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Effects of Probiotic Cells on the Mechanical and Antibacterial Properties of Sodium-Caseinate Films

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Abstract

Background and Objective: Food processing conditions such as heat, mechanical or osmotic stress can lead to considerable losses of probiotics' survival in food. Recently, the addition of probiotics into edible films has been proposed as an emerging technology for the delivery of probiotic cells. In this study, *Lactobacillus acidophilus* and *Lactobacillus casei* cells were incorporated into sodium caseinate matrix to develop a probiotic-based film which can improve food safety.

Material and Methods: Probiotic cells were separately added to the film forming solutions andthe active films were prepared by casting method. The physical, optical and mechanical characteristics of the films were examined. Color properties were determined using a colorimeterand the mechanical properties of the films were evaluated by an Instron Universal Testing Machine. The viability of *Lactobacillus acidophilus* and *Lactobacillus casei*in the films was determined during a period of 12 days. The antibacterial activities of the films were also tested against *Listeria monocytogenes*on Trypticase Soy Agar medium at 4°C.

Results and Conclusion: Results demonstrated that lactic acid bacteria cells remained viable during a storage period of 12 days (> 4 Log CFU cm $^{-2}$). The incorporation of lactic acid bacteria cells into the film polymer had no significant effect on tensile strength ($p>0.05$) whereas it significantly improves the appearance of films. Indeed, samples covered with the lactic acid bacteria film displayed higher antilisterial activity than the control group on day 6 of preservation ($p\leq0.05$). These findings show that the sodium caseinate film containing lactic acid bacteria cells can be used as a new effective packaging method for improving food safety.

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1. Introduction

Non-biodegradable petroleum-based plastic materials present a major environmental concern, which is hence increasing the absolute must of finding alternative sustainable solutions. To this aim, considerable efforts have been made in recent years to produce biodegradable packaging for the food processing. These biopolymers are being considered as eco-friendly with antimicrobial properties enhancing food safety and decreasing the risk of foodborne pathogens [1,2].

Listeria (*L.*) *monocytogenes* is an important foodborne pathogen causing listeriosis, a serious invasive infection with a high mortality rate (10-40%). Because of the frequent occurrence of *L. monocytogenes* in food, a great deal of research has been focused on this pathogen [3,4].

Nowadays, with increasing demands for probiotics and food safety, a novel active film containing the viable cells of probiotics has been proposed [5,6]. The addition of viable probiotics into film solution was first suggested in 2007 by Tapia et al. [7]. In contrast to a large amount of information on the antimicrobial efficiency of active films containing antimicrobials, to our knowledge, very limited information is available on the development of active films containing the probiotics, and the majority of these studies were reviewed by Espitia et al. [8,9].

There is a growing interest for non-dairy probiotic products, owing to dietary restrictions, allergies, and cholesterol levels.

As a result, the development of novel probiotic carriers will promote human health [6]. Food processing conditions such as heat, osmotic or mechanical stress can reduce the viability of probiotic cells. To overcome these problems, several strategies have been proposed over the last years. The application of edible films as a carrier for probiotic cells is a bioactive food packaging system, which can provide health benefits to consumers and a higher viability [6,9].

In the past decade, several researchers developed alginate films containing *Bifidobacterium* (*B.*) *animalis* subsp. *lactis*, *Carnobacter-iumaltaromaticum* and *Lactobacillus* (*L.*) *rhamnosus*, agar films containing *Lactobacillus* (*L.*) *paracasei* and *B. lactis*, sodium caseinate films containing *L. sakei*, *Lactobacillus reuteri* and *Lactobacillus acidophilus*and also gelatin films containing *L. rhamnosus*, *L. acidophilus* and *Bifidobacterium bifidum* [7,8,10,11-13]. As probiotic viability is important to induce health benefits and every biopolymer matrix has its own biological limitations for supporting probiotic cells, it is vital to examine the efficiency and viability of probiotics in the different food packaging materials. On the other hand, the mechanical changes of films, due to the addition of various probiotics, need to be investigated. To date, no published data are available on the incorporation of *L. acidophilus* and *L. casei* population into sodium caseinate matrix in order to determine the antibacterial effectiveness of the film against *L. monocytogenes* growth.

Therefore, the objectives of this research were (1) to develop a bioactive film based on the addition of *L. acidophilus* and *L. caseiviablecells* into sodium caseinate polymer; (2) to evaluate the survival of these viable cells in the films; (3) to investigate the effects of protective cultures on the physical properties of the films; and (4) to determine the antimicrobial activity of the probiotic-containing films against *L. monocytogenes* growth during 9 days storage at 4°C.

2. Materials and Methods

2.1. Bacterial strains

L. monocytogenes (PTCC 1163), *L. acidophilus* (PTCC 39392) and *L. casei* (PTCC 1608) were obtained from the Iranian Research Organization for Science and Technology (Persian Type Culture Collection, Tehran, Iran). The bacterial strains were stored at-20°C in Tryptic Soy Broth (TSB, Liofilchem, Italy) with 30% glycerol.

2.2. Preparation of the bioactive films

The bioactive films were prepared by dissolving sodium caseinate 4% (w v^{-1}) (Sigma-Aldrich, Spain) in distilled water at 65°C for 10 min and then cooled to room temperature. Next, glycerol was added [14]. The sodium caseinate: glycerol mass ratio was 1:0.4. Afterward, the samples were homogenized at 5500 g for 10 min and the final film-forming solution was degassed in order to remove bubbles. Two LAB species, *L. acidophilus* and *L. casei*, were selected to formulate the bioactive films. These strains were reactivated from a stock culture. The LAB strains were cultured into 10 ml MRS broth and incubated for 24 h at 37°C. The probiotic cells were separated from MRS by triple centrifugation at 4000 ×g for 5 min. The LAB cell count was determined by the optical density technique (OD at 600 nm) in physiological saline. Lactic acid bacteria were added separately into the film forming solutions to attain a final concentration of 10^5 CFU ml^{-1}. Finally, the control (without LAB) and bioactive films were obtained by casting method at 40°C in a forced-air oven for 24 h. The final control and bioactive films were stored in desiccators (containing saturated magnesium nitrate solution) at room temperature for 2 days.

2.3. Determination of physical properties of bioactive films

2.3.1. Measurement of film thickness

The thickness of the control and probiotic edible films was determined with a manual digital micrometer (0.001 mm, Mitutoyo, Mizonokuchi, Japan). At least ten random locations for each film sheet were measured.

2.3.2. Moisture content

The moisture content of the edible films was measured by drying the samples in an oven at 110°C until a constant weight was reached (dry sample weight).

2.3.3. Mechanical properties

The mechanical properties of the control and bioactive films were evaluated by analyzing the elongation at break (%, EB) and tensile strength (MPa, TS) using an Instron Universal Testing Machine (Model A1 70 0, Gotech, Taiwan). The method of Vejdan et al [15] and Shojaee-Aliabadi et al. [16] was adopted for this test.

Water vapor permeability: Water vapor permeability of the bioactive films was measured at 20°C according to the ASTM E96-92 gravimetric method as described by Vejdan et al. [15] and Casariego et al. [17].

Optical properties: Color properties were determined using a colorimeter (BYK-Gardner, Columbia, MD, USA). The film samples were placed on a standard white-color plate (L*= 94.63, a*= -0.88 and b*= 0.65). Color parameters such as L (lightness), a (green red^{-1}), and b (blue $yellow^{-1}$) values were evaluated from the average of eight readings [15,17]. The total color difference (ΔE) of the bioactive films was calculated as follows:

$$\Delta E = \sqrt{(L^* - L)^2 + (a^* - a)^2 + (b^* - b)^2}$$

Where L, a, and b are the color parameter values of the sample.

2.4. Antimicrobial activity of films

The methodology was adapted from Kristo et al. with some modifications. Briefly, aliquots of TSB were poured into Petri dishes [18]. After culture medium solidified, properly diluted overnight culture from *L. monocytogenes* was inoculated on the agar surface. The control and bioactive films (containing or not containing LAB) were placed onto the inoculated agar surfaces. Finally, the plates of control and edible films were covered with parafilm and stored at 4°C for 9 days.

2.5. Enumeration of Listeria and lactic acid bacteria

For microbial analysis, the agar was removed from Petri dishes and was homogenized with sterile physiological serum. Serial dilutions were made, and counts of Listeria and LAB were determined by pouring 0.1 ml of serial dilutions onto Oxford agar and MRS agar, respectively. Plates were incubated at 37°C for 24-72 h before colonies were counted.

2.6. Statistical analysis

The K-S test was used to check the normal distribution of obtained data. Statistical analysis of data was done by One-way ANOVA (SPSS 17.0). When the F-value was significant ($p \leq 0.05$), the Duncan test (post hoc analysis) was also used [19].

3. Results and Discussion

3.1. Water vapor permeability

Water vapor permeability (WVP) of the active films is one of the most important factors defining film functionality [15]. Low levels of water vapor permeability are desirable to reduce weight losses of packaged products, which affect food quality [12]. Table 1 shows WVP and physical properties of the bioactive films.

Owing to hydrophilic nature of the protein and polysaccharides, the biopolymer films are highly permeable to water vapor [20]. In this study, no differences were found between water vapor permeability values among the control and LAB active films ($p>0.05$; Table 1). Our results were similar to those of Gialamas et al.[8] which did not report significant differences among the barrier properties of sodium caseinate films containing LAB strains. They concluded that the small biomass of the LAB cells, compared to the film mass, can explain why the permeability is not affected by the presence of the bacteria. Similar findings have been reported in the study of Kristo et al. [18] that the addition of antimicrobial agents at low concentrations did not cause significant changes in the barrier properties of active films. In general, WVP of the films depends on the hydrophilic nature of protein and polysaccharides. However, the results of the present study are not in agreement with those reported by Sánchez-González et al. [20]. They observed significant differences among the barrier properties of active films. This could be attributed to differences in the LAB cells and the experimental conditions. Moreover, this might be due to the presence of LAB cells, which causes discontinuities in the biopolymer matrix.

3.2. Mechanical behavior

The effects of LAB cells on the mechanical properties of NaCas based films are shown in Table 1. Tensile strength shows the film's resistance to elongation, and elongation at break (E%) is a measure of film's capacity for stretching [20]. The thickness of active films varied between 0.107 and 0.126 mm, as shown in Table 1. The sodium caseinate control film had the tensile strength value 2.57 MPa. Only a slight reduction in TS was observed in *L. acidophilus* films ($p>0.05$), and pure NaCas films had the highest value of TS. In general, the incorporation of LAB into sodium caseinate films had no significant effect on tensile strength ($p>0.05$). These findings come in agreement with the study of Gialamas et al. [8] who stated that the incorporation of LAB cells to films has no significant effect on the tensile properties, probably as a result of the insignificant mass of added cells. Similarly, in the study of Sánchez-González et al. [20] no significant changes were reported in terms of tensile strength of protein films after the addition of probiotic cells.

In this study, pure NaCas films had the highest value of elongation (E%), and a significant reduction in E% was observed in the LAB films ($p \leq 0.05$). The results of Gialamas et al. [8] study revealed that water content in the sodium caseinate films exhibits a significant role in increasing film flexibility (E%). In the Gialamas et al. [8] study, at a moisture level of 20%, the measured E % was about 15-25, which is lower than that of the present study. As shown in Table 1, the moisture content of control film was significantly higher than that of bioactive films ($p>0.05$). In the study of Sánchez-González et al. [20] a reduction in E% was observed when LAB cells were incorporated into sodium caseinate, isolate pea protein, and methylcellulose films; however, these changes were not statistically significant.

Table 1. Thickness, physical properties, film solubility, and moisture content of the edible films.

Film	Thickness (mm)	MC (%)	Solubility (%)	WVP ($\times 10^{-10}$ g(m^1 s^1 Pa)$^{-1}$)	TS (MPa)	E (%)
control	0.107 ± 0.006 ^{a*}	33.74 ± 1.56 ^{a}	48.06 ± 1.30 ^{a}	2.059 ± 0.11 ^{a}	2.57 ± 0.49 ^{a}	201.44 ± 22.42 ^{a}
Lactobacillus. casei	0.121 ± 0.006 ^{a}	32.39 ± 0.65 ^{ab}	29.58 ± 4.83 ^{b}	2.076 ± 0.48 ^{a}	2.12 ± 0.05 ^{ab}	153.80 ± 21.96 ^{b}
Lactobasillus. acidophilus	0.126 ± 0.006 ^{a}	31.063 ± 1.52 ^{b}	34.02 ± 4.74 ^{b}	1.65 ± 0.162 ^{a}	1.96 ± 0.18 ^{a}	129.75 ± 16.39 ^{b}

*Different superscripts within the same column show significant differences among formulations ($p< 0.05$).
(MC): Moisture content, (WVP): Water Vapor Permeability, (TS): Tensile Strength, (E): Elongation

Studies show that plasticizer content and relative humidity can significantly affect the mechanical properties of films. For example, Audic and Chaufer [21] determined the mechanical behavior of NaCas based films versus a different ratio of protein: Plasticizer and relative humidity conditions. In that study, sodium caseinate films with low plasticizer content (25% glycerol) exhibited poor elongation (55%); however, elongation of films containing 50% glycerol increased to over 100%. The relative humidity condition had also a significant effect on the mechanical properties of films. In the Kristo et al. [22] study, a sharp increase in elongation (%) was observed due to increasing the moisture content of the films. Therefore, mechanical properties of sodium caseinate films (TS and E) are highly dependent on plasticizer content and relative humidity. Because the combination of plasticizers with the polymer matrix cases the chains to move apart and a reduced rigidity of structures, thus improving film flexibility and the mechanical properties.

3.3. Optical properties

The optical properties of films have an important impact on the food appearance and may influence the acceptability of the food product by the consumer [15]. The spectral distribution of the internal transmittance, which shows film transparency, is provided in Fig. 1. The results display that the addition of bacterial cells enhances the film transparency. Furthermore, the addition of protective cultures increased the film lightness (L), whereas no changes were observed for a (green red^{-1}) and b (blue yellow^{-1}) parameters (Table 2). Similar results were observed in the Sánchez-González et al. [12] and Sánchez-González et al. [20] studies. Therefore, the addition of protective culture into the sodium caseinate matrices significantly improves the appearance of films. Moreover, a more transparent film will be achieved with higher lightness, because of the addition of protective cultures.

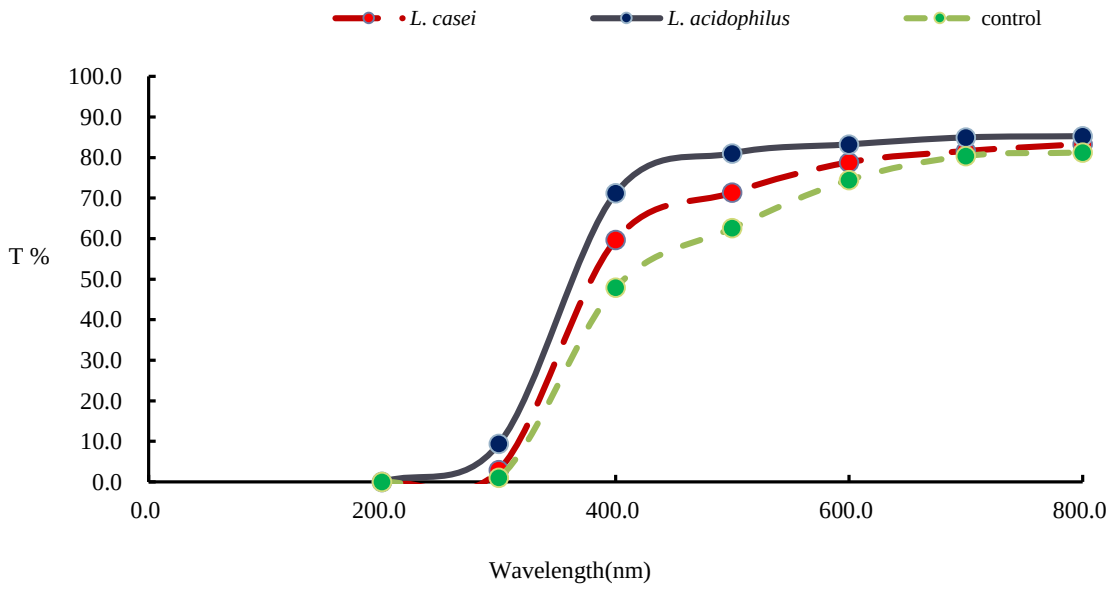

Fig. 1 Effect of probiotic cells on the internal transmittance

Table 2. Surface color parameters of bioactive edible films.

Film	L	a	b	E*Δ
Control	78.36 ± 2.21 ^{b*}	4.38 ± 0.72 ^{a}	2.06 ± 0.68 ^{a}	16.92 ± 2.35 ^{a}
Lactobacillus. casei.	81.52 ± 0.82 ^{a}	3.90 ± 0.57 ^{a}	1.92 ± 0.44 ^{a}	13.75 ± 0.87 ^{b}
Lactobacillus. acidophilus.	79.84 ± 1.31 ^{ab}	3.90 ± 0.57 ^{a}	1.58 ± 1.34 ^{a}	15.38 ± 1.26 ^{ab}

*Different superscripts within the same column show significant differences among formulations ($p < 0.05$). *(ΔE): Color difference, (L. C.): *Lactobucillus. casei*, (L. A.): *Lactobacillus. acidophilus*

3.4. Viability of lactic acid bacteria

The viability of *L. casei*and *L. acidophilus* added to sodium caseinate polymer was evaluated throughout a storage period of 12 days at 4°C. Fig. 2 provides the viability of LAB during the storage time. For *L. casei*, a sharp reduction of the initial cells occurred in the first 3 days of storage, which shows that this bacterium is more sensitive. However, the majority of the bacteria were viable up to the end of storage at 4°C. The observed decline in the number of LAB cells and their viability could be due to the adaptation to the new substrate and the low storage temperature [13]. Most of the earlier studies reported a similar positive effect of NaCas matrices on the survival of LAB cells, *L. plantarum, L. sakei, L. reuteri*, and *L. acidophilus* [8,12, 20]. Therefore, it seems that the sodium caseinate matrix is a good carrier of probiotics. In addition to sodium caseinate, other edible films could be used to incorporate lactic acid bacteria. For example, in the study of Lacey et al. [13] a gelatin film was successfully used for incorporating *L. acidophilus* and *Bifidobacterium bifidum* cells. In the study of Soukoulis et al. [23] the effect of the physicochemical properties of different compounds on *L. rhamnosus* viability was investigated. A 3- to 7-fold increase in the viability of *L rhamnosus* GG was reported in the presence of proteins, with sodium caseinate–rice starch based films.

3.5. Antimicrobial activity of probiotic-containing films

The antilisterial effects of bioactive films were evaluated on TSA medium at 4°C. The antibacterial effects of films are provided in Fig 3. NaCas films without bacterial cells were used as a control group. The initial *L. monocytogenes* population of the control group increased from 1.47 ± 0.35 to 3.88 ± 0.04log CFU $(cm^2)^{-1}$ at the end of the storage time. On day 6 of preservation, samples covered with the LAB films displayed higher antilisterial activity than the control group ($p \leq 0.05$), but there was no significant differences between the control sample and LAB films on day 3 ($p > 0.05$). After 9 days of storage, the population of *L. monocytogenes* in the *L. acidophilus* film was significantly lower than the control group ($p < 0.05$), whereas no significant effect was observed between the *L. casei* film and the control sample ($p > 0.05$).

Bacteriocins are natural compounds produced by some bacteria that can be incorporated into active films to control foodborne pathogens. The effect of *L. casei* in the control of *L. innocua*in MRS medium and meat slurry during the storage at 8°C and 4°C was investigated by Castellano et al. [24]. The maximum bacteriocin activity was observed at 8°C after 10 days (2130 AU ml^{-1}), while at 4°C maximum activity was obtained by 14 days (690 AU ml^{-1}). In the study of Martinez et al. bacteriocin production and antilisterial effect of *L. sakei* were lower at 4°C than that of 15°C. Therefore, at 4°C the bacteriocin production decreased because of the decrease in cell growth at refrigerated temperatures [25]. However, when the LAB cells were directly incorporated into the polymer solution, in addition to bacteriocins, other antimicrobial compounds such as lactic acid, diacetyl, and hydrogen peroxide can be released from the surface of the film into the food mass during the storage period.

Beside to improving food safety, the incorporation of probiotics into biopolymeric matrices provides health benefits (i.e.including modulation of immune response, strengthening of intestinal barrier, and antagonism of pathogenic microorganisms [9].)

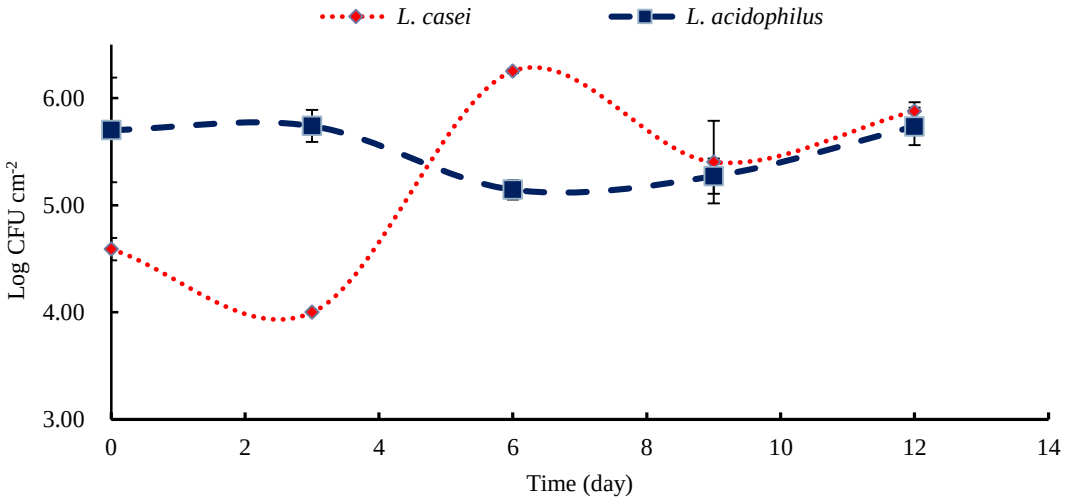

Fig. 2 The survival of *Lactobacillus acidophilus* and *L. casei* in the film during 12 days storage at 4 °C

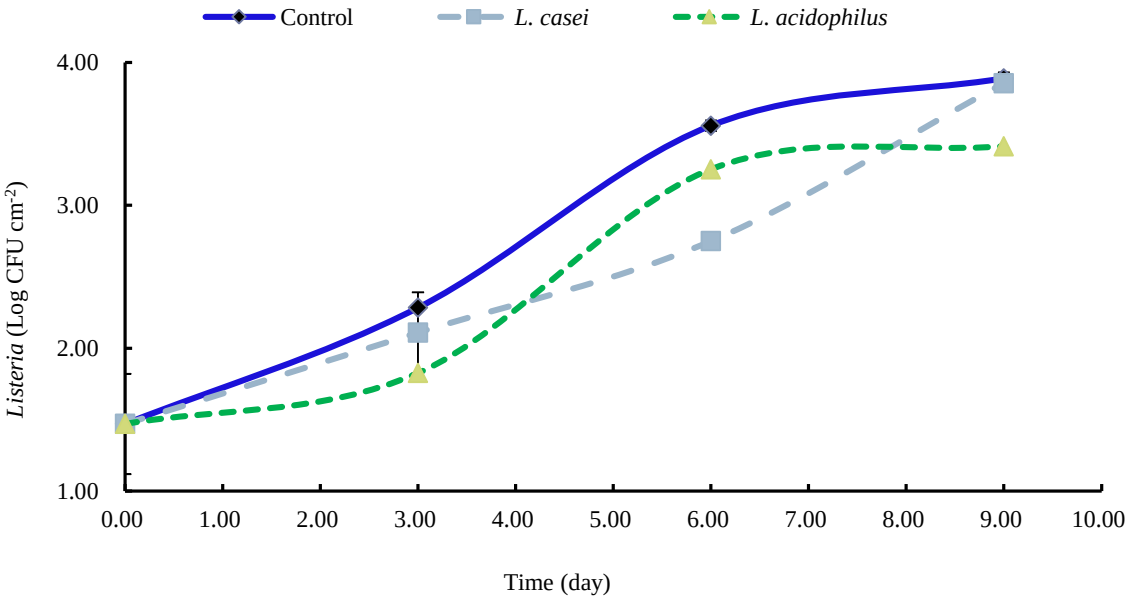

Fig. 3. The antilisterial activity of bioactive films on TSA medium during 9 days storage at 4°C

4. Conclusion

Incorporating lactic acid bacteria into edible films has been proposed as a novel technology, which provides health benefits to the consumer. The incorporation of LAB cells into films had a no significant unwanted effects on the optical and mechanical properties. Indeed the added cells were viable during the storage period. Moreover, the sodium caseinate films containing bioactive cultures displayed an antilisterial effect during refrigerated storage.

5. Acknowledgements

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6. Conflict of Interest

The authors declare that there is no conflict of interest.

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اثرات سلول های پروبیوتیک[1] بر خصوصیات مکانیکی و ضدباکتریایی فیلم سدیم کازئینات

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چکیده

سابقه و هدف: شرایط فرآوری غذا نظیر حرارت، استرس‌های مکانیکی و اسمزی می تواند منجر به کاهش قابل توجه زندمانی زیست‌یارها در مواد غذایی شود. اخیراً، افزودن زیست‌یارها به فیلم های خوراکی به عنوان یک فناوری نوظهور برای حمل سلول‌های زیست‌یار مطرح شده‌اند. در این مطالعه، سلول‌های زنده *لاکتوباسیلوس اسیدوفیلوس* و *لاکتوباسیلوس کازئی* در ماتریکس سدیم کازئینات مخلوط شدند تا فیلمی بر پایه زیست‌یار برای بهبود ایمنی غذایی تولید شود.

مواد و روش‌ها: سلول‌های پروبیوتیک به صورت مجزا به محلول‌های تشکیل‌دهنده فیلم افزوده و فیلم‌های فعال با روش کستینگ تهیه شدند. ویژگی‌های فیزیکی، مکانیکی و نوری فیلم‌ها مطالعه گردید. ویژگی‌های نوری با دستگاه رنگ‌سنج و خصوصیات مکانیکی فیلم‌ها با استفاده از دستگاه اینسترون ارزیابی شد. زنده‌مانی *لاکتوباسیلوس اسیدوفیلوس* و *لاکتوباسیلوس کازئی* در فیلم‌ها طی یک دوره ۱۲ روزهتعیین شدند. فعالیت ضد میکروبی فیلم‌ها نیز علیه باکتری *لیستریا مونوسیتوژنز* بر روی محیط TSA در دمای ۴ درجه سلسیوس مورد بررسی قرار گرفت.

یافته‌ها و نتیجه‌گیری: نتایج نشان داد که سلول‌های باکتری‌های لاکتیک اسید (LAB) طی دوره نگهداری ۱۲ روزه زنده باقی می‌مانند (بیش از ۴ Log CFU cm^{-2}) الحاق سلول‌هایLAB به فیلم‌های پلیمری تاثیر معنی‌داری بر استحکام کششی آن نداشت ($p>0/05$)، اگرچه ظاهر آن را به طور قابل توجهی بهبود دادند ($p>0/05$). در واقع، در روز ششم نگهداری نمونه‌های پوشیده شده با فیلم های حاوی LAB، اثر ضد لیستریایی قوی‌تری نسبت به گروه شاهد داشتند($p\leq0/05$). این یافته‌ها نشان می‌دهد که فیلم‌های حاوی سلول‌های LAB می توانند به عنوان روشی بسته‌بندی موثر جدید برای بهبود ایمنی غذایی مورد استفاده قرار گیرند.

تعارض منافع: نویسندگان اعلام می‌کنندکه هیچ تعارض منافعی وجودندارد.

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واژگان کلیدی

- بسته بندی فعال
- لاکتوباسیلوس اسیدوفیلوس
- لاکتوباسیلوس کازئی
- لیستریا مونوسیتوژنز
- فیلم سدیم کازئینات

***نویسنده مسئول**

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[1]Probiotic Cells-سلول‌های زیست یار