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Filled chocolate supplemented with Lactobacillus paracasei

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ABSTRACT: Nowadays, regarding to humans' need to functional and probiotic foods and the extend use of these foods, considerable attention is donated to production and development of probiotic products which eventually can play an important role in improvement of society health. Additionally, many investigations have identified the chocolate as a proper carrier for probiotic. In the present study, probiotic chocolate containing Lactobacillus paracasei was manufactured and then some of physicochemical parameters including pH and water activity (aw) together with survival assessment of Lactobacillus paracasei in chocolate were investigated. Addition of Lactobacillus paracasei to chocolate haven't had any adverse and significant effects on physicochemical parameters such as pH and water activity during 6 months storage time at ambient and refrigerated temperatures (P>0.05). According to obtained results, the amount of water activity in probiotic chocolate was higher than control chocolate (P<0.05). Moreover, the survival of Lactobacillus paracasei in probiotic chocolate didn't change significantly during 6 months storage at two different temperatures of 4 and 22 ˚C (P>0.05). The obtained results declared that the enriched chocolate with Lactobacillus paracasei can be stored at ambient temperature without having any unfavorable changes in its physicochemical properties such as pH and water activity. Also, even with slight decrement in population of probiotic bacteria of Lactobacillus paracasei during the 6 months storage time at temperatures of 4 and 22 ˚C, but the final product which included 10^8 CFU/g probiotic cells can be considered as a functional probiotic foodstuff.
Keywords: Probiotic, Survival, Chocolate, Lactobacillus paracasei.

INTRODUCTION

In recent decades, there had been significant changes in the understanding of food role in improvement of human health (Hastler, C.M. 1998; Milner, J. A. 1999). Advancements of scientific fields and achieving important and suitable results enable consumers to understand the role of foods in the increment of their life quality. The food technology has been led toward production of healthier food (Izzo, M. and Niness, K. 2001). Numerous functional foods are consumed as part of a normal diet and they provide consumers with well-documented and physiological benefits such as probiotic bacteria (Ramakrishna, S. 2013). A probiotic is a viable microbial dietary supplement that beneficially influences the host through its effects in the gut (FAO/WHO, 2002).

Probiotics are typically incorporated in a range of dairy products or fruit juice. An alternative strategy to increase the efficacy of a probiotic treatment would be to use a food matrix which naturally contains a higher content of ingredients with protective properties. As the lipid fraction of cocoa butter was shown to be protective for bifidobacteria (Lahtinen et al., 2007).

Chocolate is the noblest confectionery product of the unique sensory and textural properties. Chocolate mass, which is the suspension of particles derived from cocoa beans, sweetener and in some cases from milk in cocoa butter or its mixture with another fat is the semi-product used for chocolate manufacturing. Chocolate is also a source of magnesium and many other biologically active substances such as polyphenols and tocophenols, which are beneficial to humans (Nebesny et al., 2007).

Enrichment of chocolate with viable cells of lactic acid bacteria and development of modified technology of chocolate manufacturing to provide survival of these bacteria would contribute to enhanced beneficial impact of this product on human health. This approach is of importance because chocolate is one of favorite foodstuffs for children. Because the unique taste of chocolate is particularly valuable for consumers, its sensory attributes should remain unaltered despite the addition of preparation of lactic acid bacteria.

Physicochemical and sensory attributes of filled chocolate supplemented with live Lactobacillus casei and Lactobacillus paracesei cells and survival of these bacteria during the storage of this chocolate at different temperatures for 6 months were examined within the scope of presented work. Production of this sort of chocolate is a novel approach in the area of successful applications of lactic acid bacteria, including their probiotic strains, for manufacturing of non-dairy products.

MATERIALS AND METHODS

MATERIALS

Filled chocolates supplemented with live cells of Lactobacillus paracasei with potential probiotic properties were the examined material. Biomass of these bacteria was added in the form of lyophilizate.

Strains of lactic acid bacteria

_Lactobacillus paracasei : ŁOCK 0431. This strain was derived from CHR-Hansen Denmark.

Properties of lactic acid bacteria strains

The strains were selected on the basis of results of in vitro studies, which comprised determination of resistance to the acidity of gastric juice, resistance to bile, adherence to epithelial surfaces, and antimicrobial activity. All these studies were conducted according to the recommendations of FAO/WHO The species were classified as L. casei and L. paracasei on the basis of 16S rRNA gene sequence (similarity of 97–99%). These species rank among the typical microflora of human intestines and therefore they can be regarded as safe for manufacturing of fermented milk products and probiotic preparations. The species well tolerated pH of 3.5. Their survival after 3 h incubation at this pH was the same as at neutral pH (6.5) and was close to 100%, while at pH of 2.5 the survival reached 80–100%, dependent on the strain. More than 60% cells of the examined species survived in the presence of 4% bile salts. These strains showed the strong antagonistic activity against gram-positive and gram-negative pathogens.

Production of probiotic chocolate

Chocolate couverture contents 45% sugar, 30% cocoa butter, 8% cocoa powder,8% filler, 8% skim milk powder and 1% lecithin and vanillin was heated to a temperature 40. After the bulk liquidated, it was temperature of 26-28, and next it was slowly heated to 42.

The core of chocolate contents 50% sugar, 25% oil, 12.5% skim milk powder,10% nut powder, 1.5% cocoa powder and 1% lecithin and vanillin was grinded and separated to 2 batches. One of these batches were enriched by lyophilized probiotic bacteria. For adding LAB to core 3 gram of lyophilized probiotic bacteria solute in 50 ml of skim milk to correspond to 107 CFU of LAB per 1 gram of fresh product. Another batch goes to next step without adding LAB. Both of these batches were placed on a grid of coating machine and coated with a previously tempered couverture.

Storage of filled chocolate

Finished products were left at the temperature of 4-6 to cool down and solidify. Next products were wrapped in aluminum foil and stored at 4 and 22 for a period of time predicted as a suitable shelf life for given product, that is 6 months.

METHODS

PH

PH was measured in triplicate for each sort of chocolate using pH meter micro processor 211 produced by Hanna (Germany) . 10 grams of ground core of chocolate mixed with 100ml of distilled water and it remain for 20 minutes. pH of above liquid was measured by pH meter.

Water activity

Water activity was measured in triplicate for each sort of chocolate using hygrometer ms1 meter produced by Novasina (Switzerland). Freshly ground samples of core of chocolate (approximately 12 g) were weighed. The vial containing the sample of chocolate was opened and put on the plate. The digital probe was put on the sample and the hygrometer was switched on. Water activity and temperature of measurements were shown after approximately 2 min on the display of the hygrometer.

Microbiological analysis

Live cells of lactic acid bacteria were enumerated as colony forming units (CFU). A sample of each batch of chocolate was blended with physiological saline solution (NaCl concentration of 8.5 g/l) and a decimal dilution series was prepared. Three successive dilutions were plated in triplicate on agar MRS medium (Merc, Germany) and incubated for 48 h at 37 ◦C in atmosphere of 10% v/v CO2 in air. Survival capacity of lactic acid bacteria during the storage of chocolates supplemented with the lyophilizate of live L. paracasei cells was calculated as follows:

***survival (%) N N0 × 100**,*

where N is the log cfu/g in chocolate after the defined time of keeping, N0, log cfu/g in chocolate immediately after its production.

Statistical analysis

Statistical analysis of results was done using Microsoft Excel^{xp} program from the packet Microsoft Office^{xp}. Values of arithmetic mean of replications of assays, their standard deviation, and variance were calculated. The F-Snedecor test was used to analyze the data distribution.

RESULTS

This study reports on the some properties like pH, water activity and survival of LAB in filled chocolate supplemented with lyophilized probiotic bacteria (L. paracasei).

pH of chocolates: Supplementation of filled chocolate with lyophilized of L. paracasei did not change their pH. The amount of pH in filled chocolate supplemented with L. paracasei and the control chocolate, were similar (table 1). None statistically significant differences between the amounts of pH were found at the confidence level (α) of 0.05, within probiotic filled chocolate and control samples during 6 months storage in both temperatures.

Table 1. pH of probiotic chocolates (P.CH) and control chocolates (C.CH) at 4 and 22°^{c} during 6 months storage.

sample	Storage time(Day)									
	1	7	1 4	2 1	2 8	6 0	9 0	120	1 50	1 80
C.Ch 4˚C	6.68±0.04^{aAB}	6.68±0.02^{aB}	6.69±0.02^{aB}	6.68±0.04^{aAB}	6.67±0.03^{aAB}	6.67±0.01^{aAB}	6.68±0.05^{aAB}	6.66±0.02^{aA}	6.66±0.02^{aAB}	6.65±0.03^{aAB}
C.Ch 22˚C	6.68±0.02^{aABC}	6.70±0.01^{aBC}	6.66±0.02^{aAB}	6.68±0.03^{aABC}	6.66±0.02^{aAB}	6.69±0.02^{aABC}	6.67±0.03^{aABC}	6.65±0.02^{aA}	6.65±0.02^{aABC}	6.69±0.03^{aC}
P.Ch 4˚C	6.65±0.08^{aA}	6.64±0.08^{aA}	6.64±0.08^{aA}	6.64±0.07^{aA}	6.68±0.02^{aA}	6.63±0.06^{aA}	6.61±0.09^{aA}	6.64±0.07^{aA}	6.64±0.07^{aA}	6.65±0.08^{aA}
P.Ch 22˚C	6.66±0.03^{aAB}	6.63±0.06^{aAB}	6.63±0.06^{aAB}	6.67±0.05^{aAB}	6.65±0.08^{aAB}	6.68±0.03^{aB}	6.63±0.08^{aAB}	6.66±0.01^{aAB}	6.66±0.01^{aA}	6.67±0.02^{aAB}

*Mean ± SD (n=3)

** Different lowercase letters in each column indicate significant differences at α = 0.05 level by Tukey test (Tukey) and different capital letters in each row indicate significant differences at α = 0.05 level by least significant difference test or LSD (Least Significant Difference).

Table 2. a_w of probiotic chocolates (P.CH) and control chocolates (C.CH) at 4 and 22°^{c} during 6 months storage.

sample	Storage time(Day)									
	1	7	14	21	28	60	90	120	150	18 0
C.Ch 4˚C	0.20±0.01^{aA}	0.21±0.01^{aA}	0.20±0.01^{aA}	0.21±0.01^{aAB}	0.21±0.01^{aAB}	0.22±0.01^{aAB}	0.22±0.01^{aAB}	0.22±0.01^{aAB}	0.23±0.01^{aAB}	0.23±0.01^{aB}
C.Ch 22˚C	0.20±0.01^{aA}	0.20±0.01^{aA}	0.21±0.01^{aA}	0.20±0.01^{aA}	0.20±0.01^{aA}	0.22±0.01^{aAB}	0.22±0.01^{aB}	0.22±0.01^{aAB}	0.23±0.01^{aB}	0.23±0.01^{aB}
P.Ch 4˚C	0.27±0.01^{bA}	0.27±0.01^{bA}	0.27±0.01^{bA}	0.28±0.01^{bA}	0.27±0.01^{bA}	0.28±0.01^{bA}	0.27±0.01^{bA}	0.27±0.01^{bA}	0.27±0.01^{bA}	0.27±0.01^{bA}
P.Ch 22˚C	0.27±0.01^{bA}	0.27±0.01^{bA}	0.27±0.01^{bA}	0.26±0.01^{bA}	0.26±0.01^{bA}	0.27±0.01^{bA}	0.27±0.01^{bA}	0.27±0.01^{bA}	0.27±0.01^{bA}	0.27±0.01^{bA}

*Mean ± SD (n=3)

** Different lowercase letters in each column indicate significant differences at α = 0.05 level by Tukey test (Tukey) and different capital letters in each row indicate significant differences at α = 0.05 level by least significant difference test or LSD (Least Significant Difference).

Survival of lactobacillus paracasei in chocolate: Survival of L. paracasei cells during the storage for 6 months at 4°c were excellent and their total number was almost unchanged. After 6 months, approximately 85% of cells survived and the total number of live cells was maintained at the functional level, which ranged from 9.7×10^8 to 8.3×10^8 cfu/g. Also keeping at 22°c was almost harmless to L. paracasei cells. After 6 months of the storage, approximately 82% of cells survived. Slightly less than chocolates kept in refrigerator. But their count was maintained at the functional level from 9.7×10^8 to 8.0×10^8 cfu/g after this period.

Water activity of chocolate

Changes in water activity were presented in table 2. An increase in water activity was observed in control chocolates during the storage period (P<0.05). While none statistically significant differences between the amounts of a_w were found at the confidence level (α) of 0.05, within probiotic filled chocolate during 6 months storage in both temperatures.

Table 3. Survival of lactobacillus paracasei in probiotic chocolate at 4 and 22°c during 6 months storage.

sample	Storage time(day)									
	1	7	14	21	28	60	90	120	150	180
P.Ch 4˚C	9.7 ± 0.6^{aB}	9.3 ± 0.6^{aAB}	9.0 ± 0.0^{aAB}	8.7 ± 0.6^{aAB}	8.3 ± 0.6^{aAB}	8.0 ± 0.0^{aA}	8.3 ± 0.6^{aAB}	8.3 ± 0.6^{aAB}	8.0 ± 1.0^{aA}	8.3 ± 0.6^{aAB}
P.Ch 22˚C	9.3 ± 0.6^{aA}	8.7 ± 0.6^{aA}	8.3 ± 0.6^{aA}	8.3 ± 0.6^{aA}	8.3 ± 0.6^{aA}	8.0 ± 1.0^{aA}	8.3 ± 0.6^{aA}	8.0 ± 1.0^{aA}	8.0 ± 0.0^{aA}	8.0 ± 1.0^{aA}

*Mean ± SD (n=3)

** Different lowercase letters in each column indicate significant differences at α = 0.05 level by T-test for independent samples and different capital letters in each row indicate significant differences at α = 0.05 by paired t-test for paired samples.

DISCUSSION

Monitoring of pH in probiotic chocolate is important because; first, during the storage of chocolates the range of pH was between 6.6-6.7, so according to optimum pH for L. paracasei growth (pH: 4-5), this probiotic bacteria can't be active in filled chocolate and remain in anabiosis phase. Secondly, the amount of pH is one of the sensitive factors that affect on quality and shelf life of chocolate. Results of this measurement show that adding L. paracasei into filled chocolate doesn't have any effect on pH amount. These results approved last similar studies on probiotic chocolate mousse and dark chocolate supplemented with probiotic bacteria (Aragon-Alegro et al, 2007; Nebesny et al, 2007).

The amount of water activity in the whole time of storage was in range of 0.20 – 0.28. This level of water activity allowed L. paracasei to stay in a state of anabiosis, which provided stability and high viability of probiotic microorganisms. the amount of a_w in chocolate, because of high amount of sugar and fat, is low (about 0.3). So, survival of bacteria and fungi is low and the shelf life of product is high (Zyzelewicz et al, 2010). The results of this part is the same with last studies on probiotic chocolate couverture (Saarela et al, 2000; Budryn et al, 2007).

Crucial factors affecting the viability of lactic acid bacteria in confectionery is water activity, osmotic tension, and temperature (Matilla-Sandholm, 2002). The satisfactory survival of L. paracasei in chocolates stored at 4 and 22°c (Fig. 1) can be explained as follow. These bacteria were added to core filled of chocolate in the form of lyophilized, which were in the state of anabiosis.

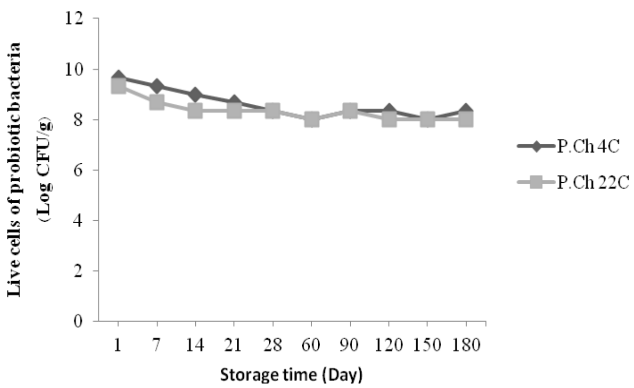

Figure 1. survival of lactobacillus paracasei in probiotic chocolate stored at 4 and 22˚C after 6 months.

Furthermore, oxygen is harmful to their cells and the interior of filled chocolate is virtually anaerobic because of very limited penetration of oxygen. Thus, lactobacillus cells were entrapped inside the chocolate, in the medium with low water content and high concentration of fat and sweetener. Another crucial factor is very low water activity of chocolate. The minimum water activity, at which gram-positive bacteria, including lactic acid bacteria, can grow, is 0.91 (McFarlane I, 1994). Water activity of the all examined samples of chocolate ranged from 0.26 to 0.28. These values fall within the so termed monolayer range. So small water activity and high concentration of sugars in core filled chocolate practically eliminate the growth of bacteria. These results approve the same reports about survival of lactobacillus bacteria in confectionary products (Da Silva et al, 2013; Possemiers et al, 2010).

Our researches provided evidence that chocolate supplemented with live cells of L. paracasei could be stored at ambient temperature. It is very important for tradesmen and consumers. But keeping the chocolate in refrigerator provides survival of higher number of bacterial cells.

CONCLUSION

The trend to supplement new foods with live lactobacillus cells is a novel and promising approach to application of lactic acid bacteria for food production. Lyophilized preparation of live cells of L. paracasei can be considered a product with more pronounced health-promoting properties than the traditional chocolate. It is not only attractive for consumers who consciously select foods with good sensory attributes but is also a source of unique bioactive and functional substances such as polyphenols, isomalt, and live cultures of lactic acid bacteria. Our study proved that chocolate was the good carrier for L. paracasei cells. Their number was kept at the relatively stable level for 6 months at 4˚C.

Consequently, these chocolates can be stored at ambient temperature together with other confectionery. However, more LAB cells survived in refrigerated chocolate. Enrichment with live cells of L. paracasei entails neither purchase nor the construction or application of additional equipment. Therefore, the results of this research project can be easily applied in the food industry. Confectioneries, including chocolate, are willingly consumed by children and youth. Enrichment of these products with components that benefit the health would supplement and improve the quality of their diet.

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