Postbiotics secreted by *Lactobacillus sakei* EIR/CM-1 isolated from cow milk microbiota, display antibacterial and antibiofilm activity against ruminant mastitis-causing pathogens

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**ABSTRACT**

Mastitis is one of the most important and multi-factorial diseases affecting dairy cows. This study was conducted to evaluate the effect of postbiotics secreted by lactic acid bacteria on mastitis. Within this aim, a total of 10 raw milk samples of healthy dairy cows were processed for the isolation of microbiota-derived bacteria. Following the isolation protocol, 24 isolates showing phenotypic characters similar to *Lactobacillus* species on specific MRS agar were selected and screened for their antibacterial activity against important mastitis-causing pathogens using the agar well diffusion method. According to the screening of the isolates, only one strain was found effective against all tested pathogens and selected for further assays. The isolate that exhibited a remarkable inhibitory effect was then identified as *Lactobacillus sakei* by 16S rRNA sequence analysis. The minimum inhibitory concentration of the postbiotics secreted by *L. sakei* into the culture medium was defined between the range of 40–50 mg/L. The antibacterial activity was also determined to be an acid-dependent effect, and HPLC analysis confirmed the presence of lactic acid (21.42 mg/mL) as the most common acid in the postbiotics. Among the 37 fatty acids, only oleic acid, palmitic acid, and stearic acid were detected. In addition to its antibacterial activity, postbiotics significantly reduced the biofilm formation of pathogens following the co-incubation, pretreatment, and eradication assays, confirmed by confocal-laser and scanning electron microscopy. In conclusion, postbiotics with antibacterial and antibiofilm activity against important mastitis-causing pathogens can be used as a promising and natural agent in the prevention of mastitis.

**HIGHLIGHTS**

- *Lactobacillus sakei* EIR/CM-1 was isolated from Holstein cow’s milk microbiota.
- Postbiotics from the strain EIR/CM-1 displayed the highest antibacterial activity against ruminant mastitis-causing pathogens.
- Co-incubation with postbiotics at 5 mg/mL and above concentrations reduced the biofilm formation of ruminant mastitis-causing pathogens by more than 70%.
- Lactic acid was found to be the highest organic acid in postbiotics.

**Introduction**

The dairy industry constitutes the largest and the most dynamic part of the agricultural economy in many countries (Borawski et al. 2020). In the global dairy market, cow milk is one of the most widely consumed products in the world, not only in liquid form which made up the largest share in terms of market value but also as cheese, yogurt, or butter. Although 497 million metric tons of cow milk were produced worldwide in 2015, its volume has reached around 532 million metric tons by 2020. The European Union is one of the top producers of the world after the United States and produced more than 157 million metric tons of cow milk in 2020 with over 22 million dairy cows. When statistical data are evaluated in terms of the annual consumption of fluid cow milk worldwide, it was declared that European Union consumed the most cow milk at 33.4 million metric tons after India, in 2020. Due to the rapid increase of the world population, the global dairy market is expected to increase significantly in the coming years (Shahbandeh 2021).
In view of its importance in the overall human diet, milk and milk products are an excellent source of valuable nutrients including a complex combination of macronutrients, micronutrients, and growth-promoting factors, such as caseins, bioactive peptides, fatty acids or polar lipids, lactose, calcium, phosphorous, magnesium, and vitamin D (Zhang et al. 2021). With providing a rich amount of these nutrients, milk has a wide range of important impacts on human metabolism and health with its anti-inflammatory (Panahipour et al. 2019), anti-oxidant (El-Fattah et al. 2019), anti-carcinogenic (Cichosz et al. 2020), and anti-osteoporosis (Fatchiyah et al. 2015) effects. Besides, beneficial associations were also declared for blood pressure and cardiovascular disease, Alzheimer’s, type 2 diabetes, obesity, and bone health (Willett and Ludwig 2020; Zhang et al. 2021).

Considering the importance of milk not only in our diet but also in the dairy industry, the growing global demand is driving the need to increase the average milk yield per cow (Cobirka et al. 2020). However, mastitis defined as an inflammation of the udder generally caused by infection is one of the most important diseases affecting the milk yield, milk composition, and its hygienic quality which lead to serious economic losses and public health considerations in the livestock and dairy industry, globally. As mastitis is considered a complex disease, current approaches for its treatment and control mostly involve the use of antibiotics to clear the quarter of the causative organisms (Pašca et al. 2020). However, serious specific pathogens that cause mastitis are mostly resistant to antibiotics (Holko et al. 2019). Besides, the extensive use of antibiotics develops resistance and consequent non-responsiveness to antibiotic therapy. Another important issue is the dramatic decrease in organic milk production depending on the unsuitable components and/or antibiotic residues (Pašca et al. 2020). Recent studies have been focussed on therapeutic regimens, such as immunotherapy, nanoparticle technology-based therapy, stem cell therapy, bacteriotherapy known as probiotics, bacteriophages, antimicrobial peptides, such as bacteriocins, in the treatment of mastitis (Benić et al. 2018; Sharun et al. 2021). Although several strategies have been evaluated over the years for mastitis treatment, most of the approaches lacked the efficacy for the elimination of the aetiological agent when used as a monotherapy (Sharun et al. 2021). Therefore, the development of natural and efficient formulations which can replace conventional techniques and solve the problem of emerging antibiotic resistance is still essential for the effective prevention and control of bovine mastitis.

Recent studies indeed suggested that beneficial bacteria in milk are important for the quality of resulting dairy products and for the health of the host (Albonico et al. 2020). To date, probiotics known as beneficial bacteria have been used for many therapeutic applications as well as controlling and treating mastitis as a low-cost and effective method. The two strains of lactic acid bacteria isolated from bovine milk were identified as Lactococcus lactis subsp. lactis CRL 1655 and Lactobacillus perolens CRL 1724, and exhibited a broad inhibitory activity against bovine mastitis-causing pathogens (Pellegrino et al. 2019). L. casei BL23 was found to have the immunomodulatory potential on bovine mammary epithelial cells which were infected with Staphylococcus aureus (Souza et al. 2018). Another strain of L. casei inhibited the invasion of bovine mammary epithelial cells by bovine mastitis-causing pathogens via inhibiting the adhesion sides (Bouchard et al. 2013). However, there are still some important concerns of probiotics for their viability, effectiveness, and safety on the target (Kothari et al. 2019). Apart from probiotics, postbiotics known as released by or produced through the metabolic activity of the microorganisms can also be utilised for the treatment of mastitis. Nowadays, postbiotics released by or produced through the metabolic activity of the microorganisms, have gained increased attention due to their beneficial effects on the host, directly or indirectly (Tsilingiri and Rescigno 2013; Żółkiewicz et al. 2020). Mainly lactic acid bacteria-derived crude extracts exhibited inhibition against mastitis-causing pathogens. It was demonstrated that postbiotics derived from L. plantarum, L. paraplan tarum, and L. salivarius presented an in vitro antimicrobial activity against isolates of Methicillin-resistant Staphylococcus aureus (MRSA), Streptococcus dysgalactiae, and Streptococcus uberis (Kang et al. 2017; Sangle et al. 2018). In view of this information, postbiotics as biologically active molecules with multi-functional properties can be designed as a novel, attractive, therapeutic, and preventive strategy in the prevention of mastitis. With the demand for new, natural, and safe products, this study aimed to analyse the potential of postbiotics derived from cow’s milk microbiota, in the treatment and control of mastitis.

Materials and methods

Bacterial strains and growth conditions

In this study, methicillin-resistant Staphylococcus aureus (MRSA) ATCC 43300, Streptococcus agalactiae ATCC 27956, and Streptococcus dysgalactiae subsp.
**Isolation of bacteria from cow milk microbiota**

Milk samples were obtained from lactating Holstein cows \( n = 10 \) housed in a single herd located in Ankara (Turkey). The experimental protocol was approved by the Ankara University Institutional Animal Care and Use Committee (protocol number 2017-7-60). Permission was also received from the owners of the animals to conduct and publish this research. Considering the number of animals approved for use with the decision of the ethics committee, a total of 10 raw milk samples were collected from Holstein cows between the ages of 4–6 (3rd–5th lactation period) which did not use antibiotics or similar substances in the last three months. Milk samples were collected during the early lactation period according to the rules of the National Mastitis Council (Oliver et al. 2004). Before sampling, the California mastitis test has been conducted to check the presence of mastitis, and the negative and clinically healthy animals were included in the experiments. Following the cleaning and disinfection of the udder end with 70% alcohol by the experienced veterinarian, the initial three streams of milk were discarded. Subsequently, \( \sim 5 \) mL of milk sample from one quarter per cow was collected into sterile screw-capped bottles placed in an ice bath under aseptic conditions and immediately transported to the laboratory (a period no longer than 2 h).

Beneficial bacteria were isolated from fresh and raw cow milk using microbiological methods. For bacteriological analysis, milk samples were serially diluted in saline solution, then each dilution was poured plated on De Man, Rogosa, and Sharpe agar (MRS) plates. Following the incubation at 37°C for 48 h, three colonies per milk sample showing phenotypic characters similar to *Lactobacillus* species on species-specific MRS agar media were selected and then tested for catalase and Gram-stained. Colonies that were presumed to be lactic acid bacteria were then subcultured onto fresh MRS agar to get pure isolates. Pure cultures were finally maintained in MRS broth supplemented with 50% glycerol at −80°C, for long-term storage.

**Molecular identification**

Molecular identification has been conducted according to the sequencing of the 16S ribosomal-RNA (16S rRNA) subunit gene region. Briefly, genomic DNA was isolated using a commercial kit (Qiagen, Germany) according to the manufacturer’s instruction, and the 16S rRNA was amplified through the polymerase chain reaction (PCR, Thermal Cycler, Bio-Rad, USA) using the universal primers [27F (5’-AGAGTTTGATCCCTGCGACT-3’) and 1492R (5’-GTTACCTTGTTACGACTT-3’)] (Lane 1991). The PCR product was then purified from the agarose gel using a purification kit (Qiagen, Germany) and sequenced by the commercial services of BM Labosis Inc (Ankara, Turkey). Obtained sequences were analysed using the Basic Local Alignment Search Tool (BLASTn) to identify the highest nucleotide sequence similarity within the GenBank database.

**Screening of the isolates for their inhibitory activity**

To determine the antibacterial activity, postbiotics as derivatives of randomly selected isolates were tested against ruminant mastitis-causing pathogens by the agar well diffusion method (Savadogo et al. 2004). Postbiotics secreted into the culture medium were obtained from the overnight cultures of each isolate following the centrifugation (4000 g, 20 min, 4°C). Filter-sterilised (0.45 μm pore size; Millipore, Burlington, MA, USA) postbiotics were then lyophilised (freezing conditions of −20°C, the vacuum pressure of 0.120 mB and a condenser temperature of −58°C; Christ freeze dryer, Germany), and dissolved in ddH2O. The obtained solutions including postbiotics were spotted on the agar plates overlayed with each pathogenic strain adjusted to 0.5 McFarland standard (Biomeireux, France), respectively. Following the incubation at 37°C for 24 h, plates were examined for inhibition zones and the diameters were measured as mm. Isolate exhibiting the maximum inhibition zone [ranked as high (>15 mm)], was selected for further analysis.

To find out whether a bacteriocin-like compound or organic acids played a role in the antibacterial activity against the pathogens, the inhibitory effect was also analysed following the treatment of the postbiotics with the 1 mg/mL proteinase K and 1 mg/mL catalase, and neutralisation, respectively (Bhunia et al. 1988). Their effects on the antibacterial activity were tested as described before.
**Determination of minimum inhibitory concentration (MIC)**

Minimum Inhibitory Concentration (MIC) of the lyophilised postbiotics against all pathogenic strains were determined using microtiter plate assay, according to Clinical and Laboratory Standards Institute guidelines (Clinical Laboratory Standards Institute 2012). Two-fold dilutions of postbiotics (1.024–0.25 mg/L) and the overnight culture of each pathogen, adjusted to 0.5 McFarland, were added to each well in the microtiter plate, respectively. Wells containing only bacteria but not postbiotics were used as the positive control, while wells only including the medium were used as the negative control. Following the incubation period at 37°C for 24 h, bacterial growth was measured spectrophotometrically at absorbance 620 nm using a microplate reader (BioTek, USA). Results were compared with the controls and the lowest concentration that completely inhibited the bacterial growth was determined as MIC value (Clinical Laboratory Standards Institute 2012).

**Assessment of antibiofilm activity**

Biofilm formation was assessed in bottom polystyrene 96-well microplates (LP Italiana, Italy) according to the crystal violet binding methodology of Stepanovic et al. (2000) with slight modifications. The overnight cultures of each pathogen adjusted to 0.5 McFarland standard were added into the microplate wells containing modified TSB medium supplemented with 0.5% glucose and 3% NaCl for MRSA 43300 and TSB medium supplied with 2.0% glucose for S. agalactiae and S. dysgalactiae which were determined as the optimised culture conditions for their ideal biofilm formation with our previous studies (Onbas et al. 2019). Following the incubation period at 37°C for 24 h, the planktonic counterparts were removed by rinsing the wells three times with physiological serum solution (0.85% NaCl) under aseptic conditions. Then wells were fixed with 200μL of 95% methanol for 15 min and stained with 200μL 0.1% crystal violet solution. After 30 min incubation period at room temperature, wells were re-washed with distilled water for removing the unbound dye and then air-dried. Finally, the biofilm-bound dye was resolved with acetone: ethanol (30:70 v/v) solution and each biomass was quantified by measuring the absorbance at 595 nm using a microplate reader (BioTek, USA). Modified TSB media without pathogens were used as the negative control.

Antibiofilm activity of postbiotics against ruminant mastitis-causing pathogens was evaluated within three approaches: Co-incubation (1); pre-treatment (2), and post-treatment (eradication) (3) assays (Onbas et al. 2019). In all assays, the modified medium without pathogen was used as the negative control, whereas the modified medium including only the pathogenic strain was used as the positive control. For the first strategy, pathogens adjusted to 0.5 McFarland standard in modified TSB medium were co-incubated with different concentrations of postbiotics and with its viable cells in microplate wells, for 24 h at 37°C, separately. Biofilm formation was then analysed as described before and calculated as a percentage inhibition with the formula of \(\frac{[C - B] - (T - B)]}{[C - B]} \times 100\) (C, well including the pathogenic strain; B, well including the modified TSB medium; T, well including the pathogenic strain and postbiotics, together).

For the pre-treatment assay, different concentrations of postbiotics were added to the microplate wells and incubated 24 h at room temperature. After the incubation period, postbiotics were removed and each well washed with phosphate-buffered saline (PBS, pH:7.2), then pathogenic strains adjusted to 0.5 McFarland standard in modified TSB medium were added to each well, separately. For the eradication assay, pathogenic bacteria were grown in microplate wells at 37°C for 24 h. Following the aspiration and removal of planktonic cells from each well, different concentrations of postbiotics were added. After the incubation period at 37°C for 24 h for each assay, biofilm formation and its inhibition were analysed as described before.

**Biofilm evaluation by microscopy**

To investigate the effects of postbiotics on bacterial biofilm matrix, the most effective approach which was selected considering the biofilm assays was performed on glass-made coverslips and then analysed by LIVE/DEAD BacLight Bacterial Viability Kit (L7007, Thermo Fisher, USA) according to the user manual. Imaging was performed with a confocal laser scanning microscope (a Plan-Neofluar 40X/1.3 DIC objective; Carl ZeissMicroscopy). The green and red fluorescence of SYTO 9 and propidium iodide (PI) was excited with an argon laser beam at 488 nm (5% intensity) and a helium/neon source at 543 nm (5% intensity), respectively. To separate the two fluorochromes, the emitted fluorescence was recorded at BP 488/543 nm and LP 585 nm in two different diachronic mirrors. For each biofilm sample, two (x and y only: 230.34 × 230.34 μm) or three (x: 230.34 μm, y: 230.34 μm, and z: 1 μm)
dimensional stacks of horizontal plane images of areas were acquired. 3-D projections of the biofilm structure were derived using the software Carl Zeiss Zen 3.3 (version Blue). The effects of postbiotics on bacterial biofilm matrix were also visualised using Scanning Electron Microscopy (SEM, Zeiss, EVO 40) according to Kiran et al. (2015).

**Characterisation of the metabolites in postbiotics**

Organic acids, fatty acids, vitamins, and amino acids in postbiotics were detected by chromatographic tools and analysed by LabSolution Data Acquisition and Post-run software systems (Shimadzu, Kyoto, Japan). Organic acids (lactic acid, oxalic acid, tartaric acid, malic acid, maleic acid, succinic acid, formic acid, acetic acid, citric acid, and butyric acid) were determined by establishing a high-performance liquid chromatography (HPLC, Shimadzu Prominance Series-LC-20A, Kyoto, Japan) using an 87H-3 column (250 × 4.6 mm, 5 µm; Transgenomic; USA). 0.08 N H₂SO₄ solution was used as a mobile phase with an isocratic pump program, and the substances were detected with a UV-visible detector (210 nm). The column oven temperature was set at 37°C and the flow rate was set at 0.6 mL/min. For quantification of the organic acids in the postbiotics, standard calibration curves obtained from the standard organic acids supplied by Merck (Germany) were used.

For the analysis of fatty acids in the postbiotics, Gas chromatography-mass spectrometry (GC-MS) based analytical strategies have been carried out following the preparation of Fatty Acid Methyl Esters (FAMEs) (Wong et al. 2015). The FAMES were analysed on a capillary column (100 m × 0.25 mm × 0.25 µm; Restek, RTX2560) using GC-MS (Shimadzu QP2020, Kyoto, Japan). Operating conditions were as follows: injector, 225°C; ion box, 225°C; carrier gas, helium; flow rate, 0.75 mL/min; split ratio, 20:1. Calibration curves obtained from the reference standards of FAMEs (Restek 35077, Food Industry FAME Mix, Restek, USA) were used for the normalisation, calibration, and quantitation purposes of FAMES.

Multi-vitamin analysis including water-soluble and water-insoluble vitamins was performed on a highly selective and sensitive high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) system (Shimadzu LC-MS 8045, Kyoto, Japan) with a column biphenyl (50 × 2.1 mm, 1.8 µm, Restek, USA) at a flow rate of 0.3 mL/min. The mobile phase was a multistep linear solvent gradient system consisting of (A) 0.1% formic acid in water and (B) methanol. All standards (Merck, Germany) and 10 mg postbiotics were diluted in methanol, filtered with 0.22 µm Teflon filter (GLScience, Japan), and injected as 1 µL. The calibration curves obtained from standard vitamins were used for calibration and quantitation purposes of vitamins in the postbiotics.

HPLC with an RF-20AXS fluorescence detector (Shimadzu Prominance Series, LC-20A, Kyoto, Japan) has been used to analyse the amino acid content of postbiotics. One gram of lyophilised postbiotics was dissolved in 100 mL of 0.1 N HCl. Derivatisation has been carried out by mixing the solutions with 90 µL of 3-Mercaptopropionic acid, 44 µL of ortho-phthalaldehyde, and 20 µL of 9H-fluorenly methylxocarbony. The separation was performed using a mobile phase [potassium-phosphate buffer (20 mM, pH: 6.9), and acetonitrile/methanol/water (45:45:10%), respectively] with a gradient pump program, and with an InertSustain C18 analytical liquid column (150 × 4.0 mm, 3.5 µm; GLScience, Japan). The flow ratio of the mobile phase was 1.0 mL/min, and the system worked under the controlled temperature at 35°C. The wavelengths used for detection were between 266 nm for excitation and 450 nm for emission. Data were analysed by comparison with the calibration curve of reference standards (Sigma, USA).

**Statistical analysis**

All assays were performed with three independent experiments (biological replicates) and each measurement was carried out in triplicate (technical replicates). Data were analysed using SPSS version 22.0 (IBM, New York, NY, USA), by one-way analysis of variance (ANOVA) followed by Dunnett’s test and unpaired t-test (GraphPad Prism v.3.0, GraphPad Software, San Diego, CA, USA). All results were presented as a mean ± standard deviation and p < .05 was used to indicate a significant difference.

**Results**

**Antibacterial activity**

In the present study, no bacteria were grown in two raw milk samples, following the isolation protocol. On the other hand, three colonies/per raw milk sample (a total of 24 colonies) showing phenotypic characters similar to Lactobacillus species on species-specific MRS agar media were selected for further analysis. All the isolates displayed no catalase activity and were found Gram-positive. Twenty-four isolates from Holstein cow’s milk microbiota were then tested for their
antibacterial activity against ruminant mastitis-causing pathogens. According to our results, nine of 24 isolates showed no inhibition, while twelve isolates were found inhibitory to at least one or two pathogenic strains. Overall, three of 24 isolates displayed a weak to strong activity against all of the tested bacteria (Supplementary Table 1). Among the isolates, only one rod-shaped and Gram-positive isolate exhibited a remarkable inhibitory effect against all tested pathogens was selected for further analysis. Its highest antibacterial activity has been observed with an inhibition zone of 20 ± 0.4 mm against *S. agalactiae* ATCC 27956, 19 ± 0.6 mm against *S. dysgalactiae* subsp. *dysgalactiae* ATCC 27957, and 17 ± 0.8 mm against MRSA ATCC 43300 (Figure 1). MIC value of the postbiotics secreted into the culture medium of the selected isolate was determined as 50 mg/mL for MRSA ATCC 43300 and 40 mg/mL for the rest of the two pathogens tested.

The effects of postbiotics were also analysed in different ways to find out the components, such as organic acids, hydrogen peroxide, or bacteriocin-like compounds responsible for the antibacterial activity. Although the inhibitory effect was not affected by proteinase K and catalase treatment, the activity was disappeared after neutralisation (data not shown), which may be related to their acidity, since the postbiotics had a pH of 4.01.

**Molecular identification**

The selected isolate for further analysis was identified according to its 16S rRNA gene region (1.492 bp) sequencing followed by the BLAST search against GenBank Bacteria and Archaea 16S ribosomal RNA sequence database. According to the results obtained, the isolate most closely matched with *Lactobacillus sakei* with a similarity of 99% and registered in the National Centre for Biotechnology Information (NCBI) as *Lactobacillus sakei* EIR/CM-1 under the accession number of MW600530.

**Antibiofilm activity**

The antibiofilm activity of postbiotics against ruminant mastitis-causing pathogens was evaluated within three approaches and the results were summarised in Table 1. Following the co-incubation protocol, the use of 7.5 mg/mL and above doses of postbiotics were found sufficient to inhibit the biofilm formed by all pathogens more than 85%. Pre-treatment with 7.5 mg/mL and above doses of postbiotics were also reduced the biofilm formation of *S. agalactiae* ATCC 27956 and *S. dysgalactiae* subsp. *dysgalactiae* ATCC 27957 with the range of 86.44 ± 2.18% and 95.10 ± 1.89%. Conversely, post-treatment of postbiotics were failed to abolish the biofilm formation. In addition to the data given in Table 1, biofilm formation rates of the mastitis-causing pathogens following the treatment with postbiotics secreted by *L. sakei* EIR/CM-1 were shown in Figure 2. Although all pathogens were regarded as strong biofilm producers, they lost their ability to form biofilm and became no biofilm producer when co-incubated with the sub-MIC value of postbiotics secreted by *L. sakei* EIR/CM-1 (Figures 2(a–c)). Remarkably, the lowest dose (1.25 mg/mL) used in this study was found to be significantly most effective to *S. agalactiae* ATCC 27956 than MRSA ATCC 43300 (*p* < .001). However, most of the tested concentrations except 2.5 and 1.25 mg/mL were also found to be effective on the inhibition of MRSA 43300 biofilm formation (*p* < .05). When the results of the pre-treatment assay were evaluated, it was seen that the biofilm formation rates were correlated with the results of the co-incubation assay (Figures 2(d–f)). Upon pre-treatment with

![Figure 1](image-url). Evaluation of the antibacterial activity of postbiotics secreted by *Lactobacillus sakei* EIR/CM-1 against mastitis-causing pathogens, by agar well diffusion method.
postbiotics in varying concentrations between 25 and 7.5 mg/mL for 24 h, *S. agalactiae* ATCC 27956 and *S. dysgalactiae* subsp. *dysgalactiae* ATCC 27957 lost their ability to form their biofilm (>90%). Nevertheless, <50% biofilm removal was detected for MRSA ATCC 43300 upon pre-treatment with doses of 5 mg/mL and below. In contrast to the co-incubation and pre-treatment approaches, postbiotics secreted by *L. sakei* EIR/CM-1 were found less effective for biofilm eradication (Figures 2(g–i)). No valuable biofilm reduction was observed for *S. agalactiae* ATCC 27956 and *S. dysgalactiae* subsp. *dysgalactiae* ATCC 27957 following the post-treatment assay. Besides, significant biofilm eradication (70.34 ± 4.20%) for MRSA ATCC 43300 was determined when the 2X MIC value of postbiotic concentration has been used (*p* < .05) In all biofilm experiments, MRS was used as control and no inhibitory effect on biofilm reduction of all pathogens tested, was observed.

In addition to the postbiotics secreted by *L. sakei* EIR/CM-1, the effects of its viable cells on biofilm reduction were also evaluated. Although no significant effect has been observed for *S. agalactiae* ATCC 27956, biofilm formation of *S. dysgalactiae* subsp. *dysgalactiae* ATCC 27957 and MRSA ATCC 43300 have been significantly reduced (*p* < .05), following the co-incubation assay (Figure 3).

Microscopic examinations were also set up to investigate the structural organisation of biofilm formed by ruminant mastitis-causing pathogens and to confirm the results of postbiotics on biofilm inhibition. For confocal laser microscopy, the dead cells were labelled with propidium iodide whereas live cells were stained with SYTO 9, which produced red and green fluorescence, respectively. According to the confocal laser microscopy images (Figure 4), biofilm production in the control groups was extremely intense at the end of 24 h. Considering the intensity of SYTO 9 fluorescence, bacterial viability was striking in the control samples. Although the 20 mg/mL did not completely inhibit the biofilm production of all three pathogens, it significantly delayed the accumulation of biomass on the surface. Considering the effects of the postbiotics on the biofilm formation of the mastitis-causing pathogens, the concentration of 20 mg/mL proved to be quite effective.

Scanning Electron Microscopy was also used to illustrate the effect of the co-incubation approach on the topology and the thickness of the biofilm developed by ruminant mastitis-causing pathogens. The micrographs displayed a well-grown biofilm along with adhering bacterial cells in controls, while dispersed bacterial cells in treated samples. It was also shown that the biofilm formed by the strains also exhibited a looser structure and lower density following the co-incubation with postbiotics (Figures 5–7).

### Table 1. The effects of postbiotics on the biofilm reduction (% of mastitis-causing pathogens.

| Co-incubation with different postbiotic doses | *S. agalactiae* | *S. dysgalactiae* | MRSA 43300 |
|---|---|---|---|
| 25 mg/mL | 93.48 ± 1.58 | 92.16 ± 1.90 | 96.21 ± 1.20 |
| 20 mg/mL | 93.87 ± 1.19 | 92.05 ± 1.74 | 95.16 ± 1.66 |
| 17.5 mg/mL | 94.10 ± 1.47 | 92.65 ± 0.94 | 95.87 ± 1.18 |
| 15 mg/mL | 94.53 ± 1.40 | 89.87 ± 3.22 | 96.02 ± 0.77 |
| 12.5 mg/mL | 93.73 ± 1.16 | 88.90 ± 4.51 | 92.45 ± 3.73 |
| 10 mg/mL | 93.44 ± 0.85 | 89.11 ± 4.07 | 91.47 ± 2.69 |
| 7.5 mg/mL | 94.04 ± 1.89 | 88.36 ± 4.53 | 90.30 ± 3.66 |
| 5 mg/mL | 93.89 ± 1.06 | 84.35 ± 4.69 | 71.14 ± 13.26 |
| 2.5 mg/mL | 92.19 ± 2.63 | 77.67 ± 6.23 | 20.37 ± 7.51 |
| 1.25 mg/mL | 84.10 ± 7.32 | 52.19 ± 11.21 | 6.65 ± 3.77 |

| Post-treatment with different postbiotic doses | *S. agalactiae* | *S. dysgalactiae* | MRSA 43300 |
|---|---|---|---|
| 25 mg/mL | 95.10 ± 1.89 | 92.20 ± 1.45 | 75.29 ± 2.49 |
| 20 mg/mL | 93.85 ± 1.23 | 91.55 ± 1.95 | 75.21 ± 1.19 |
| 17.5 mg/mL | 94.62 ± 1.12 | 90.90 ± 2.46 | 76.74 ± 0.43 |
| 15 mg/mL | 94.42 ± 1.98 | 89.67 ± 4.67 | 72.76 ± 4.76 |
| 12.5 mg/mL | 98.1 ± 1.81 | 91.52 ± 1.04 | 72.30 ± 4.11 |
| 10 mg/mL | 91.54 ± 2.01 | 91.74 ± 1.68 | 72.72 ± 4.49 |
| 7.5 mg/mL | 90.77 ± 1.58 | 91.19 ± 1.20 | 60.52 ± 1.73 |
| 5 mg/mL | 86.44 ± 2.18 | 88.88 ± 0.43 | 53.33 ± 1.08 |
| 2.5 mg/mL | 83.17 ± 1.79 | 84.97 ± 4.95 | 50.27 ± 5.48 |
| 1.25 mg/mL | 79.90 ± 2.11 | 73.51 ± 2.57 | 33.36 ± 0.76 |

Metabolites in postbiotics

Metabolites in postbiotics identified by the chromatographic analysis were listed in Table 2. The concentration of lactic acid (21.42 mg/mL) was found to be the highest organic acid, followed by formic acid and tartaric acid, with concentrations of 3.48 and 1.36%, respectively. However, oxalic acid and succinic acid were not detected (Supplementary Figure 1(a)). When the presence and the concentration of 37 fatty acids in the postbiotics were examined by GC-MS, only oleic acid (C18:1, cis-9; 0.014%), steaetic acid (C18:0; 0.087%), and palmitic acid (C16:0; 0.032%) were detected, while the other fatty acids could not be detected (Supplementary Figure 1(b)). Amongst the identified vitamins, the high-intensity of vitamins in the postbiotics are found as B-complex vitamins (B1 thiamine, B2 riboflavin, B5 pantothenic acid, and B12 cobalamin). In addition to B-complex, vitamin E 1 tocopherol was defined as one of the most common vitamins in the postbiotics with a concentration of 41.77 ppb (Supplementary Figure 1(c)). Isoleucine (0.45%) and...
glutamic acid (0.33%) were also identified as the major components of amino acid fractions in the postbiotics secreted by *L. sakei* EIR/CM-1 (Supplementary Figure 1(d)).

**Discussion**

Mastitis is considered one of the most frequent diseases occurring on dairy cows with its well-recognised effects on milk quality and yield, animal well-being, and public health (Gao et al. 2020; Sharun et al. 2021). Despite the effectiveness of widely used intramammary administration of antibiotics both for prophylaxis and therapy in mastitis, the possibilities of antibiotic residues in dairy products as well as the development of the emergence of antibiotic resistance have resulted in increased consumer concerns (Gao et al. 2020). Besides, common mastitis-causing pathogens

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**Figure 2.** Biofilm formation rates of the mastitis-causing pathogens following the treatment with postbiotics secreted by *Lactobacillus sakei* EIR/CM-1; (a–c) co-incubation; (d–f) pre-treatment; (g–i) post-treatment assays. **p < .001; ***p < .001; n.s.: not significant.
are also related to food-borne diseases in humans (Grispoldi et al. 2019). Intending to satisfy consumers’ demand for safe and organic products, the development of natural therapeutics to limit the use of antibiotics in dairy cows has gained attention, during the last decades. Because of this requirement, recent approaches for treating mastitis have involved the use of natural compounds, such as chitosan (Felipe et al. 2019), herbal extracts (Gomes et al. 2019; Ren et al. 2020), bacteriophages (Varela-Ortiz et al. 2018), antimicrobial peptides, such as nisin (Langer et al. 2017; Castelani et al. 2019), immunologically-based molecules, such as immunoglobulins isolated from yolks (Zhen et al. 2008), nanoparticle-based techniques like liposomes (Algharib et al. 2020), bovine mammary stem cells (Peralta et al. 2020), and propolis (Wang et al. 2016) which could serve as an alternative to antibiotic therapy. Even though several strategies have been developed over the years with the purpose of the treatment and management of mastitis, all of them lacked the efficacy to eliminate all associated aetiological agents (Sharun et al. 2021). Therefore, new therapeutic agents are still needed to replace conventional techniques and to solve the problem of emerging antibiotic resistance.

One of the alternative approaches applied for the prevention of mastitis includes the application of the lactic acid bacteria (LAB) as probiotics which are Generally Recognised As Safe (GRAS) and well-characterised with their potent antibacterial and anti-inflammatory activities (Sharun et al. 2021). Probiotics are usually used as feed supplements and can provide prevention by colonising the udder and inhibiting not only the growth of mastitis-causing pathogens but also their biofilm formation (Rainard and Foucras 2018; Wallis et al. 2018; Wallis et al. 2019). Different strains of LAB also can modulate innate immune response (Souza et al. 2018). To date, several microorganisms have been evaluated for their potent antibacterial, antifungal, and immunomodulatory activities (Rainard and Foucras 2018; Souza et al. 2018; Pellegrino et al. 2019). Besides, the intramammary application of lactobacilli-viable cells has led to a reduction in the somatic cell count (Yu et al. 2017). Similar to our study, various potential strains were isolated from indigenous raw milk to assess their antagonistic activity against pathogens and used for the prevention of mastitis (Sangle et al. 2018; Reuben et al. 2019). However, recent reports highlight some concerns of probiotics that need to be considered, such as their effectiveness, safety, allergic side effects, and activities depending on their cell viability on the target (Zółkiewicz et al. 2020).

Apart from probiotics, there is an increasing interest in using postbiotics which are defined as the metabolites of LAB, including any substance released by or produced through their metabolic activity (Tsilingiri and Rescigno 2013; Zółkiewicz et al. 2020). In recent studies, it was shown that the postbiotics derived from different microorganisms exhibited various beneficial effects (Zółkiewicz et al. 2020; Peluzio et al. 2021). One of the most promising effects is the prevention of infection-related diseases by inhibiting the pathogens as well as their biofilm formation. In this study, postbiotics as biologically active metabolites in cell-free supernatant secreted by L. sakei EIR/BG-1 were used to test its efficiency against ruminant mastitis-causing pathogens. Similar to our study, Malvisi et al. (2016) evaluated the antibacterial activity of the cell-free supernatants of twenty-five strains of Lactococcus lactis ssp. lactis against 29 strains of mastitis pathogens. Variable sensitivity to cell-free supernatant was observed among S. uberis and Enterococcus faecalis strains, and S. agalactiae, S. uberis, and E. faecalis displayed high MIC values. In one of the previous studies conducted by Pellegrino et al. (2019), twelve strains of LAB isolated from bovine milk were selected taking into account of inhibition of mastitis pathogens. Among them Lact. lactis subsp. lactis CRL 1655 and Lact. perolens CRL 1724 completely inhibited the growth of Staph. aureus ATCC 25923 and Staph. aureus RC108 after 24 h of co-incubation. Bouchard et al. (2015) also demonstrated...
the ability of LAB, isolated from bovine milk to inhibit the growth of representative strains of *Escherichia coli*, *S. uberis*, and *Staph. aureus*. Similar to our results, they reported that bacterial inhibition was found related to acidification since neutralisation of the cell-free supernatants relieves the inhibitory effect. This can be explainable with the effects of acids in the cell-free supernatants that can lead to the decrease of

Figure 4. Confocal laser microscopy images illustrating the effect of postbiotics on the cell viability of pathogens and their biofilm formations.
intracellular pH and dissipation of cell membrane potential (Konings et al. 1989).

In the pathogenesis of mastitis, biofilm formation is considered another important virulent factor and also a selective advantage for mastitis-causing pathogens.

The ability to grow on their biofilms leads pathogens to develop an innate resistance to almost all therapeutic agents that cannot reach the target site due to the biofilm layer (Cheng and Han 2020). Therefore, the primary effect expected from a promising and successful treatment is not only to target the pathogens but

Figure 5. Scanning electron micrographs of methicillin-resistant *Staphylococcus aureus* ATCC 43300 biofilm treated or not with postbiotics secreted by *Lactobacillus sakei* EIR/CM-1; (a,b) untreated control; (c) MIC$_{10}$: 10% of the MIC, arrows show the biofilm formed.

Figure 6. Scanning electron micrographs of *Streptococcus dysgalactiae* subsp. *dysgalactiae* ATCC 27957 biofilm treated or not with postbiotics secreted by *Lactobacillus sakei* EIR/CM-1; (a,b) untreated control; (c) MIC$_{10}$: 10% of the MIC, arrows show the biofilm formed.
also to control the biofilm formation. In our study, postbiotics secreted from \textit{L. sakei} ERI/BG-1 have also been evaluated for their efficiency on the biofilm reduction of mastitis-causing pathogens within three different approaches. Similar to our study, Koohestani et al. (2018) demonstrated that the addition of postbiotics derived from \textit{L. casei}, \textit{L. acidophilus}, and \textit{L. casei} significantly removed the biofilm, in a concentration-dependent manner, which was also confirmed by fluorescence microscopy. The effect of postbiotics produced by \textit{L. fermentum} TCUESC01 and \textit{L. plantarum} TCUESC02, isolated during the fermentation of fine cocoa, against \textit{Staph. aureus} CCMB262 biofilm production was also reported by Melo et al. (2016). A significant reduction in the thickness of the biofilm was confirmed with 90% of the MIC value (18 mg/mL). Moreover, a decrease in the biofilm matrix following the treatment with \textit{L. fermentum} TCUESC01 cell-free supernatant (50% of the MIC) has been noticed by SEM analysis, similar to our SEM observations. In another previous report, the cell-free supernatant of \textit{L. bulgaricus} FTDC8611 inhibited the biofilm formation of \textit{Staph. aureus} biofilm, an effect attributed to organic acids (Hor and Liong 2014). Herein, the antibiofilm function could be related to the biosurfactant production that can act as signalling molecules in quorum sensing (Mishra et al. 2020).

**Table 2.** Metabolites identified in the postbiotics, secreted by \textit{Lactobacillus sakei} EIR/CM-1.

| Metabolites                  | Value     |
|------------------------------|-----------|
| Organic acids (mg/mL)        |           |
| Maleic acid                  | 0.14      |
| Tartaric acid                | 1.36      |
| Lactic acid                  | 21.42     |
| Formic acid                  | 3.48      |
| Acedic acid                  | 0.20      |
| Butyric acid                 | 0.22      |
| FAMEs (%)                    |           |
| C16:0 methyl palmitate       | 0.032     |
| C18:0 methyl stearate        | 0.087     |
| C18:1 cis-9 methyl olate     | 0.014     |
| Vitamins (ppb)               |           |
| B1                           | 138.36    |
| B2                           | 21.55     |
| B5                           | 58.04     |
| B12                          | 318.42    |
| D2                           | 0.44      |
| D3                           | 0.39      |
| E1                           | 41.77     |
| K3                           | 7.65      |
| Amino acids (%)              |           |
| Aspartic acid                | 0.09      |
| Glutamic acid                | 0.33      |
| Serine                       | 0.04      |
| Glutamine                    | 0.02      |
| Histidine                    | 0.04      |
| Glycine                      | 0.06      |
| Threonine                    | 0.12      |
| Arginine                     | 0.20      |
| Alanine                      | 0.21      |
| Tyrosine                     | 0.06      |
| Cystine                      | 0.22      |
| Valine                       | 0.04      |
| Methionine                   | 0.18      |
| Trytophan                    | 0.11      |
| Phenylalanine                | 0.23      |
| Isoleucine                   | 0.45      |
| Leucine                      | 0.27      |
| Lysine                       | 0.29      |
| Proline                      | 0.10      |

**Figure 7.** Scanning electron micrographs of \textit{Streptococcus agalactiae} ATCC 27956 biofilm treated or not with postbiotics secreted by \textit{Lactobacillus sakei} EIR/CM-1; (a,b) untreated control; (c) MIC\textsubscript{10}: 10% of the MIC, arrows show the biofilm formed.
Since probiotics may exert their beneficial effects on the host through several mechanisms, it is important to know the unique metabolic properties of the strain EIR/CM-1 which is essential to define how it will interact with the pathogens. A large number of LAB produce various secondary metabolites that inhibit the growth of bacterial pathogens and their biofilm production, such as organic acids, ethyl alcohol, bacteriocins, hydrogen peroxide, and surfactants (Kanmani et al. 2013). Therefore, regarding the metabolite spectrum involved in the beneficial effects of the strain EIR/CM-1, we analysed the metabolites in postbiotics using chromatographic tools. HPLC analysis confirmed the presence of different organic acids in the postbiotics which can be related to the antimicrobial effect due to the acidifying intracellular pH and dissipation of membrane potential (Kanmani et al. 2013). Among the vitamins we identified, B-complex vitamins were found as the most common vitamins which may act as important catalysts for enzymes in energy metabolism (Peluzio et al. 2021). Similar to our study, Lactobacilli and Bifidobacterium have been reported to synthesise vitamins, such as A and some members of the B complex, which influence the host mechanisms (Rajakovich and Balskus 2019). To elucidate the components in the postbiotics, linear saturated and unsaturated fatty acids have also been analysed. Among the FAMES tested, oleic acid secreted by the strain EIR/CM-1 may also be responsible for the antibacterial activity as suggested by Lim et al. (2018).

**Conclusion**

In the dairy industry, treatment and management of mastitis which is essential not only for economical reasons and public health concerns but also regarding animal welfare, have to be the major requirement for clean and high yield milk. Despite the various advanced strategies use in the dairy sector, there is still a need for cost-effective, easy, rapidly available, field applicable, efficient, and effective therapeutic agents that can especially solve the problem of emerging antibiotic resistance, worldwide. The present study demonstrated that the postbiotics including various fatty acids, vitamins, and organic acids as the complex mixture of metabolic by-products secreted by L. sakei EIR/BG-1, originated from milk microbiota, might be used as a promising agent for the prevention of mastitis, with its antibacterial and antibiofilm activity against important mastitis-causing pathogens. However, as the successful treatment of mastitis depends on several factors, further studies are needed to prove the efficacy of postbiotics in managing bovine mastitis, in vivo.

**Ethical approval**

The experimental protocol was approved by the Ankara University Institutional Animal Care and Use Committee (Ethical protocol number 2017-7-60).

**Disclosure statement**

We certify that there is no conflict of interest with any financial organisation regarding the material discussed in the manuscript. The authors alone are responsible for the content and writing of this article.

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**Data availability statement**

The authors confirm that the data supporting the findings of this study are available within the article and its Supplementary Materials.

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