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Evaluating the antimicrobial effect of postbiotic extract from *Lactobacillus casei* on *Escherichia coli* in commercial sterilized milk

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ABSTRACT

The antimicrobial effect postbiotic extant of *Lactobacillus casei* was evaluated against *Escherichia coli* by well propagation method and growth inhibition zone diameter was measured. The selected concentration of the postbiotic extract of *L. casei* was added to milk containing *E. coli*, followed by evaluating the changes in colony number and pH during storage (1 and 5) days at 4°C then compared with control milk. The largest growth inhibition zone was formed at a concentration of 800 µl/ml against *E. coli*. Also, the counts of *E. coli* decreased on days 1 and 5 in the evaluated milk containing postbiotic ($p \leq 0.05$). The pH of milk containing postbiotic was 6.5-6.8 ($p \leq 0.05$). The results showed that the postbiotics of *L. casei* inhibited the growth of *E. coli* in milk containing.

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1. Introduction

For the first time, the term probiotic was used in 1965 for a product excreted from one protozoan leading to the growth of another protozoan (1). Tissue extracts were also used for beneficial biological interactions, including the promotion of microbial growth.

In 1974, Parker was the first to identify probiotics as useful dietary supplements, leading to the production of healthy intestinal microbiota (2). Previous studies have provided some plausible evidence for several favorable mechanisms that have intestinal-enhancing effects (3).

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These include the term intestinal microbiota, continuous competition between the mucosa and the epithelium, functional improvement of the epithelial cell epithelial barrier, and modulation of the immune system (4). However, recent evidences suggest that the probiotic survival factors may not be necessary to achieve its health-enhancing effects, and biological compounds derived from it such as cell bodies, cell extracts, pure cell walls, and supernatants can have the same health effects and play a role through similar or different metabolic pathways (5). In this regard, a study measured the ratio of live and non-living cells of probiotic supplements, indicating that the population of non-living cells was significantly higher than living cells (6). Thus, a major part of the health effects of probiotic supplements could be related to the presence of non-living cells (Postbiotics) created during the production and maintenance of the product (7). Hence, biological compounds such as postbiotics with unique properties such as stability, non-toxicity, and safety can be introduced as an alternative to probiotics (8). The production of postbiotics usually involves cell degradation techniques such as heat and enzymatic treatments, solvent extraction, and sonication. Several steps such as extraction, centrifugation, dialysis, and freeze-drying are also used to increase the production efficiency and storage of these compounds (9). Postbiotics consist of inactivated microbial cells, including cell bodies, cell fissures (teichoic acid, peptidoglycan-derived meso peptides, cell surface proteins, and end monopoly saccharides)(10), as well as cellular metabolites (short-chain fatty acids, enzymes) (11).

Organic acids are released when microbial cells are alive or broken down in the host's intestinal tract and have health-promoting effects on the host if taken in enough amounts (12). In recent years, many scientific studies have reported the health effects of supplements containing postbiotics, while illuminating the precise functional mechanisms associated with probiotic strains, including *Lactobacillus casei* and piracies, which are cellular (13). Inactivated cells have different functional properties such as antimicrobial, antioxidant, and immune-modulating effects and are applicable in various types of pharmaceutical and food systems to achieve the goals of promoting host health (14). The protective effect of *L. casei* on the preservation of fermented foods is due to the acidic conditions that occur during the growth of bacteria in food (15). Conversion of carbohydrates to organic acids such as acetic acid, and lactic acid, along with lowering the pH, increases the shelf life and good quality of fermented foods (16). This bacterium can produce other substances such as bacitracin, hydrogen peroxide, dactyl, acetaldehyde, ammonia, and free fatty acids and inhibit the growth of many microorganisms (10, 17). They can be used as medicine if the necessary principles are observed. Because the function of *L. casei*-derived postbiotics does not depend on cell viability, their purified bacteriocins are used as biological preservatives, resulting in the longer shelf life of dairy products and food safety (18). They also have antihypertensive, antimicrobial, and immune-boosting properties by producing active peptides in dairy products.

The postbiotic derivative of *L. casei* can produce various metabolites, such as lactic acid, hydrogen peroxide, bacteriocins, dactyl, and acetone, and prevent the growth of rival bacteria, including foodborne pathogens like *E. coli* (19). Hydrogen peroxide can damage bacterial nucleic acids by increasing membrane permeability (20). Studies have shown that bacteriocins are important in preserving dairy products, including long shelf life, protection at a temperature of < 4°C, and better preservation of nutrients and vitamins (21). Also, there is now consumer pressure to reduce artificial substances and additives in foods (22). The demand for processed and fresh foods can be met in part by the use of bacteriocins because their protein compounds reduce natural antimicrobial activities by enzymatic activity (10). They can also produce enzymes due to their very high thermal stability and denaturation resistance (23). In this regard, this study aims to evaluate the antimicrobial effect of a postbiotic extract derived from *L. casei* on *E. coli* in commercial sterilized milk.

2.Materials and Methods

2.1. Bacteria Preparation and Usage

L. casei ATCC =39392 and *E. coli* ATCC= 25922 were prepared by the Scientific and Industrial Research Organization (Tehran, Iran) and activated according to these protocols.

2.2. Postbiotic Extract Production Process (Supernatant)

L. casei were cultured in MRS-broth and incubated at the temperature of 37 ± 1°C for 48 h in a CO_2 incubator. The grown bacteria were poured into a falcon and centrifuged for 20 min at 104 rpm at a temperature of 4°C to detach the postbiotics from the cells. After separating the supernatant and measuring the pH, the supernatant was placed in the dry freezer at -40°C for 24 h to get its lyophilized form. Then, the resulting lyophilized powder was stored at -80°C until use.

2.3. Method for the Evaluation of the Antimicrobial Properties of Postbiotic Extracts

2.3.1. Standardized microorganisms

Microorganisms were standardized with the 0.5 Mc Farland standard. 0.5 Mc Farland shows a cell density of 1.5×10^8 cfu/ml.

2.3.2. Well Diffusion Agar method

To evaluate the antimicrobial activity of postbiotic extract (supernatant), the *E. coli* strain was cultured by the surface culture method under standard conditions of 0.5 Mc Farland suspension. Wells were drilled on Müller Hinton agar medium with a diameter of and 4 mm depth using pipette number 5. Different concentrations of postbiotic extract (supernatant) were poured into the wells and incubated at 37°C for 24 h. The cycle appeared around the wells after the specified time and temperatures and it is diameter was measured. The diameter of the halo indicates the antimicrobial effect of the extract. The larger the halo, the more antimicrobial effects the extract will have. The diameter of the growth inhibition halo was measured by a caliper and reported in millimeters to determine the antimicrobial effect.

2.4. Inoculation of Postbiotic Extracts and Microbial Species in Milk

First, 400 ml of sterilized milk was poured into a sterile Erlenmeyer flask and used as a control sample. Then, only *E. coli* was added at a concentration of 1.5×10^8

and homogenized, followed by pouring another 400 ml of sterilized milk into another Erlenmeyer flask and testing as a sample. Both samples were incubated for 10 h and then cooled to 4-5°C and refrigerated.

2.4.1. Microbiologic Assays

The antimicrobial maintainer effective test was used to determine the antimicrobial activity of the postbiotic supernatant. The challenge microorganism was derived from the collection of the original stock cultures *E. coli*. The suspension was ready conforming to standardized methods to come to a proximate concentration of 1.5×10^{8} (cfu) per milliliter serial. Dilutions of one ml of the tested and control samples in 9 ml of 0.9 saline and dilutions up to 1×10^{-10} cfu/mL were prepared from the sample. Then 0.1 ml of each dilution in three replications was transferred to disposable plates containing an EMB culture medium to perform a surface culture. Incubation was performed at 37°C for 24 h, followed by counting the colonies. The population of *E. coli* in the inoculated sample and negative control was determined on days 1 and 5 using the pour-plate method.

2.4.2. Determining the pH

The samples pH was measured to the national standard of Iran (ISIRI No. 2852) using a pH meter on days 1 and 5. First, the pH meter was calibrated for pH 4 and 7. The pH-meter electrode was placed inside the sample for at least 45 s after adjusting and rinsing the electrode with distilled water and final drying. Ultimately, the pH of the sample (in three replications) was reported.

2.5. Statistical Analysis

All experiments were conducted in triplicate. The data were reported as Mean ± Standard Deviation for the measurements of prohibition zones in the antimicrobial activity tests, and one-way (ANOVA) was used to compare the mean values ($p\leq 0.05$).

3. Results

3.1. Antimicrobial Activity

Plates containing Müller Hinton agar were prepared to test antimicrobial activity. The wells were then drilled at a distance of three millimeters and filled with postbiotic extract (supernatant) at concentrations of 800, 600, 400, 200, and 0 µl/ml. A normal halo grew and appeared after incubation for 24 h. The diameters of non-growth halos against *E. coli* were 36, 27, 35, 29, and 0 mm **(**Fig. 1**).** According to the chart, the greatest inhibitory concentration of the postbiotic extract of *L. casei* for *E. coli* was 800 mg/ml.

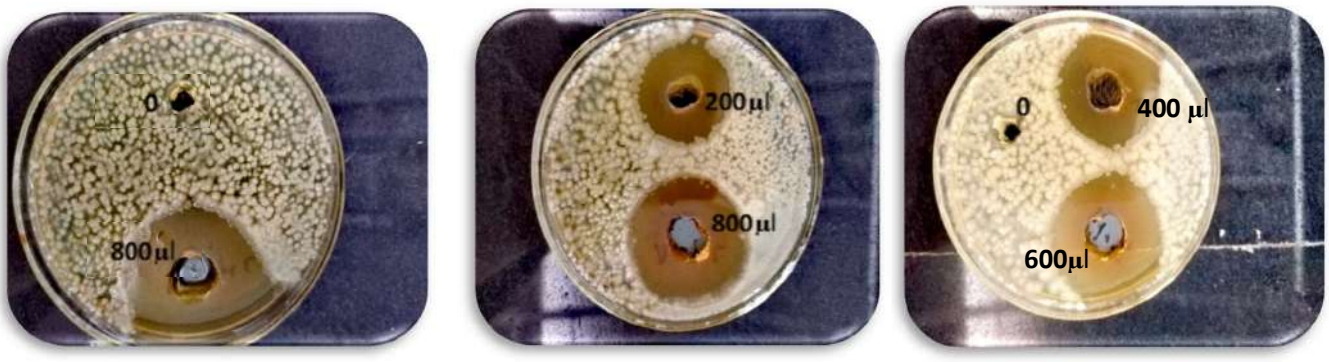

Figure 1. Antimicrobial effects of postbiotic extract (supernatant) of *L. casei* at different concentrations against *E. coli* by well method.

Table 1. Analysis of variance to evaluate the antimicrobial properties of different levels of the postbiotic extract against *E. coli* by the good method.

P-Value	F-Value	MC	df	Source
≤0.05	22.36	176.6	1	**The amount of extract from bacteria**
-	-	48.01	1	**Coefficient of variation (CV)**

Table 2. Analysis of variance of *E. coli* count in milk with and without postbiotic.

P-Value	F-Value	MC	df	Source
≤0.05	2832.17	2.30	1	**Postbiotic**
≤0.05	115.64	9.39	1	**Day**
≤0.05	117.61	9.54	1	**Postbiotic × Day**

Table 3. Mean, standard deviation, and standard error of the number of *E. coli* in the studied crops (CFU/ml), number of replicates: N, a, b, c.: In each column, the difference between the means without common letters is significant, and the difference between the means with common letters is insignificant.

	Day 1 (cfu/ml)				Day 5 (cfu/ml)			
Type of cultivation	**N**	**Mean**	**SD**	**SE**	**N**	**Mean**	**SD**	**SE**
E. coli	10	97.36×10^{6}	3.4×10^{6} a	1.78×10^{6}	10	116×10^{8} a	5.9×18^{8}	3.8×10^{8}
***E. coli* + Postbiotic**	10	66.70×10^{6}	0.127 a	0.052	10	17.2×10^{8} b	0.39×10^{8}	0.16×10^{8}

According to the results of the analysis of variance at a significance level of 95%, with increasing the concentration of the extract (supernatant), the diameter of the growth inhibition zone also increased (p≤ 0.05). (Table 1).

3.2. Variation in the Number of Bacteria in Milk Samples During refutation

In this study, 800 mg/ml of the postbiotic extract was studied against *E. coli* in milk samples on days 1 and 5. Hence, one milliliter of each dilution in three replications was transferred to disposable plates with the specific culture medium of *E. coli* EMB after preparing successive dilutions by the surface method. The plate containing the bacterial sample was incubated at 37°C for 24 h. (Table 2)

milk containing postbiotic extract (supernatant) from *L .casei* had the lowest number of *E. coli* compared to the control sample (p≤ 0.05). (Fig. 2).

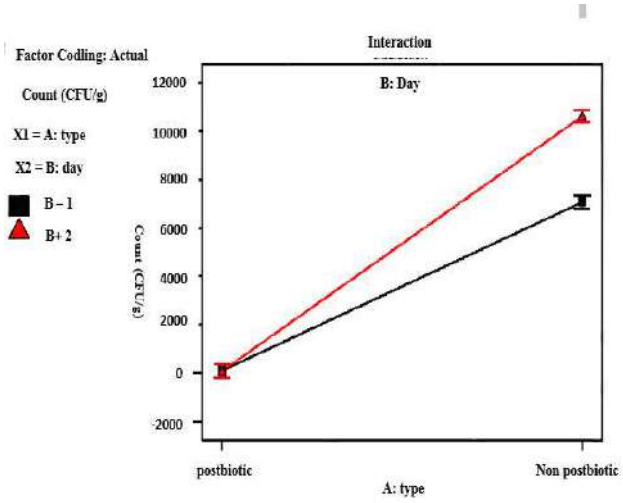

Figure 2. Evaluation of the effect of milk containing postbiotic against *E. coli* and milk without postbiotic against *E. coli* during storage

Milk containing postbiotic extract of *L. casei* inhibited the growth of *E .coli* in the first and the fifth days. However, in postbiotic-free milk extract, the number of *E. coli* increased in both the first and the fifth days. The growth of *E. coli* in each milk sample on the first and the fifth days was shown in Table 3. The population of *E. coli* in milk samples containing postbiotic extract decreased at a temperature of 4°C compared to the control sample (p≤ 0.05). The mean number of living *E. coli* cells in the milk sample containing postbiotic extract was on days 1 and 5 respectively.

3.3. pH Variation in Milk Samples During storage

The pH of all milk samples containing 800 µl/ml postbiotic and samples without postbiotics was evaluated on days 1 and 5. The experiment was factorial in a randomized complete block design with three replications (Table 4).

Table 4. Analysis of pH variance of milk with and without postbiotics against *E. coli.*

P-Value	F-Value	MC	df	Source
≤0.05	38.640	41.22	1	**Postbiotic**
≤0.05	67.4	16.0	1	**Day**
≤0.05	42	47.1	1	**Postbiotic× Day**

Based on the results samples of milk containing postbiotic extract (supernatant) and control samples, had a significant difference on days 5 and 1 (p≤ 0.05) (Fig. 3).

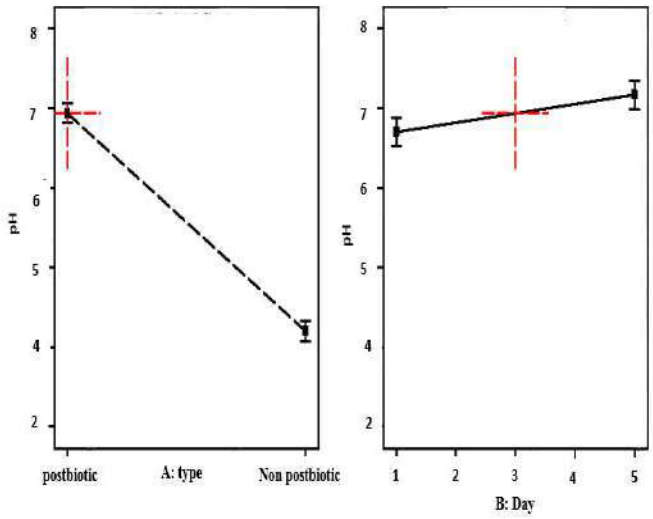

Figure 3. A: pH changes in milk containing postbiotic and *E. coli*.
B: pH changes in milk without postbiotic and only *E. coli*.

On the first day showed that the pH of the sample without postbiotic was lower than the sample containing postbiotic. This comparison on day 5 showed that the sample without postbiotics had the lowest pH value compared to the sample containing postbiotics. In general, based on the obtained results, the pH of the sample containing postbiotic was 1 ($p \leq 0.05$), which showed the growth inhibitory effect on *E. coli*. Although (*Escherichia coli*) can grow in a neutral pH medium, in this study, the presence of postbiotics has prevented the growth of this bacterium in a neutral medium. During the first and the fifth days, the pH of milk containing postbiotic extract (supernatant) did not decrease, indicating the effect of postbiotic and inhibition of E. coli growth. In general, there was a significant difference between the control sample and the postbiotic sample in the present study ($p \leq 0.05$).

4. Discussion

In recent decades, bioactive compounds such as probiotics, prebiotics, and postbiotics have attracted much attention. These compounds are related to the beneficial gut microbiota and promote the health of the host (24). Postbiotics are also functional parts of living probiotics that can perform the biological and physiological functions of their living parent cells (25, 26). Postbiotics usually exert their health and therapeutic effects through similar or different mechanisms of action from their parent cells (27). Postbiotics are less sensitive than probiotics to processing conditions, environmental factors, and gastrointestinal conditions, which adds to their economic viability (28). *E. coli* is one of the most common causes of food infection. It is also a common pathogenic bacterium between humans and animals and one of the most important bacterial agents transmitted from food (29). Because this bacterium has become resistant to antibiotic treatment, non-antibiotic antimicrobial methods such as probiotics and post-antibiotics are of great interest (30). The antimicrobial effect of *L. casei* supernatant can be attributed to the production of lactic acid because the environment becomes acidic during its growth, and pathogenic bacteria are sensitive to acidic conditions and are killed in low acidity (31).

According to the results, different concentrations of postbiotic extract (supernatant) influenced the growth of *E. coli*. The study showed that higher concentrations of postbiotic extract (supernatant) led to a larger diameter of the growth inhibition zone (32). reported that the smallest inhibitory concentrations of *L. casei* supernatant, cell wall, and cytoplasmic extract of *L.*

casei had the greatest antimicrobial impacts on *E. coli* with gastrointestinal infections (33). This study reported that the various concentrations of the postbiotic extract had an inhibitory effect on the growth of *E. coli*. The findings are consistent with a 2020 study by Mantziari et al. examined the antimicrobial effects of Lactobacillus species on *E. coli* (34), and based on the results, the evaluation of the antimicrobial properties of the postbiotic extract (supernatant) against *E. coli* was confirmed (35). Thus, the extraction and use of postbiotics, both in the laboratory and in the food industry, can prevent the growth of pathogenic microbes, increases the shelf life, and delay the spoilage of food products (36).

In this study, milk was selected as the base food medium in which the postbiotic extract (supernatant) of *L. casei* was added. The results indicated that the presence of postbiotic at a concentration of 800 µl/ml inhibited the growth of *E. coli* in commercial sterile milk. This finding is consistent with the results of Karimi et al (37), who introduced mechanisms related to the inhibitory effect of Lactobacilli by producing short-chain active fatty acids as well as consuming nutrients and bacitracin's in may suppress the pathogenic bacteria dairy products(38). It was also reported in this study that higher concentrations of postbiotic extract (supernatant) of *L. casei* in milk led to a longer shelf life at refrigerator temperature.

Based on the results, the pH in the control milk sample without postbiotic and with only *E. coli* decreased to 3.8 on the fifth day. In contrast, in the tested milk sample containing postbiotic, the pH remained around 7.2. In other words, the pH of milk samples containing postbiotics and *E. coli* was about 6.5-7.2 until the fifth day, which was significantly higher than the pH of the milk sample without postbiotics which was 3.5-5 ($p \leq 0.05$).

According to the results obtained from the antimicrobial properties of postbiotic extract (supernatant) at a concentration of 800 µl/ml in milk containing *E. coli*, the hypothesis regarding the addition of postbiotic extract (supernatant) to milk against *E. coli* was confirmed. Therefore, the use of postbiotic products in the food industry can prevent and lead to long-term storage of milk.

5.Conclusions

Today there is an increasing attention to the effects of probiotics determined using microbial metabolites as bioactive metabolites. Postbiotics are considered soluble agents (metabolic by-products), which include proteins, peptidoglycan peptides, cell surface proteins, polysaccharides, and organic acids. Due to their obvious chemical properties, postbiotics have a safe dose, long life, and anti (inflammatory, inflammatory, hypertensive) and antioxidant impacts.

Conflict of interest

The authors declare no conflict of interest.

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