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ORIGINAL PAPER

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BIO-PRESERVATION OF CHOCOLATE MOUSSE WITH FREE AND IMMOBILIZED CELLS OF *LACTOBACILLUS PLANTARUM* D2 AND LEMON (*CITRUS LEMON* L.) OR GRAPEFRUIT (*CITRUS PARADISI* L.) ZEST ESSENTIAL OILS

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ABSTRACT

Background. The bio-preservation of food products using various natural ingredients and metabolites from various types of beneficial microorganisms released during targeted fermentation is a method that simultaneously has a preservative effect on the food product and provides a model of its composition in order to ensure its functional and health properties. This double effect can be achieved by incorporating ingredients with proven preservative and functional effects into the food product, such as essential oils from various plant species and probiotic bacteria. The aim of the present research was to study the synergistic effect of selected probiotic lactic acid bacteria (LAB) and essential oils with high antimicrobial activity against pathogenic and spoilage microorganisms for the bio-preservation of chocolate mousse food emulsion.

Materials and methods. The susceptibility of the selected probiotic strain *Lactobacillus plantarum* D2 to different concentrations of the selected lemon or grapefruit essential oil was examined using the disc-diffusion method. Nine chocolate mousse variants were prepared with the inclusion of free or immobilized cells of the probiotic strain *L. plantarum* D2 and/or lemon or grapefruit zest essential oils. The chocolate mousse variants were stored for 20 days in refrigerated conditions, and changes in the concentration of viable lactobacilli cells, the pH and the microbiological purity were monitored in accordance with standard requirements by taking samples on the 0^{th}, 5^{th}, 10^{th}, 15^{th}, and 20^{th} days of storage. An organoleptic evaluation of the chocolate variants was performed on the 0^{th} day.

Results. Concentrations of up to 1% lemon or grapefruit essential oil did not affect the growth of the probiotic strain *L. plantarum* D2, which revealed opportunities for their joint application for the bio-preservation of food emulsions. The obtained chocolate mousse variants were characterized by preserved organoleptic characteristics and microbiological safety. Free or immobilized probiotic *L. plantarum* D2 cells applied alone or in combination with lemon or grapefruit essential oils provided bio-preservation of the food emulsions, maintaining a high concentration of viable cells (10^6–10^7 cfu/g) during storage under refrigerated conditions for 20 days.

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Conclusion. The combined application of free or immobilized probiotic LAB and lemon or grapefruit essential oils resulted in better bio-preservation results than in the use of probiotic LAB or essential oils alone, thus suggesting a synergistic effect between the two bio-preservative agents. Moreover, the obtained chocolate mousse emulsions can be classified as functional foods and the chocolate mousse food matrix can successfully be used as a vehicle for delivery of probiotic LAB to a wide range of food consumers. The obtained results and the developed successful bio-preservation strategy for the production of chocolate mousse food emulsions would provide grounds for the future selection of other probiotic lactobacilli strains, essential oils and synergistic combinations of them for the development of successful bio-preservation strategies for other types of food and beverage products.

Keywords: bio-preservation, probiotic, *Lactobacillus plantarum*, essential oil, synergistic combination

INTRODUCTION

Bio-preservation is a method of preserving food products that controls the number of pathogenic or spoilage microorganisms and/or increases the shelf life of products using biomolecules from animal (lysozyme, lactoferrin), plant (essential oils and plant extracts) or microbial (e.g. nisin) origin or microorganisms (most commonly LAB) that produce various metabolites with high antimicrobial activity. Each perishable food has its own specific features which require the corresponding specifics of bio-preservation, i.e. successful approaches to bio-preservation of one food product cannot be directly extrapolated to another food product, but a serious adaptation of the approach is needed depending on the specific features of the specific food product. Therefore, one of the prerequisites for the development of a successful bio-preservation technology is a knowledge of the microbial ecosystem of the respective product during its storage. Changes in the organoleptic profile of the product, including its storage, are also important for the bio-preservation process (Kostov et al., 2020).

Currently, probiotic foods are being marketed as sources of bacteria that benefit overall human health. Generally, probiotics are incorporated into dairy products and fruit juices (Possemiers et al., 2010). Confectionery products provide consumers with calories and sweetness (organoleptic properties), but they usually do not have any added value. The development of new technologies facilitating the supplementation of confectionery with probiotic LAB can lead to the development of novel functional confectionery products, enriched with health-promoting ingredients. As confectionery products are consumed not only by adults but also by children and teenagers, their supplementation with live probiotic LAB is advisable. The basic criterion of a quality evaluation of this type of product should be the maintenance of viability of the probiotic LAB cells at a functional level during technological processes and throughout the storage period. The sensory attributes are also of great importance for the acceptance of the newly developed products by consumers. Therefore, maybe it would be advisable for these products to have the same sensorial characteristics as the traditional LAB-free ones (Żyzelewicz et al., 2010). Chocolate is believed to have a higher lipid content than cocoa butter (Lahtinen et al., 2007), which could protect and preserve probiotics (Ramli et al., 2012). The incorporation of probiotics into chocolate could offer a wonderful alternative to common dairy products and allow the health claims of chocolate-based food products to be broadened. In spite of the inherent high fat and sugar contents of chocolate, its consumption provides the body with antioxidants, predominantly polyphenols, including flavonoids such as epicatechin, catechin, and procyanidins (Gadhiya et al., 2015; Hii et al., 2009; Kruszewski and Obiedziński, 2018). Maillard and Landuyt (2008) found that the incorporation of two selected specific LAB strains with documented probiotic properties into chocolate showed very good results because the probiotic strain characteristics, combined with the appropriate incorporation methodology and a suitable protective micro-encapsulation technology allowed the specific difficulties of including probiotics

in the composition of cocoa and chocolate derivatives to be overcome. Moreover, they performed quantitative and qualitative analysis of the selected probiotic strains and gut microflora evolution during the digestion process by an *in vitro* model of the human digestive tract, and the examination demonstrated that chocolate can actually be an ideal carrier for the intestinal delivery of probiotics.

The development of chocolate and chocolate-based foods enriched with probiotics requires a good understanding of the selected probiotic strains, the chocolate food manufacturing process, and various critical points of the process that might negatively affect the probiotics' survival, as well as the application of specific protective technology to increase probiotic survival during food production and storage (Gadhiya et al., 2015). Novel technologies have been developed and implemented, such as specific microencapsulation methods, in order to increase probiotic bacterial resistance and broaden the applications of probiotic bacteria from fresh dairy products to long shelf life, processed food products.

Mousse is an aerated dessert with a stabilized foamy structure that, although traditionally homemade, is nowadays produced on an industrial scale and is gaining space in the dessert market.

The chocolate mousse food emulsion is sensitive to microbial spoilage, as the aqueous phase of these products is a suitable environment for the growth of unwanted microorganisms. Controlling the growth of unwanted microorganisms, probiotic LAB and essential oils can give products antioxidant capacity as well as affecting their health benefits. For example, LAB improve the gastrointestinal microflora (Kerry et al., 2018) and skin microflora (Tkachenko et al., 2018); phytonutrients of plant extracts such as polyphenols, carotenoids, sterols and polyunsaturated fatty acids play an important role in the prevention of some diseases (Nikmaram et al., 2018). Some components of essential oils have anti-inflammatory, detoxifying, antioxidant and hormone-balancing effects, thus having beneficial effects on human health (Mariutti et al., 2011). The simultaneous application of microorganisms and essential oils can hypothetically have a synergistic effect on the bio-preservation of a given food product, and can attribute certain functional properties, but each combination of bio-preservative agents and microorganisms for a given food must be systematically tested to determine the relevant minimum processing parameters.

Plant extracts and probiotic bacteria have significant *in vitro* potential (antimicrobial and/or antioxidant), which can be used for the successful bio-preservation of various food products, including food emulsions. The lemon (*Citrus lemon* L.) zest essential oil and the grapefruit (*Citrus paradisi* L.) zest essential oil used for the preparation of chocolate mousse variants have exhibited antimicrobial activity against saprophytic (*Bacillus subtilis*, *Aspergillus niger*, *Aspergillus flavus*, *Penicillium chrysogenum*, *Fusarium moniliforme*) and pathogenic microorganisms (*Escherichia coli*, *Salmonella abony*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans*) (Denkova et al., 2020). The selected probiotic strain *L. plantarum* D2 has demonstrated significant antimicrobial activity against *E. coli*, *Salmonella* sp. and *S. aureus* examined by co-cultivation of *L. plantarum* D2 with each of the six pathogens (2 strains from each pathogen species or genus) – there were no living cells of the pathogens at the 60th hour of the co-culturing of each pathogen with *L. plantarum* D2 (Denkova-Kostova et al., 2018; Teneva et al., 2015; Teneva et al., 2017).

The aim of the present study was to study the synergistic effect of selected probiotic LAB and essential oils with high antimicrobial activity against pathogenic and spoilage microorganisms for the bio-preservation of chocolate mousse food emulsion.

MATERIALS AND METHODS

Essential oils

Essential oils – lemon (*Citrus lemon* L.) and grapefruit (*Citrus paradisi* L.) zest essential oils were used to conduct the experiments in the present study. The oils were obtained from the dried zest of *C. lemon* L. (0.0176 g EO/g DW) and *C. paradisi* L. (0.0158 g EO/g DW), respectively. 250 g from each plant material were ground and passed through a 0.5 mm sieve. The moisture content analyzed by AOAC 934.06 was established to be 1.9% for grapefruit and 1.8% for lemon (AOAC, 2007). The essential oils were extracted by aqueous distillation in a laboratory glass apparatus according to the British Pharmacopoeia (1999). The ground plant material was processed using

the following parameters: plant material:water ratio = 1:20; plant material:flask volume ratio = 1:100, a frequency of 6% and duration of 300 min. The distillation rate was maintained evenly at the beginning of the process for 5 or 10 minutes and in the middle and at the end of the process the quantity of the obtained essential oil and the distillation water were recorded every 20–30 minutes. The end of the distillation was reached when two consecutive measurements did not show an increase in the essential oil amount. The experiments were performed in triplicate.

Microorganisms

The strain *L. plantarum* D2, isolated from mayonnaise food emulsion, with proven probiotic properties (Denkova-Kostova et al., 2018; Teneva et al., 2015; Teneva et al., 2017) was used in the present study.

Media

LAPTg10-broth (Teneva et al., 2015). LAB grow in this medium. The inoculated medium was incubated at 37 ±1°C for 18 hours under microaerophilic conditions to obtain LAB culture in the exponential growth phase to be used for the immobilization procedure or for the determination of the susceptibility of *L. plantarum* D2 to different concentrations of lemon and grapefruit essential oils.

LAPTg10-agar (Teneva et al., 2015). LAB grow on this medium. The inoculated Petri dishes with the medium were incubated at 37 ±1°C for 48–72 hours under microaerophilic conditions to determine the concentration of viable lactobacilli cells.

Saline solution with Tween 80 (Teneva et al., 2015). This was used for the preparation of appropriate tenfold dilutions of the chocolate mousse variant samples.

Methods of analysis

Determination of the susceptibility of *L. plantarum* D2 to different concentrations of lemon and grapefruit essential oils – disc-diffusion method. LAPTg10-agar plates were spread plated with an overnight suspension of the probiotic strain, grown in LAPTg10-broth for 18 hours at 37 ±1°C, with a concentration of viable cells of 10^7 cfu/ml. Meanwhile, decimal dilutions of the essential oils in saline solution containing 1% (v/v) Tween 80 were prepared. The experiments were conducted with dilutions of 10^0, 10^{-1}, and 10^{-2}. Two hours after inoculation of the plates, paper discs (6 mm in diameter) were placed on the surface of the agar medium. Six µL of the corresponding dilution were pipetted onto the corresponding paper discs. Paper discs soaked in distilled water were used as blanks. The results were recorded as diameters of the clear zones around the paper discs, in millimeters, after 24–48 h of incubation of the Petri dishes at an optimal temperature for the growth of *L. plantarum* D2 – 37 ±1°C for 24–48 h. The experiments were performed in triplicate. The mean values and the standard deviations were calculated using MS Office Excel 2013 (Teneva et al., 2019).

Preparation of chocolate mousse. Chocolate mousse (CM) was prepared from 2 main ingredients: chocolate with a cocoa content of 52% (Fin Carre, Lidl) and cream (22% fat) (Milbona, Lidl). 400 g of chocolate was melted in a water bath at a temperature of 65ºC, stirring periodically and taking care that the internal temperature did not exceed 45ºC. Then the melted chocolate was allowed to cool, taking care for it not to harden. Meanwhile, 600 ml of cream was whipped using a mixer. Slightly cooled chocolate was added to the whipped cream in small portions, stirring constantly to achieve the desired texture. The chocolate mousse thus obtained was divided into portions of 50 g each, leaving 1 portion for a control sample, and the pre-selected essential oil (EO; lemon EO or grapefruit EO) and the probiotic strain in the form of free or immobilized cells were added to the other portions according to the following scheme (Table 1).

The concentrations of viable *L. plantarum* D2 cells in the used free cell suspension and the immobilized suspension were 5×10^{10} cfu/ml and 2×10^{10} cfu/ml, respectively.

The resulting chocolate mousses were stored in refrigerated conditions (0°C to +4°C) for 20 days. All chocolate mousse variants were analyzed on the 0^{th}, 5^{th}, 10^{th}, 15^{th} and 20^{th} days of storage. On each sampling day, microbiological analysis (determination of the concentration of viable lactobacilli cells, determination of the microbiological parameters) and physico-chemical analysis (pH) were carried out.

Table 1. Chocolate mousse variants composition

No	Chocolate mousse variant
1	Control chocolate mousse (CCM)
2	CCM + 0.25 ml of grapefruit EO
3	CCM + 0.25 ml of lemon EO
4	CCM + 0.25 ml of grapefruit EO + 0.25 ml of free cells of *L. plantarum* D2
5	CCM + 0.25 ml of grapefruit EO + 0.25 ml of immobilized cells of *L. plantarum* D2
6	CCM + 0.25 ml of lemon EO + 0.25 ml of free cells of *L. plantarum* D2
7	CCM + 0.25 ml of lemon EO + 0.25 ml of immobilized cells of *L. plantarum* D2
8	CCM + 0.25 ml of free cells of *L. plantarum* D2
9	CCM + 0.25 ml of immobilized cells of *L. plantarum* D2

Immobilization of LAB in an emulsion. A fresh 24 h culture of *L. plantarum* D2 was centrifuged for 15 min at 5000×g, the supernatant was removed, and the biomass sludge was washed once with saline solution. The biomass sludge was suspended to the initial volume with saline solution and was added to 20 ml of 4% alginate solution. 0.2% Tween 80 and 20 ml of 4% alginate solution with LAB were added to 100 ml of plant oil, the mixture was homogenized well with a magnetic stirrer for 10–15 minutes. 100–200 ml of 2% $CaCl_2$ solution was added to the mixture in portions. The emulsion was stirred for 30 minutes to the gelation of the alginate, then centrifuged for 15 minutes at 5000×g. The sludge was washed once with saline solution. The obtained immobilized preparation was mixed with 1–2 ml of saline solution and added to the respective chocolate mousse variant. The concentration of viable *L. plantarum* D2 cells in the immobilized suspension was 2×10^{10} cfu/ml (Bigdelian and Razavi, 2014).

Determination of the concentration of viable lactobacilli cells – spread plate method (Frank and Yousef, 2004; Denkova et al., 2013).

Determination of microbiological parameters of chocolate mousse: mesophilic aerobic and facultative-anaerobic bacteria, according to BDS EN ISO 4833:2004; yeasts and molds, according to BDS EN ISO 21527-2:2011; *Escherichia coli*, according to ISO 16649-2:2001; *Salmonella* sp., according to BDS EN ISO 6579:2003; coagulase-positive staphylococci, according to BDS EN ISO 6888-1:2005 + A1:2005.

Organoleptic evaluation. Chocolate mousse variants were rated by 10 specialists in the field of food based on 6 indicators, ranging from 0 (worst rating) to 9 (best rating): appearance, characteristic odor, color, taste, aftertaste and texture.

Processing the results

The data from the triplicate experiments were processed with MS Office Excel 2010 software, using statistical functions to determine the standard deviation and maximum estimation error at a significance level of $\acute{\alpha} < 0.05$.

The statistical processing of the results was performed with Statgraphics Centurion XV Trial version, with algorithms embedded in the software itself.

RESULTS AND DISCUSSION

The susceptibility of the probiotic strain *L. plantarum* D2 to different concentrations of lemon (*Citrus lemon* L.) or grapefruit (*Citrus paradisi* L.) zest essential oils was studied. Experimental data show that the growth of *L. plantarum* D2 was not affected by the studied essential oils in concentrations up to 1%. This in turn shows that the selected probiotic strain can be used in the preparation of food emulsions, along with lemon or grapefruit zest essential oils.

Chocolate mousse variants with free or immobilized cells of the probiotic strain *L. plantarum* D2 and/or lemon or grapefruit essential oils were prepared. There was little change in the pH values during the storage of all chocolate mousse variants (Table 2). In chocolate mousses preserved only with essential oils, the pH was maintained almost constantly, while in chocolate mousses preserved with *L. plantarum* D2 and essential oils, the pH decreased slightly. The pH values for all the chocolate mousse variants (including the control) were commensurable at the end of the storage period (pH being around pH = 6), demonstrating that the addition of the essential oils and/or probiotic *L. plantarum*

Table 2. Changes in the pH of the chocolate mousse variants

No	Chocolate mousse variant	Days				
		0	5	10	15	20
1	Control chocolate mousse (CCM)	6.080 ±0.042	6.070 ±0.037	6.180 ±0.051	6.110 ±0.022	6.165 ±0.040
2	CCM + grapefruit EO	6.055 ±0.075	6.075 ±0.067	6.105 ±0.043	5.980 ±0.025	6.170 ±0.031
3	CCM + lemon EO	5.965 ±0.063	6.110 ±0.023	6.020 ±0.045	6.075 ±0.047	6.235 ±0.032
4	CCM + grapefruit EO + free cells of *L. plantarum* D2	5.875 ±0.055	6.335 ±0.044	5.925 ±0.051	5.870 ±0.040	6.190 ±0.027
5	CCM + grapefruit EO + immobilized cells *L. plantarum* D2	5.905 ±0.018	6.330 ±0.036	6.015 ±0.069	6.005 ±0.064	6.070 ±0.014
6	CCM + lemon EO + free cells of *L. plantarum* D2	5.715 ±0.029	6.310 ±0.039	5.810 ±0.057	5.970 ±0.019	5.975 ±0.024
7	CCM + lemon EO + immobilized cells of *L. plantarum* D2	5.810 ±0.037	6.265 ±0.027	5.830 ±0.037	5.915 ±0.055	6.095 ±0.033
8	CCM + free cells of *L. plantarum* D2	5.765 ±0.066	6.170 ±0.065	5.945 ±0.071	5.805 ±0.054	6.120 ±0.050
9	CCM + immobilized cells of *L. plantarum* D2	5.800 ±0.041	6.280 ±0.066	5.890 ±0.058	5.950 ±0.020	6.075 ±0.087

D2 cells did not affect the physico-chemical parameter of the finished chocolate mousses. These results were in contrast with the research results of Aragon-Alegro et al. (2007), who reported that the probiotic chocolate mousse variant (with free probiotic *L. paracasei* cells) had significantly lower pH values when compared to the control, probably due to the presence of *L. paracasei*. According to Beresford et al. (2001), the optimal pH for growth of the most common bacteria is near to neutral, while their growth is suppressed at pH values below pH = 5.0. Thus, the slight fluctuations in the pH values in all chocolate mousse variants were not sufficient to impair the survival of the probiotic microorganism present in the chocolate mousse.

An analysis of the variables (ANOVA) for the changes in pH was performed, both between the individual chocolate mousse variants (compared to the control and compared with each other) and with respect to the storage time of the chocolate mousse variants (results not shown).

Regarding the statistical difference between the different chocolate mousse variants, the ANOVA shows that no statistically significant difference was found between the different variants. The *F*-ratio, which in this case equals 0.639536, is a ratio of the between-group estimate to the within-group estimate. Since the *P*-value of the *F*-test is greater than or equal to 0.05, there is not a statistically significant difference between the means of the 9 variables at the 95.0% confidence level.

A statistically significant difference was observed in terms of storage time. The data show that the difference becomes apparent between the 5th and 10th day after the preparation of the chocolate mousse variants. Within each column, the levels containing X's form a group of means within which there are no statistically significant differences. The method currently being used to discriminate between the means is Fisher's least significant difference (LSD) procedure. With this method, there is a 5.0% risk of calling each pair of means significantly different when the actual difference equals 0.

As the storage time is included in the study, a more detailed statistical analysis is inappropriate, as the pH values do not change significantly within 1 or 2 days.

In the three chocolate mousse variants with free probiotic *L. plantarum* D2 cells, there was a slight decrease in the concentration of viable lactobacilli cells from the 0th to the 5th day of refrigerated storage in chocolate mousse variants with grapefruit essential oil and free probiotic cells, while in the chocolate mousses

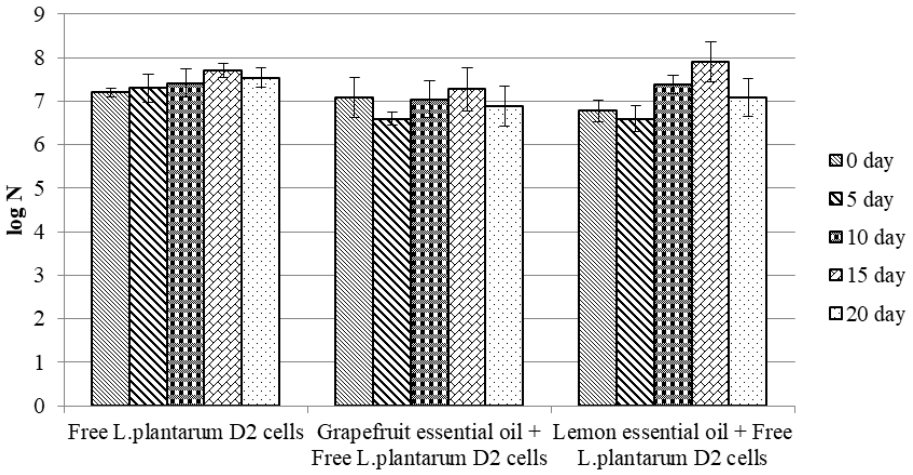

Fig. 1. Changes in the concentration of viable lactobacilli cells of the chocolate mousse variants bio-preserved with free cells of *L. plantarum* D2. Log *N* is $\log_{10} N$, where *N* is the number of viable *L. plantarum* cells, cfu/g

with free lactobacilli cells or free lactobacilli cells and lemon essential oil the number of living probiotic cells was maintained (Fig. 1). After that the concentration of viable lactobacilli cells in all three variants with free *L. plantarum* D2 cells increased up to the 15th day, followed by a slight decrease by the 20th day of storage. The final concentration of living probiotic lactobacilli cells in these 3 variants at the end of the storage period was 10^7 cfu/g (Fig. 1). These results are consistent with the research results by Aragon-Alegro et al. (2007) who reported that *L. paracasei* maintained constant populations, always above 7 log cfu/g, during the whole refrigerated storage of the probiotic and symbiotic chocolate mousses.

In the three chocolate mousse variants with immobilized probiotic *L. plantarum* D2 cells, there was an increase in the concentration of living probiotic cells from the 0th to the 5th day of refrigerated storage, followed by a decrease in the number of viable cells up to the end of the storage period. The difference in the concentration of viable lactobacilli cells between the 0th and the 20th day in these variants was around 1 log *N* (Fig. 2). Borges, Ferreira, and Costa (2004) evaluated the survival of *Lactobacillus acidophilus*, microencapsulated in a calcium alginate matrix, in chocolate mousse. The author's verified that there was a 2 log decrease in counts of the probiotic microorganism after 20 days of storage of the product when immobilized cells of the microorganism were employed. The obtained results for all chocolate mousse variants with free or immobilized probiotic *L. plantarum* D2 cells indicate good maintenance of viability of the probiotic strain in the probiotic and symbiotic chocolate mousses. According to Boylston et al. (2004), the recommended level of probiotic microorganisms in a food at the time of consumption in order to have beneficial effects on consumer's health is 10^6 cfu/g. Thus, the produced chocolate mousse variants with free or immobilized *L. plantarum* D2 cells in the present study can be considered an ideal vehicle for delivery of sufficient living probiotic *L. plantarum* D2 cells to the consumer. The results of the present research confirm the conclusions of Yonejima et al. (2015) that probiotics in chocolate were more stable against gastric acid treatment than probiotic powder and those in beverages or in yogurt. Besides, chocolate processing turned out to be useful in protecting the enzymatic activities of the probiotics against gastric acid treatment.

With regard to pathogenic microorganisms, the number of living cells of mesophilic aerobic and facultative anaerobic microorganisms and yeasts and molds in all the chocolate mousse variants met the standard requirements (Table 3). The number of living cells of mesophilic aerobic and facultative anaerobic microorganisms of the control variant increased during

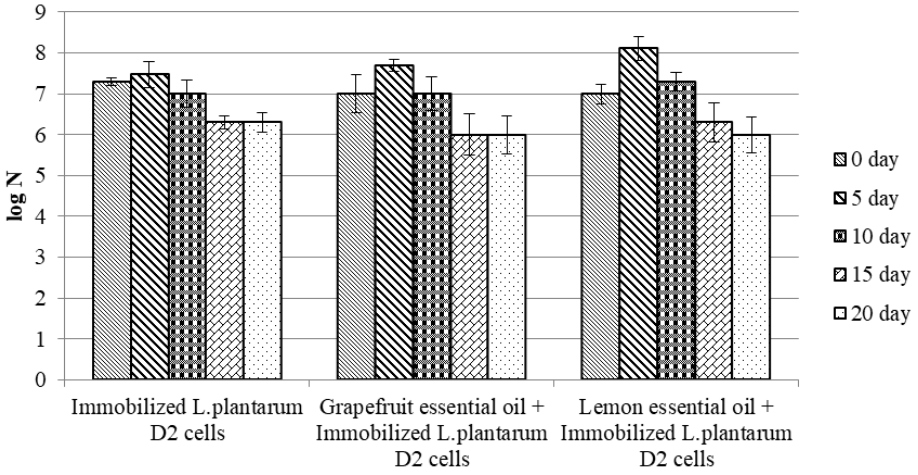

Fig. 2. Changes in the concentration of viable lactobacilli cells of the chocolate mousse variants bio-preserved with immobilized cells of *L. plantarum* D2. Log *N* is $\log_{10} N$, where N is the number of viable *L. plantarum* cells, cfu/g

Table 3. Microflora of the chocolate mousse variants bio-preserved on the 0th and 20th days of storage in refrigerated conditions (from 0°C to +4°C)

No	Chocolate mousse variant	Parameter, cfu/g									
		MAFAM		yeasts and molds		*E. coli*		*St. aureus*		*Salmonella* sp.	
		days									
		0	20	0	20	0	20	0	20	0	20
1	Control chocolate mousse (CCM)	20	68	25	53	<10	<10	<100	<100	NF	NF
2	CCM + grapefruit EO	19	15	27	<10	<10	<10	<100	<100	NF	NF
3	CCM + lemon EO	15	10	20	<10	<10	<10	<100	<100	NF	NF
4	CCM + grapefruit EO + free cells of *L. plantarum* D2	16	<10	21	<10	<10	<10	<100	<100	NF	NF
5	CCM + grapefruit EO + immobilized cells of *L. plantarum* D2	19	<10	27	<10	<10	<10	<100	<100	NF	NF
6	CCM + lemon EO + free cells of *L. plantarum* D2	22	<10	25	<10	<10	<10	<100	<100	NF	NF
7	CCM + lemon EO + immobilized cells of *L. plantarum* D2	16	<10	21	<10	<10	<10	<100	<100	NF	NF
8	CCM + free cells of *L. plantarum* D2	18	<10	24	15	<10	<10	<100	<100	NF	NF
9	CCM + immobilized cells of *L. plantarum* D2	15	<10	28	21	<10	<10	<100	<100	NF	NF

MAFAM – mesophilic aerobic and facultative anaerobic microorganisms.
NF – not found.

the storage period. In all the chocolate mousse variants preserved with lactobacilli and/or essential oils, the number of living cells of mesophilic aerobic and facultative anaerobic microorganisms and of yeasts and molds decreased by the 20th day of storage. It is noteworthy that the number of living cells of mesophilic aerobic and facultative anaerobic microorganisms decreased but did not reach the lower detection limit of the enumeration method used, while the number of yeasts and molds decreased to the lower detection limit of the enumeration method used in the chocolate mousse variants preserved with essential oils only. On the other hand, the number of living cells of mesophilic aerobic and facultative anaerobic microorganisms decreased to the lower detection limit of the enumeration method used, while the number of yeasts and molds did not reach the lower detection limit of the enumeration method used in the chocolate mousse variants preserved with free or immobilized probiotic cells of *L. plantarum* D2 only. In fact, the number of living cells of mesophilic aerobic and facultative anaerobic microorganisms as well as the number of yeasts and molds decreased to the lower detection limit of the enumeration method used only in the chocolate mousse variants preserved with essential oils and free or immobilized probiotic cells of *L. plantarum* D2 (Table 3).

As the indicators, according to the standards used, can also be non-numerical (not found; less than a given standardized value), it is not possible to perform ANOVA. However, according to the data obtained, it can be concluded that there is a statistical difference between the 0th and the 20th days, as in some of the indicators there is a twofold and threefold decrease after the storage period.

The better bio-preservation results could be explained by the synergistic effect of the antimicrobial activity of the selected probiotic strain *L. plantarum* D2 against mesophilic aerobic and facultative anaerobic microorganisms and yeasts and molds, as well as the antimicrobial action of lemon or grapefruit essential oils. These results provide grounds to suggest that the used bio-preservation solution for a chocolate mousse food matrix with the inclusion of lemon or grapefruit essential oils and/or probiotic cells of *L. plantarum* D2 was successful. In contrast, in a study by Aragon-Alegro et al. (2007), neither coliforms nor *E. coli* were detected during the whole storage period of the probiotic and the synbiotic chocolate mousse variants. They reported that, except in the synbiotic mousse, for which yeast and mold were only detected after 21 days of storage, these contaminants were detected after 14 days of storage of the chocolate mousse variants. Thus, they concluded that the production of probiotic and synbiotic chocolate mousse using a preservative or bio-preservative that is not harmful for the viability of probiotic bacteria should be considered.

A number of authors have reported that the addition of probiotic microorganisms affects the flavor of the food product to which they are being added (Bernardi et al., 2004). It was established that the different chocolate mousse variants had good organoleptic characteristics, which is one of the prerequisites for their realization on the market. There were no significant differences in the scores of the different chocolate mousse variants, i.e. the addition of probiotic cells and/or essential oils in the used concentrations did not interfere in the sensory preference of the product by consumers (Fig. 3A, 3B). The results from the conducted organoleptic evaluation are in compliance with the research results by Aragon-Alegro et al. (2007), who reported that the sensorial results of the chocolate mousse trials studied did not indicate any significant differences in preference between the probiotic chocolate mousse variant, the control chocolate mousse variant and the symbiotic chocolate mousse variant evaluated by 42 mousse consumers.

A multiple comparison was made between the nine chocolate mousse variants in terms of the overall organoleptic evaluation of the individual samples. The ANOVA table divides the variance of the data into two components: a between-group component and a within--group component. The *F*-ratio, which in this case equals 1.01665, is a ratio of the between-group estimate to the within-group estimate. Since the *P*-value of the *F*-test is greater than or equal to 0.05, there is not a statistically significant difference between the means of the 9 variables at the 95.0% confidence level. The results of the statistical analysis (not shown in the publication) show that a significant difference was observed only between chocolate mousse variants no 1 and no 3 as well as between variant no 3 and variant no 8. There was no statistically significant difference between the other chocolate mousse variants in terms of their overall performance. A more detailed comparison shows

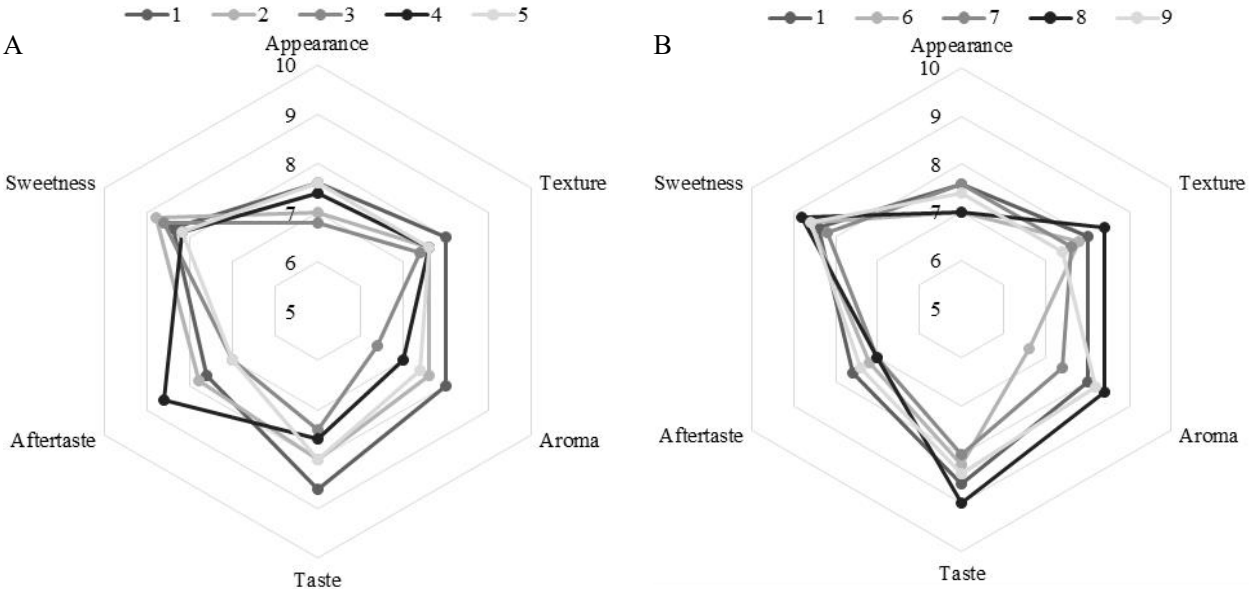

Fig. 3. Organoleptic evaluation of chocolate mousse variants bio-preserved with lemon or grapefruit essential oils and/or free or immobilized cells of the probiotic strain *L. plantarum* D2

that, as a general assessment, the samples with the inclusion of essential oils were slightly less popular than the control variant and the samples with free probiotic *L. plantarum* D2 cells, although statistical analysis shows that there is no significant difference.

The obtained results are of great importance for the production of food emulsions because by introducing essential oils and probiotic bacteria into the composition of food emulsions, additional contamination with pathogenic and saprophytic microorganisms will be avoided and the growth of unwanted microflora will be suppressed, which is especially relevant in modern food production. Moreover, changes in the technology of chocolate mousse manufacturing resulting from its enrichment with free or immobilized cells of probiotic LAB and/or essential oils entail neither the purchase nor the construction or application of additional equipment. Therefore, the results of the present research can be easily applied after the respective scaling up in the food industry.

CONCLUSION

The bio-preservation of chocolate mousse food emulsion was achieved by incorporating ingredients with proven preservative and functional effects into food products, namely grapefruit or lemon essential oils and probiotic bacteria. Concentrations of up to 1% lemon or grapefruit essential oils did not affect the growth of the probiotic strain *L. plantarum* D2, which revealed opportunities for their joint application for bio-preservation of food emulsions. The produced chocolate mousse food emulsions were characterized by preserved organoleptic characteristics and microbiological safety. Free or immobilized probiotic *L. plantarum* D2 cells applied alone or in combination with lemon or grapefruit essential oils provided bio-preservation of food emulsions, maintaining a high concentration of viable cells (10^6–10^7 cfu/g) during storage in refrigerated conditions for 20 days, and converted the resulting chocolate mousse emulsions into functional foods on the one hand, and demonstrated that the chocolate mousse food matrix was a suitable environment for probiotic lactobacilli and can be successfully used as a vehicle for probiotic delivery to a wide range of food consumers on the other. The future perspectives of the present research would be focused on the selection of other probiotic LAB strains and essential oils and the selection of synergistic combinations of them, as well as the selection and employment of different

immobilization methods using different carriers, for the development of successful bio-preservation strategies for other types of food and beverage products.

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