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Viability of *Lacticaseibacillus paracasei* in Ultrafiltered white brined cheese packaged in modified atmosphere and flexible multilayer films

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Abstract

In this study, two packaging materials, modified polypropylene (MPP) and polyethylene terephthalate/aluminum/ low-density polyethylene (PETFA-Al-LDPE) were studied under various atmospheric conditions: 100 % CO_2, 70 % N_2 - 30 % CO_2, 80 % N_2 - 20 % CO_2 for packing probiotic ultrafiltered (UF) white brined cheese. pH, titratable acidity, moisture content, *Lacticaseibacillus paracasei* viability and overall acceptability were monitored within a 12-week period. The control samples were packaged in atmospheric air. Results revealed that samples packaged in PETFA-Al-LDPE with the combination of 70 % N_2 - 30 % CO_2 had the lowest pH, highest acidity and moisture content. The viability of *Lacticaseibacillus paracasei* was reported to be $4*10^6$ CFU/g within 12 weeks. The highest and lowest overall acceptability was for the cheese packaged in 70% N_2 - 30 % CO_2 and the control sample, respectively.

Key words: MAP; Multilayer films; white brined cheese; *Lacticaseibacillus paracasei*

Introduction

White brined cheese has an extraordinary position among all kinds of cheeses in Iran and its continuous industrial production has been extending lately. During ultrafiltration, milk becomes concentrated via the filtration process. The obtained concentrated milk interacts with the starter culture and coagulates. In comparison to other fermented dairy products such as yogurt and fermented milk, cheese is a better carrier for probiotics due to specific physicochemical properties like higher pH, lower titratable acidity, higher buffer capacity and denser structure as a result of which probiotics have a safer condition and maintain their biological activity while passing the gastrointestinal system (Karimi et al., 2011; Gomes da Cruz et al., 2009).

Probiotic viability depends on various factors including final pH of the product, H_2O_2 existence, other microorganisms, incubation time, thermal treatment, probiotic strain, added stimulators, microbial growth inhibitors and finally, the applied packaging systems as well as their nature (Karimi et al., 2011; Gionchetti et al., 2003b). Production of desirable taste and odour by specific microbial strains is of utmost importance in probiotic food manufacturing. Lactic acid bacteria are used in industry for improvement of odour, taste, and structure of fermented products. Due to their inhibition effects on undesirable bacteria, it has been widely studied to use them or their pure bacteriocins for food preservation purposes (Sreekumar and Mosono, 2000). *Lacticaseibacillus paracasei* (in its free or stabilized form) has been used as the starter culture in white brined cheese production (Terpou et al., 2019). Probiotic foods are used to redress the microbial balance of gastrointestinal system, immune system stimulation, anticancer activity, lactose inefficiency treatment, irritable bowel syndrome treatment, prevention and cure of diarrhoea and cholesterol control.

Modified atmosphere packaging (MAP) has gained currency due to the increased demand of consumers for some fermented products namely yogurt and cheese. In modified atmosphere packaging systems, the natural gas of system may be altered to delay any unwanted reaction in the product. It also controls fungi reproduction and the subsequent aflatoxin toxicities. Furthermore, the storage time can increase. However, the atmosphere composition must be selected based on the product itself, production process and the packaging material (Ščetar et al., 2019; Khoshgozaran et al., 2012; Floros and Matsos, 2005). CO_2 has been considered as the principal gas in modified packaging systems. This prohibits aerobic bacteria, fungi, and yeast activity and maintains the product's texture and its sensory properties. CO_2 is soluble in water and fat and its solubility increases with temperature reduction and acts as a bacteriostatic element. N_2 used in MAP is a neutral and tasteless gas that possesses no antibacterial effect (Temiz, 2010; Floros et al., 2000; Floros and Matsos, 2005; Barukčić et al., 2020). Soft cheese becomes rapidly sour in the presence of water and this reaction gets worse while CO_2 exists in the environment. This problem can be solved by CO_2 concentration is kept under 30 % and N_2 is used as filler (Subramaniam, 1989).

In some cases, it gets highly crucial to use a multilayer packaging system to make a balance between the required conservation conditions and the packaging material, as a single layer may fail to meet all the needs simultaneously. In addition to the protection effect against light, moisture, and oxygen, lack of permeability to volatile compounds and flexibility are considered as key factors for choosing the material type. In this regard, flexible multilayer film is one of the best options.

Up to now, many studies have investigated the effect of MAP with different atmospheric conditions with or without other materials on the properties of cheese and the possible storage time enhancement (Dermiki et al., 2008; Papaioannou et al., 2007; Floros et al., 2000; Temiz, 2010; Maniar et al., 2006; Gammariello et al., 2009; Mannheim and Soffer, 1996; Conte et al., 2009; Del Nobile et al., 2009; Garabal et al., 2010; Olarte et al., 2001; Rodríguez-Alonso et al., 2011; Pintado and Malcata, 2000, 2007; Jakobsen and Risbo, 2009; Govaris et al., 2011; Rodriguez-Aguilera et al., 2011; Conte et al., 2011; Temiz et al., 2009; Alves et al., 1996; Alam et al., 2016; Chen and Hotchkiss, 1993; Gammariello et al., 2009; Felfoul et al., 2017; Mortensen et al., 2003; Del Nobile et al., 2009; Barukčić et al., 2020). However, there has been no experiment conducted on the effect of MAP with multilayer packaging material on probiotic bacteria viability. UF white brined cheese has the highest production quantity in Iran and is very popular among Iranians. Within the production process, packaging is regarded as an inseparable part which has a huge influence on the quality of the probiotic dairy products. Since most fermented products are packaged in plastic material which lets oxygen in, serious problems occur for viability of probiotic bacteria. As a result, within the present study, viability of the probiotics in UF white brined cheese in modified atmosphere packaging system has been investigated.

Materials and methods

Materials

Raw milk was bought from Zarrin Toranj Sepehr (Iran), the starter culture was purchased from Mit Co. (Germany) and $CaCl_2$ was prepared from Jiozeeung (China). *Lactobacillus lactis* (CHR Hansen R 704) was purchased from Christian Hansen (Denmark) and *Lacticaseibacillus paracasei* (CHR Hansen A/S) from collection. Co. (Australia). PETFA-Al-LDPE and MPP materials were obtained from Pan Co, (Iran). All the chemicals were of analytical grade acquired from Merck Co, (Germany).

Producing UF probiotic cheese packaged in modified atmosphere and flexible multilayer films

Having conducted the regular tests, raw milk (2 % fat) was precooled and went through clarifier and bacteriophage filters. Then, it was pasteurized at 72 °C for 15 sec. Pasteurized milk enters the preheater and reaches a temperature of 50 °C; it then enters ultrafiltration, with a concentration factor of 5X. Ultrafiltration had three loops, and the final dry matter of retentate reached 35 % after leaving the third loop. The acquired retentate was homogenized and pasteurized at 77 °C for 1 min, then cooled to 35 °C. 0.00055 percent mesophilic starter culture, *Lactococcus lactis* and probiotic *Lacticaseibacillus paracasei* (10^8-10^9 cfu/mL) was added to retentate. 0.5 hr. later, 0.02 % (w/v) $CaCl_2$ was added. Having reached the pH of 5.6, the started culture was added (0.001% w/v) in sterilized distilled water. To form a clot in the second phase of enzymatic coagulation, the temperature remained the same at 35 ° C and the retentate was filled in a 300-g package without any delay (Table 1). When the curd was formed, 3 % NaCl was poured on the samples and the lead was placed by MAP (VAC- Star S-225) (Italy). The produced cheese was kept and incubated at 30 °C (to reach pH 4.7) for 24 h and then moved to a refrigerator at 4 °C. Within 12 weeks, the products were examined for pH, acidity, moisture, *Lacticaseibacillus paracasei* viability and sensory properties.

Probiotic UF cheese samples analysis

The pH of samples was measured using a digital pH metre (Metrohm, Swiss, model 713). Titratable acidity was measured based on the titration method with NaOH and expressed in terms of % lactic acid (AOAC, 1995). Moisture content was determined based on the oven method and reported on weight percent basis (AOAC, 1995).

In order to investigate the viability of the mentioned bacteria, Rogosa agar medium culture (Merck Co, Germany) modified by acetic acid at 96 % (v/v) was implemented and pH was adjusted to 5.2 and finally incubated at 37 °C for 72 h. Viability measurement was done fortnightly according to Madureira et al. (2008).

Cheese samples were randomly coded and panellists evaluated the odour, colour, taste, texture and overall acceptability based on a hedonic scale (1= least favourable, 5=most favourable) every fortnight. 10 trained evaluators aged from 21 to 35 participated in the experiment. Prior to the test, participants were asked to fill in a form containing questions about their age and their cheese consumption history (none, less than once a month, 4-5 times a month, and more than 6 times a month). Evaluators who normally consume cheese 2-4 times a month were not selected for final analysis. Cheese slices were cut into a standard dimension ready to be bitten and were kept at a plastic chamber to reach ambient temperature 2 h before the analysis. Evaluators used distilled water to wash their mouth between samples.

All analyses were performed once a week within 12 weeks.

Statistical analysis

All treatments were conducted in completely randomized design and each treatment was performed for 3 times. Average values were compared with Duncan test and the level of significance was set at 0.05. Data was analysed by SPSS (Ver. 21).

Results and discussion

Titratable acidity and pH of probiotic UF cheese samples during cold storage

The comparison between pH and acidity of cheese samples (figs 1 and 2) showed that there is no significant difference between cheeses with various films and atmospheric composition ($p>0.05$). pH and acidity ranged from 4.78±0.96 - 5.01±0.14 and 1.38±0.01 - 1.17±0.08, respectively at the end of the 12 weeks of experiment. In almost all test intervals, cheese samples packaged in PETFA-Al-LDPE film composed of 30 % CO_2-70 % N_2 and aired PETFA-Al-LDPE films had the lowest and the highest pH, respectively. The reduction of pH at MAP may be a result of CO_2 solubility in water. Furthermore, lactose transformation to lactic acid is effective in pH reduction

Table 1. Introduction of treatments used in the research

Abbreviation	Description
PP+CO2	Modified polypropylene film + 100 % CO_2
PP+CO2+N2 (30:70)	Modified polypropylene film + 70 % N_2 - 30 % CO_2
PP+CO2+N2 (20:80)	Modified polypropylene film + 80 % N_2 - 20 % CO_2
PP	Modified polypropylene film + air (Control 1)
PETFA-PE+CO2	Polyethylene terephthalate/aluminium/ low-density polyethylene film + 100 % CO_2
PETFA-PE+CO2+N2(30:70)	Polyethylene terephthalate/aluminium/ low-density polyethylene film + 70 % N_2 - 30 % CO_2
PETFA-PE+CO2+N2 (20:80)	Polyethylene terephthalate/aluminium/ low-density polyethylene film + 80 % N_2 - 20 % CO_2
PETFA-PE	Polyethylene terephthalate/aluminium/ low-density polyethylene film + air (Control 2)

(Dermiki et al., 2008; Felfoul et al., 2017). pH reduction was lower in samples with 100 % CO_2 compared to 30% CO_2/70 % N_2 samples which may be a consequence of atmospheric pressure reduction after CO_2 absorbance and moisture loss in cheese samples. In 30 % CO_2/70 % N_2 samples, the inert N_2 gas prohibits atmospheric pressure drop, hence moisture loss too. Generally, with the increase in in CO_2, pH reduces and acidity increases. In this study, CO_2 effect alone led to a 0.4-unit reduction in pH. The results are in agreement with similar studies (Dermiki et al., 2008; Temiz et al., 2009; Felfoul et al., 2017).

Along with the progress of storage time, pH reduced and acidity increased. The pH of cheese samples is an indicator of the activity of starter and non-starter lactic acid bacteria. However, pH reduction rate is varying in different chesses (McSweeney et al., 1993). Decline in pH and increase in acidity were mainly due to lactic acid formation from lactose via lactic acid bacteria (LAB), acidic amino acids and free fatty acid production through proteolysis and lipolysis by the starter culture. It is also likely that short chain fatty acids, which are released as final metabolites as probiotic bacteria, are influential in this matter (Yerlikaya and Ozer, 2014; Chevanan and Muthkumarppan, 2007; Dermiki et al., 2008; Osman et al., 2008; Rotaro and Clementi, 2008).

Cheese pH is not solely dependent on lactic acid produced by bacteria and the curd's buffer capacity, which is related to casein content, citrate and phosphate levels, is also important in this regard. The increase in moisture content leads to a decrease in H^+ ion concentration which ends in higher values of pH. Based on the production process, pH change ranges from 4.38-5.94 in the ripening period. Similarly, the pH reduction was reported in Turkish white cheese and Armada by Öner et al. (2006) and Lemya et al. (2010) which was attributed to metabolic reactions in LAB and organic acid such as lactic acid. Maniar et al. (2006), Dermiki et al. (2008) and Felfoul et al. (2017) also reported an increase in acidity level of the cheese packaged in MAP.

Moisture of probiotic UF cheese samples during cold storage

The effect of packaging and atmospheric condition on the moisture content was not significant (P>0.05). Results revealed that PETFA-Al-LDPE film with the composition of 30 % CO_2/70 % N_2 had the highest moisture content (Fig. 3). Having compared the moisture content of samples, it can be concluded that the largest moisture loss occurred in the control samples which is related to moisture evaporation. In comparison to PETFA-Al-LDPE, MPP maintained higher moisture content, most probably due to lower permeability of PETFA-Al-LDPE. 30 % CO_2/70 % N_2 also preserved higher moisture content in comparison to the other compositions. As mentioned earlier, N_2 gas prohibits pressure loss restricting thereby moisture reduction. Dermiki et al. (2008) and Felfoul et al. (2017) also reported the lack of MAP effect on the moisture content of cheese samples.

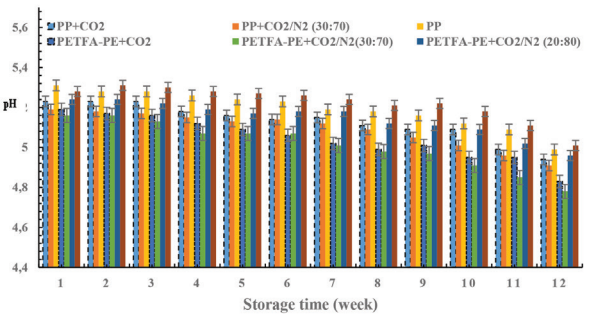

Figure 1. pH changes of probiotic UF cheese samples during cold storage

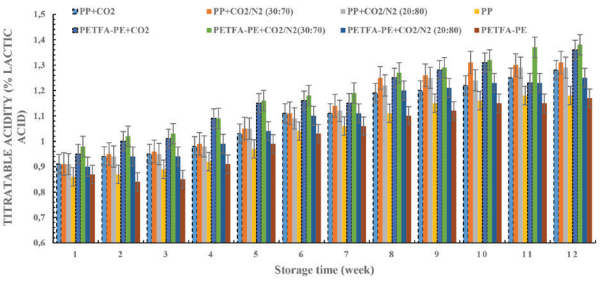

Figure 2. Titratable acidity changes of probiotic UF cheese samples during cold storage

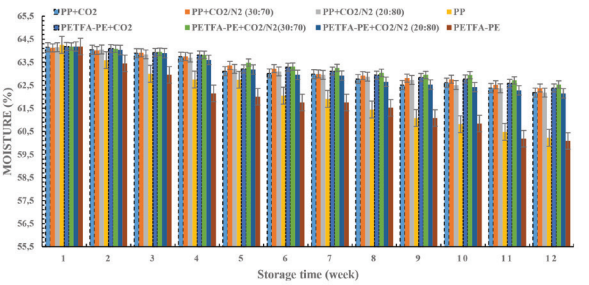

Figure 3. Moisture changes of probiotic UF cheese samples during cold storage

Moisture content reduced during storage which is due to surface evaporation and the permeable film. Acid production by LAB helps the pH reduction trend within storage time which consequently increases whey and moisture loss (Caridi et al., 2003). Therefore, during the storage period, moisture content decreased and reached the minimum after 12 weeks. This moisture loss during ripening was also observed in hard cheeses such as Parmigiano Reggiano (D'Incecco et al., 2020).

Lactobacillus paracasei viability of probiotic UF cheese samples during cold storage

The effect of packaging and atmospheric composition on viability of *Lacticaseibacillus paracasei* was not significant (p>0.05). Results revealed that with the increase in CO_2 concentration in films, *Lacticaseibacillus paracasei* viability showed a greater reduction (Fig. 4) since CO_2 inhibits microbial growth because of its bacteriostatic

properties. Not only does MAP retain the quality properties of cheese, but it also enhances its shelf life (Temiz, 2010; Dermiki et al., 2008; Gammariello et al., 2009; Conte et al., 2009; Khoshgozaran et al., 2012; Del Nobile et al., 2009; Conte et al., 2011; Temiz et al., 2009; Mannheim and Soffer, 1996). Papaioannou et al. (2007) reported mesophilic bacteria growth restriction in CO_2-N_2 composition which subsequently resulted in longer shelf life. In addition, packaging type affected *Lacticaseibacillus paracasei* viability. The lowest and highest viability was observed in MPP with 100 % CO_2 and PETFA-PE with 30 % CO_2/ 70 %N_2. On the other hand, high moisture content is crucial in microorganism growth and higher moisture in PETFA-Al-LDPE packages helped *Lacticaseibacillus paracasei* growth in 30 % CO_2/70 % N_2 packages. Dermiki et al. (2008) also reported an increase in the total number of LAB in cheese samples during a 50-day period in modified CO_2-N_2 system. However, Papaioannou et al. (2007) reported a decrease in mesophilic bacterial growth in Greek cheese in CO_2-N_2 packages during 40 days. Maniar et al. (2006) also observed an ascending pattern in LAB in cottage cheese samples packaged in air and a stable pattern in MAP.

Lacticaseibacillus paracasei viability decreased in cheese samples during storage ($p>0.05$). LAB are natural residents of cheese microflora which play a key role in cheese ripening (Tornadijo et al., 1995) and increase the amount of short-chain peptides, free amino acids and free fatty acids (Arenas et al., 2004). LAB reduction in ripening period is dependent on factors such as salt, sensitivity of starter cultures, water activity and autolysis ability of breeds (Vassiliadis et al., 2009). Slower metabolism rate of *Lactobacillus* sp. and their ability to cope with harsh conditions (acidity, low water activity, and high sanity) is a major reason for their survival until the end of storage time in comparison with the other LAB (Arenas et al., 2004). During the fermentation period of cheese, various metabolites like lactate, citrate, glycerol, and amino acids are produced and are efficiently used by *Lactobacillus* sp. (Öner et al., 2006). Lactococcus bacteria rapidly ferment lactose and are numerous within the first days, so they create high acidity (Arenas et al., 2004). According to some previous studies, health effects of probiotics occur only if at least 10^6-10^7 bacteria per g or mL of food sample enter the intestine (Tharmaraj and Shah, 2004). These results confirm the viability of probiotics till 56 days of storage in all samples in $8x10^6$-$3x10^7$ cfu/g level as in 30 % CO_2/70 % N_2 samples, the number of bacteria was still in probiotic range after 84 days. Generally, probiotic viability in fermented products is rather low due to low pH and high acidity as these factors are the major restricting elements for their viability (Kitazawa et al., 2001; Dinakar and Mistry, 1994). So, the reduction in their viability is natural. Xong-Xin et al. (2015) and Abd El.Salam et al. (2012) also reported *Lactobacillus casei* reduction in Cheddar and Domiati during storage. *Bifidobacterium animalis* subsp. *lactis* BB-12 viability in the Mascarpone-type cheese declined throughout the whole storage period (de Almeida et al., 2018). The results of Cárdenas et al. (2014) also confirmed *Lactobacillus salivarius*

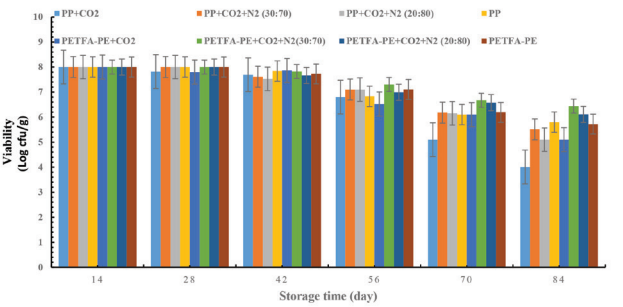

Figure 4. *Viability changes of probiotic UF cheese samples during cold storage*

reduction after 28 days. Also, viability of encapsulated *L. acidophilus* reduced in Requeijão cremoso processed cheese formulations during 45 days (Povetta et al., 2020). On the contrary, Barros and De Carvalho Delfino (2014) reported no meaningful difference in *Lactobacillus casei* viability during 30 days in petit-Suisse cheese as the pH did not pass 4.2 during this time due to buffer capacity of nutrients in milk (phosphates, citrate, peptones). They also reported that *lactobacillus casei* BGP 93 was resistant to acid (up to pH=3), consequently, it was not affected by acidity. Yerlikaya and Ozer (2014) reported no reduction or increase in *Lacticaseibacillus paracasei* viability during 28 days in white cheese. Madureira et al. (2008) observed that *Lactobacillus casei* in cheese sample made of sweet whey protein had a slight increase after 21 days. In Cheddar cheese, *Lactobacillus casei* was sustained in its initial level and then decreased slightly during the following 32 weeks. *Lacticaseibacillus paracasei* reduced up to week 8, increased in week 12 and then decreased again up to week 32 (Phillips et al., 2006). The results of Bodzen et al. (2020) also confirmed this important fact that atmosphere air in *Lactobacillus casei* preparation triplicates final viability in intestine simulated condition with $N_2H_2CO_2$.

Overall acceptance of probiotic UF cheese samples during cold storage

Packaging and atmospheric composition had a significant effect on overall acceptance of the cheese samples ($p<0.05$). In both films, after 12 weeks of storage, cheese packaged in 30 % CO_2/70 % N_2 and the control sample showed the highest and the lowest overall acceptance (Fig 5). Higher viability and moisture content were the reason of higher acceptance in 30 % CO_2/70 % N_2 samples. Samples with PETFA-Al-LDPE system showed higher acceptance in comparison to MPP samples. At week 6, the score for this test was 4.7±0.01 to 4.61±0.02 for PETFA-Al-LDPE with 30 % CO_2/70 % N_2 and MPP film with air, respectively. This pattern in modified atmosphere can be explained by different variations in structure, colour, taste and odour of cheese.

The influence of storage time on the acceptance of the samples was meaningful ($p<0.05$). This score initially

increased and then decreased and the highest point was observed in weeks 5 and 6, and the minimum was attributed to week 12. After 12 weeks, PETFA-Al-LDPE with 30 % CO_2/70 % N_2 and MPP with air content exhibited the highest and the lowest scores, respectively. Improved odour and taste as result of microorganism activity and their proteolysis and lipolysis, reduced moisture content and textural properties led to the best scores for weeks 5 and 6.

The best atmospheric composition was different in various studies based on the cheese type. Temiz (2010) reported that 40 % CO_2/60 % N_2 and 100 % CO_2 had the same acceptability score at the end of the experiment. Maniar et al. (2006) claimed satisfactory acceptance for cottage cheese with 100 % CO_2. Dermiki et al. (2008) reported that 60 % CO_2/ 40 % N_2, 40% CO_2/60 % N_2 saved the sensorial quality of Myzithra Kalathaki cheese for 30 days. After 14 days of storage, fresh Cameros cheese of goat milk packaged on 40 % CO_2/60 % N_2 and 50 % CO_2/50 % N_2 had a satisfactory odour and taste although their appearance and texture were not favourable (Olarte et al., 2001). Rodríguez-Alonso et al. (2011) declared that 80% N_2/20 % CO_2 and 100 % N_2 compositions are not suitable for preserving the sensorial properties of semi-hard "Arzua-ulloa" cheese. Temiz et al. (2009) concluded that the taste and odour of Turkish whey cheese (Lor) packaged in modified atmosphere (40 % N_2, 60 % CO_2, and 30 % N_2/70 % CO_2 was sustained after 45 days. Mozzarella cheese overall acceptability after 43 days of storage in 50 % CO_2/50 % N_2 was lower in comparison with 100 % CO_2 (Alves et al., 1996). Gammariello et al. (2009) proposed 75 % CO_2/25 %N_2 composition for maintenance

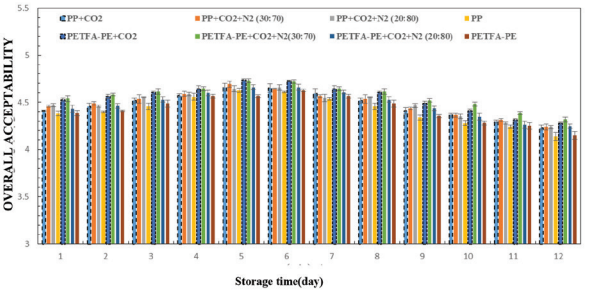

Figure 5. Changes in overall of probiotic UF cheese samples during cold storage

of sensorial properties and prolonged shelf life of Italian cheese Giuncata. Felfoul et al. (2017) reported that 40 % Co_2/ 60 % N_2 was successful for saving Mozzarella cheese sensorial features.

Conclusion

Packaging systems and their related materials influence probiotic bacteria. In this study, the effect of modified atmosphere and multilayer films on pH, acidity, moisture, overall acceptability and viability of *Lacticaseibacillus paracasei* in ultrafiltration cheese was analysed based on the collected data, UF Feta cheese with PETFA-Al-LDPE and 30 % CO_2/70 % N_2 was the best option due to better moisture preservation, higher probiotic viability and the higher overall acceptance.

Preživljavanje soja *Lacticaseibacillus paracasei* u ultrafiltriranom siru u salamuri pakiranom u modificiranoj atmosferi i fleksibilnim višeslojnim filmovima

Sažetak

U ovom istraživanju je ispitivana mogućnost primjene dvaju ambalažnih materijala - modificiranog polipropilena (MPP) i polietilen tereftalata/aluminij/polietilen niske gustoće (PETFA-Al-LDPE) u različitim uvjetima modificirane atmosfere (100 % CO_2, 70 % N_2 – 30 % CO_2, 80 % N_2 - 20 % CO_2) za pakiranje probiotičkog sira iz salamure proizvedenog iz ultrafiltriranog (UF) mlijeka. Tijekom 12 tjedana čuvanja uzorcima sira su određivani pH, titracijska kiselost, sadržaj vlage, preživljavanje soja *Lacticaseibacillus paracasei* i prihvatljivost. Kontrolni uzorci su pakirani u normalnoj atmosferi (zrak). Dobiveni rezutati ukazuju kako uzorci pakirani u PETFA-Al-LDPE i modificiranoj atmosferi 70 % N_2 - 30 % CO_2 imaju najniži pH, te najveću kiselost i sadržaj vlage. Preživljavanje soja *Lacticaseibacillus paracasei* je bilo oko 4x10^6 CFU/g tijekom 12 tjedana. Najnižu i najvišu prihvatljivost su imali uzorak pakiran u atmosferi 70% N_2 - 30 % CO_2 te kontrolni uzorak.

Ključne riječi: MAP; višeslojni film; sir u salamuri; *Lacticaseibacillus paracasei*

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