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# *In vitro* Antimicrobial Effect of Probiotic Films Based on Carboxymethyl Cellulose-Sodium Caseinate Against Common Food-Borne Pathogenic Bacteria

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**ABSTRACT**

**Introduction:** Consumption of proper amounts of probiotic microorganisms through food products has several health benefits for the host. Recently, growing research has been focused on the characterization and verification of the potential use of probiotic films in food industry. The present study aimed to investigate the *in-vitro* antimicrobial properties of probiotic carboxymethyl cellulose-sodium caseinate (CMC-SC) films containing *Lactobacillus acidophilus*, *L. reuteri*, and *Bifidobacterium bifidum* against *Listeria monocytogenes*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Escherichia coli* O157:H7.

**Methods:** CMC-SC composite films were prepared using the casting method. The *in-vitro* antibacterial properties of the CMC-SC films were evaluated using the agar disk diffusion and broth microdilution methods.

**Results:** The antimicrobial properties of the probiotic films were as follows (inhibition zone diameter and log differences in the population, respectively): *S. aureus* (2.13-5.65 mm, -0.79--3.82)>*L. monocytogenes* (1.76-5.32 mm, -0.65--3.34)>*S. typhimurium* (2.13-4.33 mm, -0.34--2.79)>*E. coli* O157:H7 (1.88-3.86 mm, -0.18--2.62). The optimal antimicrobial effects against the mentioned bacterial pathogens were observed in the films supplemented with *L. acidophilus* + *L. reuteri* + *B. bifidum.*

**Conclusion:** According to the results, incorporation of some probiotic strains into edible films could result in remarkable antimicrobial effects against *S. aureus*, *L. monocytogenes, S. typhimurium*, and *E. coli* O157:H7, which is an effective solution to the issue of safety in the food industry.

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## Introduction

In recent decades, food industries have changes enormously for the better monitoring of food quality and safety properties as demand for 'ready-to-eat' and 'easy-to-consume foodstuffs' increases [1]. In general, consumers are interested in the purchase of fresh foodstuffs containing lower chemical preservatives and additives [2]. However, the control of pathogenic microorganisms in foods commonly requires the utilization of chemical synthetic antimicrobial agents; some of these microorganisms include *Listeria monocytogenes*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Escherichia coli* O157:H7, as well as spoilage bacteria, such as Enterobacteriaceae, *Pseudomonas* spp., *P. fluorescens*, and *Shewanella putrefaciens* [3]. Reports suggest that the growth of microbial spoilage agents on the surface/depth of fresh food products may alter the main organoleptic properties of these products (odor, color, taste, and other sensory characteristics [4]. Furthermore, it has been reported that 30-40% of the population in developed countries and 30-40% of the general population are affected by foodborne diseases each year, especially due to the consumption of foods containing *S. aureus*, *E. coli* O157:H7, and *S. typhimurium* [2]. Therefore, the maintenance of food products through the utilization of novel procedures should be investigated in order to address the concerns regarding food spoilage and safety.

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The term 'probiotics' has been proposed by the Food and Agriculture Organization (FAO) and World Health Organization (WHO), which is defined as the appropriate amount of living organisms that have numerous beneficial properties for human and animal health. Indeed, the health benefits of probiotic microorganisms are provided through the improved properties of indigenous microflora [2]. As a result, the consumption of these microorganisms through food could positively influence the health of the host by decreasing bacterial, viral, and antibiotic-associated diarrhea, irritable bowel syndrome, inflammatory bowel disease, lactose intolerance symptoms, atopic allergies, and low-density lipoprotein-cholesterol, as well as the recovery of ulcerative colitis and improvement of the immune function [5]. To date, probiotic microorganisms (especially *Lactobacillus*, *Bifidobacterium*, *Streptococcus thermophilus*, and *Saccharomyces boulardii*) have been used as additives in several foodstuffs, such as fresh or low-acid cheeses, dairy desserts, ice cream, and yogurt [6]. Moreover, previous studies in this regard have confirmed the antimicrobial activity of probiotic microorganisms in dairy products [7], seafood [8], and meat products [9].

According to the literature, probiotics could also be carried by polymeric materials, such as sodium caseinate (SC), sodium alginate, starch, and methylcellulose, which are commonly used in the food packaging industry [1, 10-12]. Among various biopolymer materials, carboxymethyl cellulose (CMC) and SC are considered to be optimal candidates in this regard. CMC is a water-soluble cellulose, which is obtained from the carboxymethyl groups ($CH_2COONa$) that are linked to some of the OH groups on the cellulose backbone [13].

Owing to their low secondary chemical structure (alpha-helix and beta sheets), caseins are random-coil polypeptides with high molecular flexibility, which enables them to develop critical intermolecular interactions [14]. Caseinates are developed through subjecting acid-coagulated casein to natural pH (6.7) using sodium, calcium or potassium hydroxide [15]. SC-based films have also been reported to be an appropriate matrix to supplement nisin-loaded liposomes and nanoclay without changing the physical and mechanical properties of the film [16]. CMC and SC enriched with antimicrobial compounds have been widely investigated regarding their ability to extend the shelf life of various food products and inhibitory effects against foodborne pathogens, especially *L. monocytogenes* and *E. coli* O157:H7 [1, 13, 16-18].

The present study aimed to evaluate the *in-vitro* antimicrobial properties of CMC-SC-based composite films containing *Lactobacillus acidophilus*, *Lactobacillus reuteri*, and *Bifidobacterium bifidum* against *L. monocytogenes*, *S. typhimurium*, *S. aureus*, and *E. coli* O157:H7 using the broth microdilution and agar disk diffusion methods.

## Materials and Methods

### *Experimental Materials*

In this study, CMC (medium molecular weight: 250 KDa) and SC powders (medium molecular weight: 200 KDa) were purchased from Sigma-Aldrich (UK). *L. acidophilus* (PTCC 1643), *L. reuteri* (PTCC 1655), *B. bifidum* (PTCC 1644), *S. aureus* (ATCC 6538), *L. monocytogenes* (ATCC 19118), *E. coli* O157:H7 (ATCC 10536), and *S. typhimurium* (ATCC 14028) were obtained from the culture collection of the Iranian Research Organization for Science and Technology (IROST) in Tehran, Iran. All the culture microbial agar/broth, solvents, and chemicals were of an analytical grade and obtained from Merck Company (Germany). In addition, distilled water was used to prepare all the media and solutions.

### *Preparation of Microorganisms*

Pathogenic and probiotic strains were cultured in the brain heart infusion (BHI) and De Man, Rogosa, and Sharpe (MRS) broth, respectively, incubated at the temperature of 37±1ºC for 24-48 hours, and stored with 20% glycerol at the temperature of -20ºC until further analysis. All the pathogenic strains were sub-cultured in the BHI broth twice, incubated as described earlier, and diluted to 7 log CFU/ml using 0.1% peptone water before further analysis. *L. acidophilus*, *L. reuteri*, and *B. bifidum* were prepared in the MRS broth at the temperature of 37±1ºC for 48 hours in aerophilic/anaerobic conditions and diluted to 11 log CFU/ml using 0.1% peptone water.

### *Preparation of Probiotic CMC-SC Films*

CMC-SC composite films were prepared in accordance with the methods proposed by Gialamas et al. (2010) [1] and Khezrian and Shahbazi (2018) [13]. Accordingly, CMC (1% w/v) and SC (5% w/v) were dispersed in sterile

distilled water and homogenized at 600 rpm at room temperature. Glycerol was applied as a plasticizer and added at 0.75 ml/g (based on the amount of CMC/SC) to both solutions, followed by the continual stirring of the mixtures for 10-15 minutes. At the next stage, the solutions were combined at the ratio of 50:50 and stirred again for one hour. Afterwards, one milliliter of each probiotic strain (11 log CFU/ml) was added to 100 milliliters of the solution and stirred again for one hour. Finally, eight groups were designated, including control film (no probiotic microorganisms), film *L. reuteri*, film + *L. acidophilus*, film + *B. bifidum*, film + *L. acidophilus* + *B. bifidum*, film + *L. reuteri* + *B. bifidum*, film + *L. acidophilus* + *L. reuteri*, and film + *L. acidophilus* + *L. reuteri* + *B. bifidum*. All the investigated films were prepared by casting approximately 50 milliliters of the mixtures on 12-centimeter glass plates and drying in ambient conditions for 48-72 hours.

### *In-vitro Antimicrobial Activity of the Probiotic CMC-SC Films*

The *in-vitro* antibacterial properties of the CMC-SC films were examined using the agar disk diffusion and broth microdilution assays [19]. In brief, 15-20 milliliters of melted BHI agar was poured into 90-millimeter sterile petri dishes, and 100 microliters of each bacterial suspension were cultured individually. Following that, the designated films (diameter: 7 mm) were placed on the surface of inoculated BHI agar. After overnight incubation at the temperature of 37ºC, the diameters of the inhibition zone were recorded.

In the broth microdilution assay, 96-well sterile microdilution plates with U-bottom wells were used by casting 180 microliters of the prepared probiotic films to form the mixtures, as well as 20 microliters of the bacterial suspension containing 7 log CFU/ml of the pathogenic microorganisms. The last row of the wells was used as a parallel positive control, which contained 180 microliters of the BHI broth with no film-forming solution and 20 microliters of the inoculum. Afterwards, the plates were sealed with sterile plate sealers. The contents of each well were shaken on a plate shaker for 30 seconds and incubated overnight at the temperature of 37°C. At the next stage, sampling was performed from each well using ten-fold serial dilutions with the BHI broth, followed by plating on the BHI agar and incubation at the temperature of 37°C for 24 hours. After incubation, the bacterial colonies were counted, and the obtained results were expressed in terms of the differences in the population (DP) using the following equation:

$$Log\ DP = log\left(\frac{N}{N_0}\right) = log\ N - log\ N_0$$

Where $N$ and $N_0$ represent the bacterial population (CFU/ml) at the $t$ (after 24 hours of incubation at 37±1 ºC) and zero time, respectively.

### *Statistical Analysis*

All the experiments were conducted in triplicate. Data analysis was performed in triplicate in SPSS version 16 (SPSS, Chicago, IL, USA) using Dunnett T3 test to compare the mean values between various products. In all the statistical analyses, P-value of less than 0.05 was considered significant.

## Results and Discussion

Tables 1 and 2 show the *in-vitro* antimicrobial properties of the probiotic films containing *L. acidophilus*, *L. reuteri*, and *B. bifidum*. Accordingly, the control CMC-SC film had no antimicrobial effects against *S. aureus*, *L. monocytogenes*, *E. coli* O157:H7, and *S. typhimurium* as evidenced by the growth of the corresponding pathogens after 24 hours of incubation (range: 0.47-1.33 log DP). Similarly, previous studies have also denoted that CMC and SC films exhibited no inhibitory effects against pathogenic and spoilage microorganisms [18,20, 21].

**Table 1.** *In-vitro* Antibacterial Activity of Probiotic Films (inhibition zone diameter in mm)

| Film | ***S. aureus*** | ***L. monocytogenes*** | ***S. typhimurium*** | ***E. coli* O157:H7** |
|---|---|---|---|---|
| Control (no probiotics) | ND | ND | ND | ND |
| *L. reuteri* | 3.71±0.22$^{b}$ | 3.51±0.01$^{b}$ | 3.06±0.04$^{b}$ | 2.87±0.31$^{b}$ |
| *L. acidophilus* | 2.96±0.01$^{c}$ | 2.49±0.03$^{c}$ | 2.13±0.14$^{b}$ | 1.88±0.02$^{bc}$ |
| *B. bifidum* | 2.13±0.03$^{c}$ | 1.76±0.02$^{c}$ | ND | ND |
| *L. acidophilus + B. bifidum* | 4.01±0.05$^{b}$ | 3.56±0.01$^{ab}$ | 3.37±0.52$^{ab}$ | 3.05±0.03$^{ab}$ |
| *L. reuteri + B. bifidum* | 4.34±0.01$^{a}$ | 4.03±0.03$^{a}$ | 3.85±0.02$^{a}$ | 3.36±0.11$^{a}$ |
| *L. acidophilus + L. reuteri* | 5.12±0.22$^{a}$ | 4.88±0.14$^{a}$ | 4.31±0.05$^{a}$ | 3.45±0.01$^{a}$ |
| *L. acidophilus + L. reuteri + B. bifidum* | 5.65±0.01$^{a}$ | 5.32±0.13$^{a}$ | 4.33±0.04$^{a}$ | 3.86±0.01$^{a}$ |

*ND: Not determined; Different lowercase letters in same column indicate significant difference (P<0.05)*

According to the results of the present study, the CMC-SC films supplemented with *L. acidophilus*, *L. reuteri*, and *B. bifidum* reduced the growth of all the pathogens with the following patterns (inhibition zone diameter and log DP, respectively): film + *L. acidophilus* + *L. reuteri* + *B. bifidum* (3.86–5.65 mm, -2.62–-3.82)>film + *L. acidophilus* + *L. reuteri* (3.45–5.12 mm, -2.24–-3.11)>film + *L. reuteri* + *B. bifidum* (3.36–4.34 mm, -1.89–-2.65)>film + *L. acidophilus* + *B. bifidum* (3.05–4.01 mm, -1.23–-2.11)>film + *L. reuteri* (2.87–3.71 mm, -0.88–-1.67)>film + *L. acidophilus* (1.88–2.96 mm, -0.76–-1.21)>film + *B. bifidum* (1.76–2.13 mm, -0.18–-0.79) (tables 1 & 2).

**Table 2.** *In-vitro* Antibacterial Activity (log DP*) of Probiotic Films

| Film | ***S. aureus*** | ***L. monocytogenes*** | ***S. typhimurium*** | ***E. coli* O157:H7** |
|---|---|---|---|---|
| Control (no probiotics) | 0.47±0.02$^{c}$ | 0.73±0.03$^{d}$ | 0.98±0.01$^{c}$ | 1.33±0.03$^{d}$ |
| *L. reuteri* | -1.67±0.03$^{b}$ | -1.23±0.02$^{c}$ | -1.09±0.05$^{b}$ | -0.88±0.02$^{c}$ |
| *L. acidophilus* | -1.21±0.01$^{b}$ | -1.11±0.05$^{c}$ | -0.95±0.02$^{b}$ | -0.76±0.03$^{c}$ |
| *B. bifidum* | -0.79±0.07$^{b}$ | -0.65±0.01$^{c}$ | -0.34±0.03$^{b}$ | -0.18±0.07$^{c}$ |
| *L. acidophilus + B. bifidum* | -2.11±0.02$^{a}$ | -1.88±0.05$^{b}$ | -1.65±0.01$^{b}$ | -1.23±0.02$^{b}$ |
| *L. reuteri + B. bifidum* | -2.65±0.06$^{a}$ | -2.32±0.02$^{a}$ | -2.11±0.03$^{a}$ | -1.89±0.04$^{b}$ |
| *L. acidophilus + L. reuteri* | -3.11±0.01$^{a}$ | -3.02±0.03$^{a}$ | -2.53±0.06$^{a}$ | -2.24±0.01$^{a}$ |
| *L. acidophilus + L. reuteri + B. bifidum* | -3.82±0.06$^{a}$ | -3.34±0.07$^{a}$ | -2.79±0.02$^{a}$ | -2.62±0.03$^{a}$ |

**Log DP= log ($N/N_0$)=log N – log $N_0$ where N and $N_0$ denote bacterial population at t and zero time,respectively; Different lowercase letters in same column indicate significant differences ($P<0.05$)*

Numerous synergistic mechanisms have been confirmed for the natural antimicrobial constituents produced by probiotic bacteria (e.g., bacteriocins), including the sequential inhibition of a common biochemical pathway, suppression of the protective enzymes, combinations of cell-wall biological agents, and utilization of active cell-wall agents to increase the uptake of other antimicrobials [2].

Previous studies have also reported the antimicrobial effects of probiotic films on various food models *in-vitro* [1, 10, 11]. For instance, Gialamas et al. (2010) [1] stated that the application of SC films enriched with *Lactobacillus sakei* in a laboratory medium (Tryptose soy agar) and a food model system (fresh beef) inoculated with *L. monocytogenes* could remarkably suppress pathogen growth compared to the controls. In another study, Pavli et al. (2017) [10] reported that sodium alginate edible films containing *Lactobacillus plantarum* B282, *L. plantarum* L125, and *Lactobacillus pentosus* L33 might act as antimicrobial materials in ready-to-eat ham slices, which rendered them proper candidates for food packaging in order to delay the growth of microbial spoilage in the treated ham samples compared to the untreated samples.

In another research in this regard, Sánchez-González et al. (2014) [11] investigated the antilisterial effects of SC and methylcellulose films supplemented with *L. acidophilus* and *L. reuteri*, reporting that the films resulted in the complete inhibition of *L. innocua* for one week in Tryptose soy agar. Moreover, other studies have confirmed the effects of lactic acid bacteria or their metabolites on the growth inhibition of pathogens in cooked ham, commercial barley soups, and raw beef patty [22-24].

Several studies have investigated the application of sodium lactate, potassium sorbate, stearic acid, and nisin in fresh foodstuffs using biodegradable films, demonstrating that the enrichment of the films with nisin could increase inhibitory activity more significantly compared to the direct addition of antimicrobial agents to a food model system [25, 26]. A common mechanism that may be responsible for such effect is product acidification through lactic acid production using lactic acid bacteria (critically *L. acidophilus* and *L. reuteri*), while another hypothesis in this regard is focused on competition for nutrients [27].

The antimicrobial properties of *B. bifidum* may be correlated with *Bifidobacterium*-associated bacteriocins, including bifidocin B, bifidin I, and lantibiotic bisin [28]. Bacteriocins are classified into major categories, including class I (lantibiotics distinguished based on post-translational modification) and class II (bacteriocins as unmodified peptides that could be divided into four subgroups of class IIa-d, with the class IIa subgroup containing the peptides that often reduce potent antimicrobial properties, with 37-48 amino acids that are positively charged) [29, 30]. Recent studies have indicated that bacteriocins affect the cell

membrane of pathogens, thereby leading to significant morphological damage, decreased pH gradient across the bacterial cell membrane, and the subsequent suppression of the bacterial barrier function [24, 30]. Antimicrobial properties of the probiotic films were as follows (inhibition zone diameter and log DP, respectively) (tables 1 & 2): *S. aureus* (2.13-5.65 mm, -0.79 - -3.82)>*L. monocytogenes* (1.76-5.32 mm, -0.65 - -3.34)>*S. typhimurium* (2.13-4.33 mm, -0.34 - -2.79)>*E. coli* O157:H7 (1.88-3.86 mm, -0.18 - -2.62). The highest antimicrobial activity of the probiotic films against gram-positive bacteria was mainly attributed to the lack of a lipopolysaccharide layer in these microorganisms [31].

## Conclusion

According to the results, the incorporation of *L. acidophilus*, *L. reuteri,* and *B. bifidum* into the CMC-SC films was a viable strategy to deliver probiotics into food products. The highest antibacterial activity belonged to the combination of film + *L. acidophilus* + *L. reuteri* + *B. bifidum*, followed by film + *L. acidophilus* + *L. reuteri*, film + *L. reuteri* + *B. bifidum*, film + *L. acidophilus* + *B. bifidum*, film + *L. reuteri*, film + *L. acidophilus* and film + *B. bifidum*, respectively. Therefore, the designated probiotic films could be used as antimicrobial compounds against common foodborne pathogens, including *S. aureus*, *L. monocytogenes, S. typhimurium*, and *E. coli* O157:H7.

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## Conflicts of interest

None declared.

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