast milk at all or its amount is only a few nanograms per litre. The situation is much worse in African countries and in the Middle East, where 10–100% of breast milk were tested positive for AFM1, and the volume even exceeded levels of up to 200–1,000 ng/L. In the United States and the European Union, the maximum limit for the toxin content of breast milk is 25 ng/L, which is significantly exceeded by the results of these other referred countries. It can be therefore said that in Europe the toxin exposure of infants to breast milk is negligible, while the situation may be critical for some African and Near East countries.

Very sensitive methods are available to determine the AFM1 content of milk. After proper preparation and concentration, most researchers use the HPLC-MS and ELISA methods with the help of which even up to 10 nanograms of toxins can be detected. In addition to the very low detection level, the advantage of HPLC methods is that they allow the detection of several toxins from one sample. ELISA's popularity lies in the fact that, in addition to being very specific, it is extremely fast, and that several samples can be classified with it very quickly following proper preparations carried out even on site.

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Bioprotective potential of lactic acid bacteria

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Abstract. Acidification in lactic-fermented foods is realized by lactic acid bacteria as an added starter culture or by autochthonous strains. These microbial strains possess different prominent features that define the technological, organoleptic, nutritional, and microbial safety aspects of the product. The bioprotective effect of the bacterial strains may be related to antagonistic properties against food spoilage and/or pathogenic strains. The aim of the present study is to determine the antimicrobial properties of three different food-grade lactic acid bacteria in order to use them as bioprotective cultures. Our findings show that the *Lactobacillus pentosus*, *Enterococcus faecalis*, and *Pediococcus parvulus* exerted a bacteriostatic effect on *Escherichia coli* and *Bacillus cereus*, whereas the *Saccharomyces cerevisiae* growth was not inhibited, which made them susceptible agent for co-culture systems.

Keywords and phrases: lactic acid bacteria, bioprotective, antagonistic

1 Introduction

One of the most common and ancient methods of food preservation is fermentation, and that process is driven by microorganisms. The acidification in fermented foods is caused by the formation of organic acids as primary metabolites, e.g. the lactic acid is synthetized by lactic acid bacteria (LAB) as added starter culture or by autochthonous strains. These bacterial strains possess different prominent features that define the technological, organoleptic properties as well as nutritional and microbial safety aspects of the product (*Altieri et al.*, 2017; *Ruiz-Rodríguez et al.*, 2017). The bioprotective potential of the bacterial strains is related to antagonistic properties. The LAB exert their protective activity mainly via three modes: displacement/exclusion, competition for nutrients, and production of antimicrobial metabolites (*Ben Said et al.*, 2019). The antimicrobial metabolites, however, may act through different mechanisms such as the inhibition of the spoilage microorganisms resulting in the membrane destabilization in spoilage microorganisms, proton gradient interference, enzyme inhibition, or creation of reactive oxygen species (*Siedler et al.*, 2019).

The inhibitory effect of LAB is associated with metabolic compounds like primary metabolic products as different organic acids or complex compounds derived from protein metabolism (*Rodríguez et al.*, 2017). It was shown that lactic acid and acetic acid derived from central carbon metabolism comprise an antimicrobial spectrum, which includes some Gram-positive and some Gram-negative organisms and yeasts. Hydrogen peroxide, acetaldehyde, and acetoin have an antimicrobial spectrum, which includes also Gram-positive and some Gram-negative organisms and yeasts (*Siedler et al.*, 2019). The short-chain fatty acids are the prominent factor in the antagonistic phenomena (*Gao et al.*, 2019). Due to the production of acetic acid, the pH decreases, and the different undesirable microorganisms are deactivated. The other mechanism that may prevail is the weak acid theory, resulting in the acidification of cytoplasm. Additionally, the acids may trigger other disorders in cell such as energy competition, intracellular anion accumulation, and inhibition or induction of the synthesis of different macromolecules. It was shown that acetic acid has an inhibitory effect against *Saccharomyces cerevisiae* (*Gao et al.*, 2019). Synthetised or hydrolysed proteinaceous compounds are also responsible for antimicrobial activities. The bacteriocins are effective against most spoilage bacteria and foodborne pathogens (*Zhang*, 2019; *Todorov & Chikindas*, 2020). The antifungal peptides derived from the hydrolysis of food proteins show an inhibitory effect against moulds (*Siedler et al.*, 2019). Competitive exclusion

as a novel antimicrobial mechanism is also associated with fungal growth inhibition. Exhaustion of manganese is an inhibitory effect of LAB against yeast and moulds (*Siedler et al.*, 2020).

LAB possess antimicrobial activity against foodborne pathogens and spoilage yeast (*Narbad & Wang*, 2018). The supernatant of LAB liquid cultures and different combinations of LAB effectively inhibited the *Escherichia coli* serotypes, what may represent a public health concern. This bacterium is involved in the faecal contamination of fermented foods and may cause foodborne diseases (*Gao et al.*, 2019). Another studied microbe was the *Bacillus cereus*, being a common food-borne pathogen that contaminates plant and dairy products. These bacterial strains are thermoduric spore formers. Toxins produced by these bacteria, such as cereulide, cytotoxin K, haemolysin BL, or non-hemolytic enterotoxin, cause food poisoning (*Laslo & György*, 2018). Two types of foodborne diseases are attributed to these bacteria: an emetic intoxication and diarrheal infection (EFSA, 2005). Different probiotic strains exert antibacterial effects on these bacteria (*Zhang et al.*, 2016).

Considering the functional aspects of LAB, these microorganisms may represent a biological alternative to the use of synthetic additives in food. The aim of the present study is to determine the antimicrobial and bacteriostatic properties of food-grade lactic acid bacteria in order to provide evidence for or confirm them as bioprotective cultures to highlight their potential as an alternative to chemical additives.

2 Materials and methods

Determination of the antagonistic activity of LAB

The antagonistic activity of LAB was analysed through growth curve analysis. We determined the effect of the selected three food-grade LAB on the growth of *Escherichia coli*, *Bacillus cereus*, and *Saccharomyces cerevisiae* and then inoculated them with different inoculum sizes. The three LAB strains were *Lactobacillus pentosus* and *Enterococcus faecalis* originated from whey and *Pediococcus parvulus* originated from sauerkraut juice. The LAB were inoculated in MRS broth and incubated at 37 °C for 48 hrs. The cell-free supernatant was recovered by centrifuge at 14000 rpm for 10 min.

The tested bacterial species, *Escherichia coli* and *Bacillus cereus* liquid culture, were grown for 12 hrs at 28 °C and inoculated in 180 μl nutrient broth with 1%, 1.5%, and 2%. Also, 50 μl of the cell-free supernatant of LAB was added, and the absorbance values at the wavelength of $\lambda = 595$ nm were

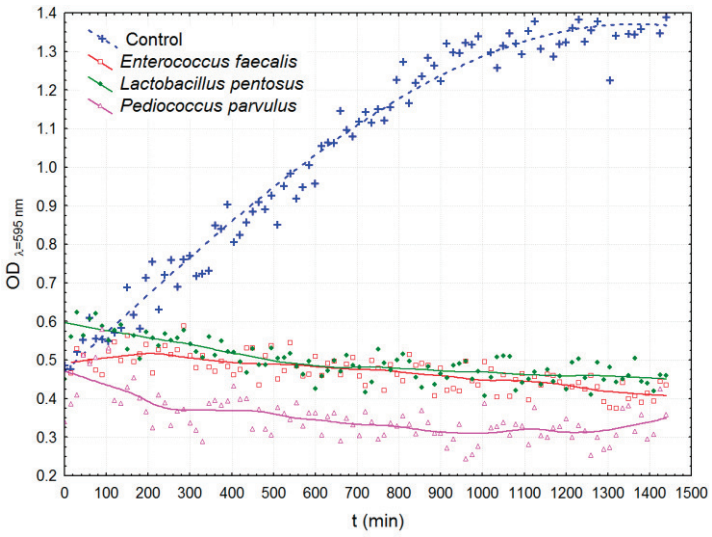
recorded by Fluostar Optima Microplate Reader (BMG Labtech, Ortenberg, Germany) in every 15 min for 25 hrs.

The tested *Saccharomyces cerevisiae* liquid culture was grown for 12 hrs at 28 °C and inoculated in 180 μl complex broth with 1%, 1.5%, and 2%. Also, 50 μl of the cell-free supernatant of LAB was added, and the absorbance values (at $\lambda = 595$ nm) were recorded by Fluostar Optima Microplate Reader every 15 min for 25 h. The measurement was repeated five times. The growth curve representation was performed with Statistica 8.0 (StatSoft, Inc., Oklahoma, USA).

3 Results and discussions

One of the beneficial effects of LAB is related to its antagonistic activity against other microorganisms. The antagonistic effects of the three different food-grade LAB was evaluated during the growth of the tested bacteria inoculated with different concentrations. In the case of *E. coli*, the used supernatants of the LAB liquid culture exerted a growth inhibition effect. The inoculation percentage affected the growth kinetics.

The LAB supernatant effect on the growth of *E. coli* inoculated with different inoculum concentrations is shown in *Fig. 1*.



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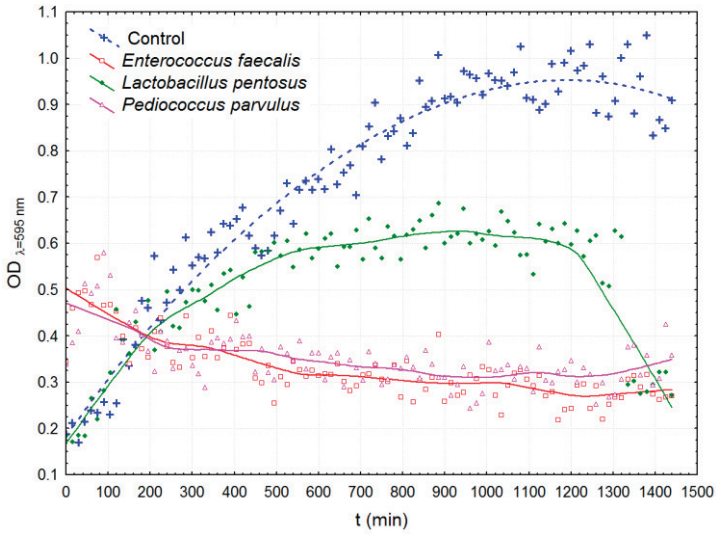

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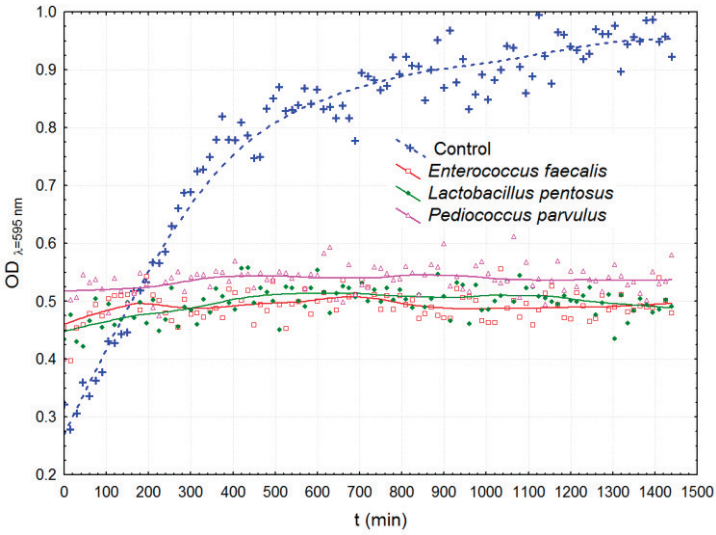
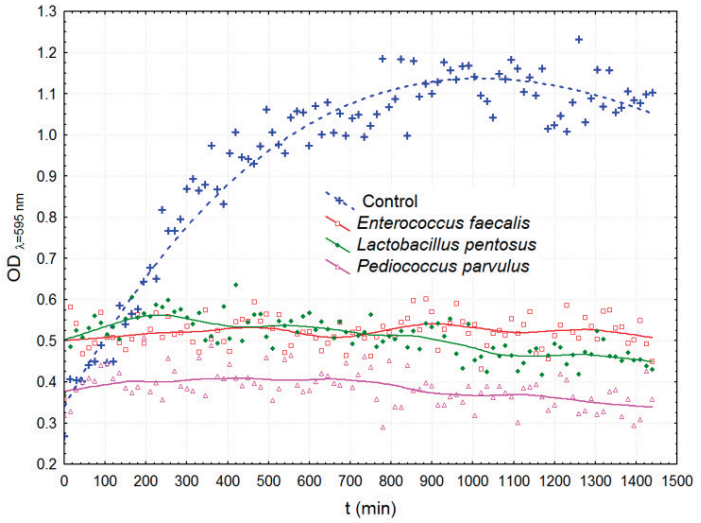


(c)

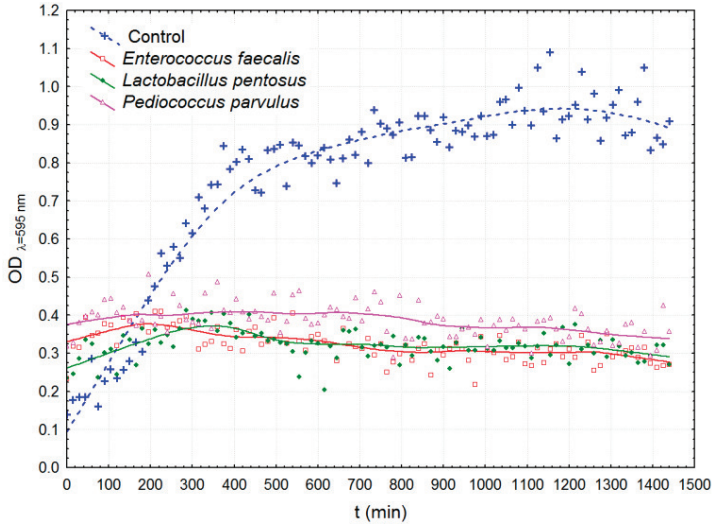
Figure 1. Growth curves of *E. coli* 1% (a), 1.5% (b), and 2% (c) in the presence of supernatants of LAB

In the presence of *Pediococcus parvulus*, the supernatant of *E. coli* with 1.5% inoculum presented a slight growth, but ultimately the death phase appeared.

The antibacterial effect was also found against *B. cereus*. The LAB supernatant effect on the growth of *B. cereus* inoculated with 1% inoculum is shown in *Fig. 2*.



(a)



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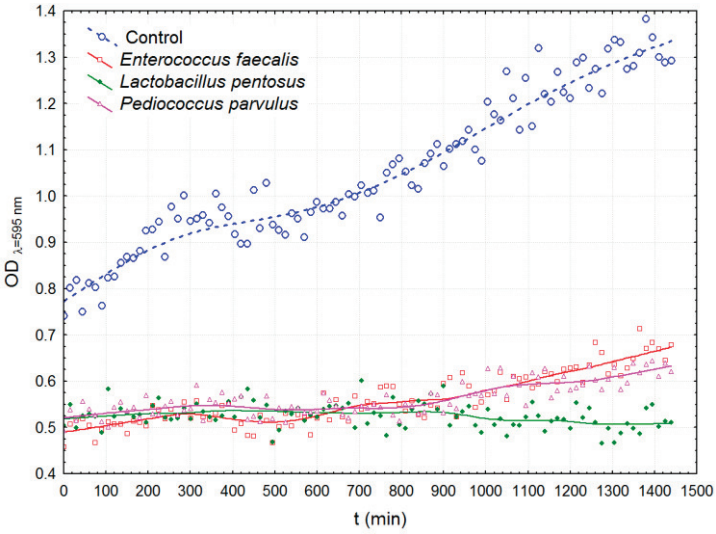


(c)

Figure 2. Growth curves of *B. cereus* 1% (a), 1.5% (b), and 2% (c) in the presence of supernatants of LAB

Lactobacillus pentosus is involved in vegetable fermentation, such as the case of olive, but it can be also detected in different traditional dairy products. Different strains of these bacteria exhibit probiotic characteristics providing health benefits (*Belicová et al.*, 2013; *Montoro et al.*, 2016).

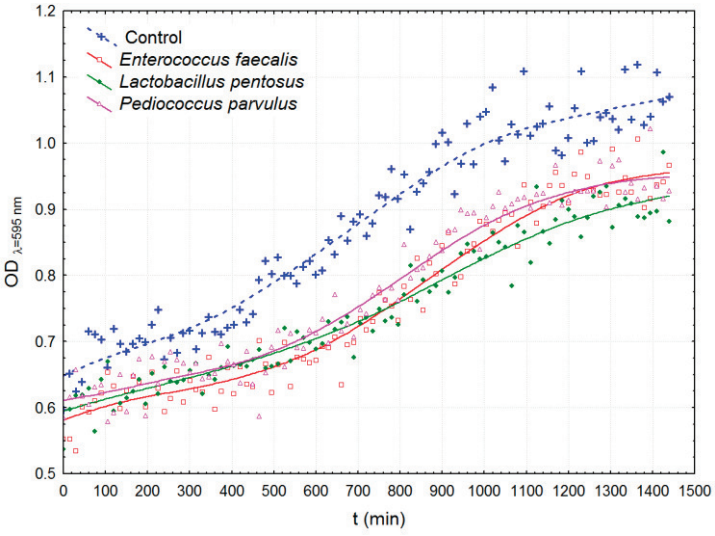
Yi et al. (2020) found that *L. pentosus* was an appropriate candidate for the biocontrol of food-borne pathogens such as *E. coli*. It has been shown that these food-grade bacteria produced antibacterial peptides. *L. pentosus* 22C originated from traditional yoghurt with a small peptide pentocin 22C production capacity, and it exerted antagonistic activity against *B. cereus* (*Motahari et al.*, 2017).

Bacterial strains belonging to genus *Enterococcus*, such as *Enterococcus faecalis*, are widespread in nature. This genus comprises pathogenic and beneficial strains too. Some species of *Enterococcus faecalis* are involved in food preservation, possessing various beneficial traits. It can be found in all types of fermented foods as adjunct starter cultures from vegetables through dairy to meat products. It was shown that these strains are able to produce enterocins, and antimicrobial peptide with an active role in the growth inhibition of food-borne pathogenic and spoilage bacteria (*Hanchi et al.*, 2018; *Baccouri et al.*,

2019). Due to antimicrobial activity, *E. faecalis* is proposed as food-grade protective bacteria in dairy industry (*Silvetti et al.*, 2014).

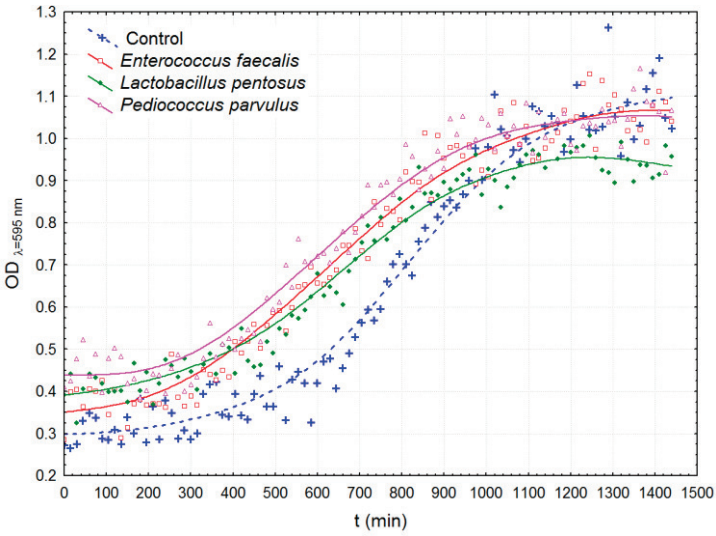
P. parvulus, an obligate homofermentative bacterium, belongs to the *Pediococcus* genus. These bacteria appear in different fermentation environments, such as wine, brewery, and meat, and plant fermentations such as olive (*Wade et al.*, 2018). *Heperkan et al.* (2014) proposed *P. parvulus* (E42) as a potential adjunct culture in traditional fermented beverage making such as boza. *Immerstrand et al.* (2010) highlighted that *P. parvulus* is a good candidate for a protective culture, and, besides the technological aspects, it exerts an antibacterial effect on *B. cereus*. Apart from the peptides, different organic compounds with antagonistic activity in LAB supernatant were identified (*Siedler et al.*, 2019).

The effect of the LAB supernatants on the growth of yeast is presented in *Fig. 3*.

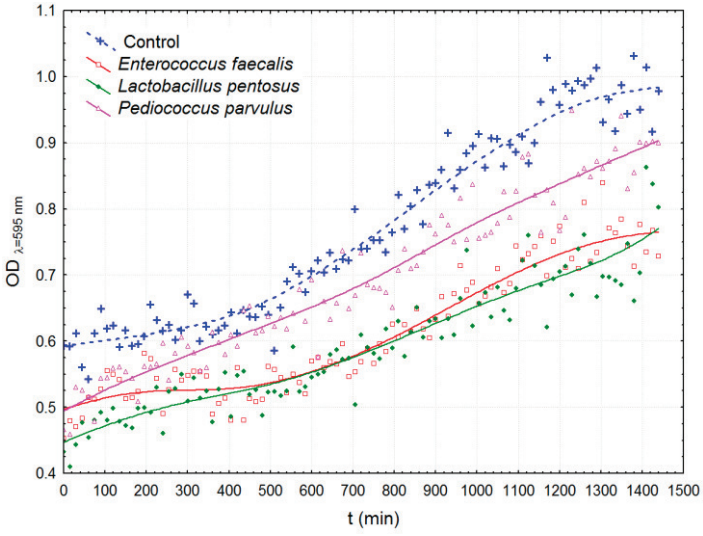


(a)

Our results show that the supernatant of the LAB does not inhibit the growth of the *Saccharomyces cerevisiae*. In the case of 1.5% inoculum, the growth was even stimulated (Fig. 3b). A similar result was found in dairy products, where the stimulated growth of *Saccharomyces boulardii* was observed and its survival was assured (*Lourens-Hattingh* & *Viljoen*, 2001).



(b)



(c)

Figure 3. Growth curves of *Saccharomyces cerevisiae* 1% (a), 1.5% (b), and 2% (c) in the presence of supernatants of LAB

Tristezza et al. (2016) revealed compatibility between *S. cerevisiae* and LAB strains during wine making. The positive effect of the yeasts on the growth of

LAB is attributed to the fact that *S. cerevisiae* favours the growth of lactic acid bacteria (*Sieuwerts et al.*, 2018). Our findings reveal the mirror effects as in this case the supernatant of LAB favoured (or did not inhibit) yeast growth. The practical use of this is the occurrence and co-cultivation of these two microbes in different fermented foods (*Ponomarova et al.*, 2017).

The differences in the mechanism of LAB activity against microbial growth have been attributed to the diversity in the gene expression or molecular structures of tested bacterial strains, which result in different traits and adaptations (*Gao et al.*, 2019).

4 Conclusions

Based on these results, the lactic acid bacterial isolates, originating from the different ecology of fermented food products, showed an antibacterial (bacteriostatic) effect against two food-borne pathogen strains. In the case of yeast, they showed compatibility. *Lactobacillus pentosus*, *Enterococcus faecalis*, and *Pediococcus parvulus* exerted an antibacterial bacteriostatic effect on *Escherichia coli* and *Bacillus cereus* growth, whereas the *Saccharomyces cerevisiae* yeast growth was not inhibited, which makes them potential agents for co-culture systems. It can be concluded that lactic acid bacterial strains from diverse fermented food ecosystems possess a bioprotective potential that may contribute to their application as adjunct culture in different cheese and vegetable fermentations.

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Antibacterial activity of plant extracts against *Listeria monocytogenes* isolated from ready-to-eat salads

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Abstract. Ready-to-eat salads are becoming more and more popular. However, due to their ingredients, they represent a suitable growth environment for different microbes. In the prevention of foodborne diseases, hygienic food preparation and appropriate storage conditions are very important. During this study, ten different ready-to-eat salads were analysed for the presence of *Listeria monocytogenes*. Five different selective agar mediums were used for the enumeration and isolation of *Listeria monocytogenes*. The isolated bacterial strains were subjected to morphological and biochemical confirmation tests. The antibacterial effects of five different freshly squeezed vegetable juices (carrots, celery, beets, horseradish, and onions) and of five essential oils (dill, thyme, oregano, lemongrass, and sage) were determined against *Listeria monocytogenes*, *Listeria innocua*, and *L. monocytogenes* strains isolated from

Keywords and phrases: ready-to-eat salad, fresh vegetable juices, essential oils

ready-to-eat salads. Based on the results obtained from fresh vegetable juices, carrot juice exerted the highest antibacterial effect, while the others showed no or slight inhibitory effect (horseradish, beets, onions) against *Listeria* species. Among the essential oils, thyme, lemongrass, and oregano showed the strongest antibacterial effect against the studied *Listeria* species.

1 Introduction

The genus *Listeria* has 17 species; six among them show high genetic relatedness: *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, *L. innocua*, and *L. marthii*. *L. monocytogenes* is pathogenic to humans and ruminants. On rare occasions, *L. ivanovii*, which is pathogenic to ruminants, may infect humans, causing foodborne outbreaks (*Bhunia*, 2018).

L. monocytogenes is a causative agent for listeriosis disease, affecting primarily the immunocompromised populations (pregnant women, neonates, human immunodeficiency virus-infected patients, and organ transplant recipients); on rare occasions, it causes gastroenteritis in immunocompetent persons (*Bhunia*, 2018). These bacteria can invade intestinal epithelial cells and multiply in phagocytic cells. They are able to enter the bloodstream, causing septicaemia or meningitis; additionally, the infection of the foetus may lead to miscarriage. The severity of listeriosis is associated with a high mortality rate, reaching 25%-30% (*Deák*, 2006).

L. monocytogenes is widely distributed in nature, can grow at 3-4 °C, and is able to survive freezing and drying temperatures in food. It enters the body through different foods, unwashed vegetables, contaminated milk, dairy products, and meat (*Deák*, 2006). Examination of the prevalence of these bacteria in fresh agricultural products revealed that *L. monocytogenes* was detected in the case of cucumbers, cabbage, carrots, tomatoes, and lettuce, while in the case of fruits it was detected in sliced apples and peaches. Thus, freshly consumed fruits and vegetables can be associated with human listeriosis (*Grumezescu & Holban*, 2018). The ability of *L. monocytogenes* to survive under extreme conditions and to form a biofilm is a food safety issue. Removal of these bacteria from food processing industries presents difficulties. Because *L. monocytogenes* forms part of the natural gut microbiota, its presence in slaughterhouses, in meat-processing factories, or in the retail trade can lead to cross-contamination. The risk of food contamination can be reduced effectively by the application of workers' hygiene and sanitation practices in food preparation plants and also by knowledge about how bacteria spread (*Kurpas*

et al., 2018). Meat and poultry products are the main carriers of *L. monocytogenes*. Among these products, the most common source are ready-to-eat (RTE) products (*Bhunia*, 2018).

RTE foods are food products that have undergone different preparation steps and can be used without any additional bactericidal treatment such as reheating. The production of RTE sandwiches, salads, and meats involves human handling (cutting or slicing), which can lead to cross-contamination. Because of the increasing demand and consumption of these types of foods and the fact that they are not further processed, the microbiological risks to consumers have also risen. The number of diseases transmitted by certain allochthonous microbes in RTE foods is on the rise; in some cases, food infection or poisoning may have fatal consequences. The genetically encoded survival mechanism of *L. monocytogenes* against a number of preservation conditions (heating, cooling, salting, pH reduction) and the high mortality rate of listeriosis highlight its importance in RTE foods. Antibiotic-resistant *Listeria* species have been detected in raw and RTE foods. *Listeria monocytogenes* isolates were resistant to ampicillin, penicillin, tetracycline, rifampicin, and sulphamethoxazole trimethoprim (*Marian et al.*, 2012).

The ecological and physiological characteristics of *L. monocytogenes* allow its colonization of food environments, and so it is able to grow and multiply during processing and storage. Their stress resistance is due to their biofilm-forming ability, and the formation of persistent cells increases their ability to survive under environmental stress conditions (*Buchanan et al.*, 2017).

The food industry currently needs innovative processing technologies and preservation methods to meet consumer demands for fresher and safer RTE products. For these purposes, the use of natural antimicrobial compounds is an alternative method. The antimicrobial and antioxidant properties of plant essential oils, phenolic and related compounds are known, and it is important to highlight their potential use in the active packaging. Antimicrobial-based food packaging systems are based on two principals: the antimicrobial agent migrates into the food and the antimicrobial agent is incorporated into the packaging material (*Siddiqui & Rahman*, 2015). From the essential oils, for example, oregano and thyme essential oil can be used as natural preservation methods due to their significant antibacterial properties (*Bhagat et al.*, 2016). Clove essential oil, whose main ingredient is eugenol, affects cell structure and causes irreversible damage to the cell membrane as well as leakage of three biological macromolecules (protein, ATP, and DNA) and may lead to the decreased activity of two intracellular enzymes (β-galactosidase and AKP). Clove oil affects the respiratory metabolism of *Listeria monocytogenes*, reduces the

activity of enzymes involved in the citrate cycle (isocitrate dehydrogenase, citrate synthase, and α-ketoglutarate), and eugenol alters the structure of DNA by forming eugenol-DNA chimeras. The minimal inhibitory concentration of clove essential oil on *Listeria monocytogenes* was 0.5 mg/ml, resulting in a 95.82% reduction after 4 hours and 99.99% after 8 hours (*Cui et al.*, 2018).

Cranberry juice concentrate was used in the preservation of RTE, which did not affect the organoleptic properties of red pepper and exhibited antibacterial activity against *Escherichia coli* O157: H7, *Listeria monocytogenes*, and *Salmonella typhimurium* (*Harich et al.*, 2017). Lemon essential oil showed good results in reducing the number of *L. monocytogenes* in fruit-based salads (*Hwang & Huang*, 2010). The aim of the present study is to determine the antimicrobial effect of five different freshly squeezed vegetable juices and of five essential oils against *Listeria monocytogenes*, *Listeria innocua*, and *L. monocytogenes* strains isolated from RTE salads on five different selective agar media.

2 Materials and methods

During our work, ten different RTE salads (*Table 1*) were analysed for the presence of *Listeria monocytogenes*.

Table 1. Ingredients of RTE salads examined for *Listeria monocytogenes*

Sample	Ingredients
1	lettuce, cucumber, tomato, onion, olive, pizza crust
2	cucumber, tomato, onion, olive, Feta-like cheese – Telemea, red paprika
3	lettuce, cucumber, tomato, onion, tuna, egg, lemon, corn, olive, pizza crust
4	lettuce, tomato, chicken meat, peas, corn, olive
5	olive, Feta-like cheese – Telemea, tomato, red paprika, cucumber, onion, lettuce, oil
6	bread cubes, yellow paprika, cucumber, tomato, chicken breasts, mayonnaise
7	lettuce, cucumber, chicken meat, cottage cheese, corn, tomato, carrot, red cabbage
8	mushrooms, olive, lettuce, cheese, carrot, tomato, red cabbage
9	tomato, cucumber, paprika, cottage cheese, spices
10	cabbage, onion, tomato, corn, carrot, lettuce

From the stock suspension (10 g sample and 90 ml physiological solution), prepared as a first step, 1-1 ml was spread onto five different selective agar media for the enumeration and isolation of *Listeria monocytogenes* (Listeria mono Differential Agar, Listeria Oxford Medium Base, Listeria mono Differential Agar Base, Listeria Identification Agar Base (Palcam), ChromoBio®Listeria Plus Base). After incubation, colonies with a morphology typical of *Listeria spp.* were isolated and streak plates were made. The isolated bacterial strains were subjected to confirmation and biochemical tests. During the microscopic morphological observations, the cell shape, motility, and Gram type with 3% KOH test of the bacterial isolates were determined. The selected *Listeria* isolates were subjected to catalase test, indole test, hydrogen sulphide production, and carbohydrate fermentation test on TSI medium. Also, the growth capacity of bacterial isolates in the presence of 16% NaCl was determined.

The next step of the research was the determination of the antibacterial effect of 5 different freshly squeezed vegetable juices (carrots, celery, beets, horseradish, onions) and 5 essential oils (dill, thyme, oregano, lemongrass, sage) against *Listeria monocytogenes*, *Listeria innocua* strains, and *L. monocytogenes* isolates from RTE salads with agar diffusion method. *Listeria monocytogenes*, and *Listeria innocua* were used for positive control.

Commercially available vegetables and essential oils used for the study were purchased from a local supermarket. The selection of the essential oils took into account that they come from herbs with culinary applications. In a sterilized Petri dish, the solidified Nutrient Agar and Listeria Enrichment Agar mediums were inoculated on the surface with a 0.1 ml suspension of the tested bacterial strains and isolates (10^8 CFU/ml). At the centre of all of the inoculated media, an 8 mm diameter hole was cut with the help of a sterile test-tube. In this hole, 0.2 ml of vegetable juice and essential oil was dropped. The incubation was carried out at the temperature of 37 °C for 24 hours. Following incubation, the inhibition zones were measured in mm.

3 Results and discussions

Based on the results, the highest number of *Listeria monocytogenes* was detected in samples 5, 7, 8, and 10, whereas the lowest occurrence of these bacteria was detected in sample 6 (*Table 2*).

Table 2. Occurrence of *Listeria monocytogenes* in analysed samples on the different selective mediums

Ready-to-eat salad samples	Listeria mono Differential Agar CFU/g	Listeria Oxford Medium Base CFU/g	Listeria mono Differential Agar Base CFU/g	Listeria Identification Agar Base (PALCAM) CFU/g	ChromoBio® Listeria Plus Base CFU/g
1	$5.7 \cdot 10^2$	$1.51 \cdot 10^3$	$6.9 \cdot 10^2$	$2.8 \cdot 10^2$	$3 \cdot 10$
2	$1.4 \cdot 10^2$	$3.2 \cdot 10^2$	$8.6 \cdot 10^2$	$9.3 \cdot 10^2$	$2 \cdot 10$
3	$1.16 \cdot 10^3$	$9.5 \cdot 10^2$	$8.5 \cdot 10^2$	$2.2 \cdot 10^2$	$1.7 \cdot 10^2$
4	$4.54 \cdot 10^3$	$6 \cdot 10$	$5.3 \cdot 10^2$	$3.73 \cdot 10^3$	< 10
5	$4.67 \cdot 10^3$	$3.66 \cdot 10^3$	$3.95 \cdot 10^3$	$4.52 \cdot 10^3$	< 10
6	< 10	$1 \cdot 10$	$2.2 \cdot 10^2$	$1.5 \cdot 10^2$	$4 \cdot 10$
7	$4.03 \cdot 10^3$	$3.02 \cdot 10^3$	$5.46 \cdot 10^3$	$4.55 \cdot 10^3$	$3 \cdot 10$
8	$3.29 \cdot 10^3$	$1.05 \cdot 10^3$	$6.43 \cdot 10^3$	$6.12 \cdot 10^3$	$4 \cdot 10$
9	$1.89 \cdot 10^3$	$3 \cdot 10$	$1.94 \cdot 10^3$	$2.21 \cdot 10^3$	$7 \cdot 10$
10	$6.17 \cdot 10^3$	$6.77 \cdot 10^3$	$6.39 \cdot 10^3$	$6.23 \cdot 10^3$	$1 \cdot 10$

From the typical *L. monocytogenes* colonies, developed on the selective agar mediums, 56 pure cultures were obtained. According to the results of morphological confirmation tests, 11 *Listeria* isolates were selected. These isolates are Gram-positive, motile rod-shaped bacteria, which are the most common characteristics of *Listeria* species. According to the results of biochemical confirmation tests, 10 out of 11 isolates possessed typical characteristics of Listeria such as glucose utilization, non-indole- and non-hydrogen sulphide production, and the ability to grow in the presence of 16% NaCl (*Table 3*).

Most bacterial isolates with typical characteristics of *Listeria monocytogenes* were isolated from Listeria Oxford Medium Base, which was found to be a highly selective medium. Regarding selectivity, this was followed by Listeria Identification Agar Base (PALCAM) and, finally, by Listeria mono Differential Agar Base.

Food-borne listeriosis has been associated with the consumption of dairy products, seafood, meat products, fresh vegetables and fruits, and RTE foods. Among RTE foods, raw foods by non-thermal processing (salads, vegetables, fruits, dairy products) pose an increased health risk to consumers. For food-producers, *Listeria monocytogenes* represents a challenge in this context and a priority because it is widely distributed in nature and is able to grow at low temperatures (*Ziegler et al.*, 2019). Because of the changes in lifestyle, RTE foods are still prominent. Consuming these foods raw or minimally processed, *L. monocytogenes* may be present due to their high survival rate, psychrophilic

character, ability to form biofilm in food-processing equipment, and resistance to most disinfectants (*Szymczak et al.*, 2020). Regulation of *L. monocytogenes* in RTE foods differs from country to country, ranging from minimum level as zero tolerance (0 CFU in 25 g) for all RTE foods to maximum level (100 CFU/g) for foods which do not promote growth (*Dong et al.*, 2021).

Table 3. Biochemical confirmation test results of *Listeria* isolates

Bacterial isolate	**Glucose utilization**	**Hydrogen sulphide production**	**Indole production**	**Growth in the presence of 16% NaCl**
Li 1 LOM	+	-	-	+
Li 2 LMDAB	+	-	-	+
Li 3-1 LOM	+	-	-	+
Li 3-2 LOM	+	-	-	+
Li 4 LOM	+	-	-	+
Li 7 LOM	+	-	-	+
Li 9-1 PA	+	-	-	+
Li 9-2 PA	+	-	-	+
Li 9-1 LOM	+	-	-	+
Li 9-2 LOM	-	-	-	+
Li 10 PA	+	-	-	+

Notes: Li: *Listeria*; 1, 2, 3, 4, 7, 9, 10: RTE salad sample numbers; LOM: Listeria Oxford Medium Base; LMDAB: Listeria mono Differential Agar Base; PA: Listeria Identification Agar Base (PALCAM)

Analysing the ingredients of various salads, *Listeria sp.* was detected in marinated and smoked fish, cabbage, carrots, and dairy products (e.g. Feta cheese) (*Szymczak et al.*, 2020). In an outbreak of listeria infection in a hospital, as a vehicle for *L. monocytogenes* contamination celery, an ingredient of chicken salad, was mentioned (*Sahu et al.*, 2017). Different factors significantly influence the growth of *L. monocytogenes*, such as the food matrix, storage temperature, or storage time. Reduction of the storage temperature in the market to 5 °C coupled with the product's shelf life could contribute to reducing the risk of *L. monocytogenes* in RTE salads (*Ziegler et al.*, 2019). The duration of the LAG phase can be influenced by the pH value of mayonnaise, for example, in seafood salad; however, the most important factor influencing the rate of reproduction is storage temperature (*Skalina & Nikolajeva*, 2010). The presence of low levels of *L. monocytogenes* in sample 6 may be associated with the sample ingredients. This RTE salad contained less raw ingredients and contained mayonnaise, pickled cucumber, toasted bread cubes,

and cooked chicken breast.

Based on the results of the antimicrobial activity of freshly squeezed vegetable juice, celery had no antibacterial effect against the tested *Listeria* species. Beet juice had no inhibitory effect on the growth of *L. monocytogenes* and *L. innocua*. In the case of *Listeria* isolates, a small zone of inhibition could be observed (*Table 4*).

White onion juice showed a slight antibacterial effect against five *Listeria isolates*; no zone of inhibition was found in the case of the other tested bacteria. Horseradish juice exerted slight inhibition on most *Listeria* isolates, but no inhibition was detected against *L. monocytogenes* and *L. innocua*. According to numerous studies, carrot juice possesses an antimicrobial effect against *L. monocytogenes* and other *Listeria* species (*Deák*, 2006). Based on our experiments, an inhibition zone was detected against *L. monocytogenes*, *L. innocua*, and all tested *Listeria isolates*.

Among the studied essential oils, the strongest antimicrobial effect was shown by thyme and lemongrass, followed by oregano (*Table 5*). Sage essential oil exhibited a small inhibition zone, but some *Listeria* isolates originated from fresh salads were more sensitive than laboratory strains. Dill essential oil did not inhibit *L. monocytogenes*, and in the case of *L. innocua* a small zone of inhibition was found (1.50 ± 0.52). Among the bacterial isolates, there were susceptible strains where complete inhibition also occurred.

Thymus vulgaris essential oil presented inhibitory activity against pathogenic bacteria *S. aureus* and *L. monocytogenes*, which are often associated with fresh and low-ripened cheese (*Julliane de Carvalho et al.*, 2015). In particular, cinnamon and oregano showed strong activity against seven out of ten *L. monocytogenes* strains although they showed a lower efficacy against *Salmonella* strains (*Mazzarrino et al.*, 2015). *Melissa officinalis* has an antimicrobial effect against *Bacillus subtilis*, *Clostridium botulinum*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhimurium*, and *Staphylococcus aureus* (*Tajkarimi et al.*, 2010). The essential oil of *Salvia officinalis* showed strong bactericidal and bacteriostatic effects against both Gram-positive and Gram-negative bacteria. Among Gram-positive pathogens, *Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis*, *Enterococcus faecalis*, *Listeria monocytogenes*, and *Staphylococcus epidermidis* show high sensitivity to *S. officinalis* (*Ghorbani & Esmaeilizadeh*, 2017). Essential oils of *Apium graveolens* showed antimicrobial activity against *Saccharomyces cerevisiae*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella sp.*, and *Escherichia coli* (*Gupta et al.*, 2012).

Table 4. The effect of the vegetable juice on the growth of the studied bacteria (inhibition zone in mm, average ± S.D., n = 10)

Studied bacteria	**Celery**	**Beets**	**Onions**	**Horseradish**	**Carrots**
Listeria monocytogenes	No inhibition	5.80 ± 0.91			
Listeria innocua	No inhibition	No inhibition	No inhibition	No inhibition	4.40 ± 0,69
Li 1 LOM	No inhibition	2.60 ± 0.61	No inhibition	No inhibition	7.00 ± 1.15
Li 2 LMDAB	No inhibition	3.35 ± 0.74	2.35 ± 0.41	3.25 ± 0.58	9.00 ± 1.33
Li 3-1 LOM	No inhibition	2.43 ± 1.15	2.15 ± 0.41	4.85 ± 1.00	9.10 ± 2.99
Li 3-2 LOM	No inhibition	1.65 ± 0.74	No inhibition	No inhibition	5.70 ± 0.67
Li 4 LOM	No inhibition	3.00 ± 0.66	3.25 ± 0.85	2.70 ± 0.53	6.30 ± 2.05
Li 7 LOM	No inhibition	1.92 ± 0.44	No inhibition	4.00 ± 0.78	6.10 ± 1.37
Li 9-1 PA	No inhibition	2.35 ± 0.47	2,00 ± 0,23	2.70 ± 0.34	2.50 ± 0.47
Li 9-1 LOM	No inhibition	2.25 ± 0.58	3.45 ± 0.55	3,60 ± 0.93	8.80 ± 2.34
Li 10 PA	No inhibition	2.20 ± 0.94	No inhibition	3.55 ± 0.79	8.10 ± 1.79

Table 5. The effect of the essential oils on the growth of the studied bacteria (inhibition zone in mm, average ± S.D., n = 10)

Studied bacteria	**Dill**	**Lemongrass**	**Sage**	**Oregano**	**Thyme**
Listeria monocytogenes	No inhibition	21 ± 1.41	2.15 ± 0.62	19.9 ± 1.10	Total inhibition
Listeria innocua	1.50 ± 0.52	Total inhibition	4.90 ± 0.56	Total inhibition	Total inhibition
Li 1 LOM	Total inhibition	20.81 ± 1.47	No inhibition	Total inhibition	Total inhibition
Li 2 LMDAB	10.1 ± 1.85	Total inhibition	19.7 ± 0.82	19.1 ± 1.28	25.8 ± 1.22
Li 3-1 LOM	15.7 ± 4.59	20.4 ± 2.01	5.20 ± 0.91	18.3 ± 1.05	25.9 ± 3.28
Li 3-2 LOM	Total inhibition	15.5 ± 0.97	22.1 ± 2.92	26.4 ± 2.01	Total inhibition
Li 4 LOM	7.6 ± 2.36	24 ± 4.39	5.30 ± 0.48	20.9 ± 2.46	29.9 ± 3.57
Li 7 LOM	6.5 ± 1.77	Total inhibition	26.5 ± 2.79	22.6 ± 2.11	18.8 ± 1.03
Li 9-1 PA	9.5 ± 2.36	Total inhibition	12.8 ± 2.09	16.6 ± 1.64	Total inhibition
Li 9-1 LOM	6.3 ± 2.26	26.2 ± 2.25	14.0 ± 2.94	20.9 ± 2.84	24.4 ± 2.06
Li 10 PA	7.6 ± 2.27	Total inhibition	23.4 ± 1.77	17.8 ± 1.98	22.6 ± 2.11

4 Conclusions

As ready-to-eat salads contain many raw ingredients, the presence of *Listeria monocytogenes* needs to be taken into account. Therefore, it is very important to maintain hygiene during the processing of raw materials and manufacture and to ensure adequate storage conditions throughout the shelf life. Among the selective media used in our investigation, the highly selective medium for the isolation of *Listeria monocytogenes* was found to be the Listeria Oxford Medium Base. Results from our study demonstrated that carrot juice exerted the highest antibacterial effect on the *Listeria* species. Among the essential oils, thyme, lemongrass, and oregano showed the strongest antimicrobial effect against *L. monocytogenes*, *L. innocua*, and *Listeria* isolates originated from salads. The use of natural antimicrobials (fresh vegetable juices or essential oils), which can also be used for gastronomic purposes, can contribute to the production of safe and healthy food.

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