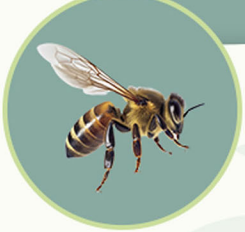




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## Anti-*Listeria* activity of postbiotics of *Lactiplantibacillus sakei* in beef fillet using aerosolization technique

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### Abstract

In recent years, the use of probiotics and their metabolites, known as postbiotics as natural preservatives has received increasing attention in the food industry. This study aimed to prepare and characterize postbiotics of *Lactiplantibacillus sakei* and to investigate its application as an anti-*Listeria* solution on beef fillets using an aerosolization technique. The functional groups, including organic acids, polysaccharides and other minor metabolites, were identified by Fourier transform infrared (FTIR) in the postbiotics. The 2, 2'-diphenyl-1-picrylhydrazyl radical scavenging activity of the postbiotics was reported as 0.82 mg mL<sup>-1</sup>. The antimicrobial test using the agar well diffusion method revealed a zone of inhibition of 27.00 ± 1.20 mm. Application of an aerosolized postbiotics solution resulted in a significant reduction in *Listeria monocytogenes* counts on beef fillets, reaching 3.30 log<sub>10</sub> CFU g<sup>-1</sup> over a 15-day storage period at 4.00 ± 1.00 °C. The results of this study revealed that the postbiotics of *L. sakei* was an effective antimicrobial additive for controlling foodborne pathogens in beef fillets and aerosolization is a promising method for developing an antimicrobial coating on meat to enhance meat safety.

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### Introduction

Meat and meat products constitute a major part of human food and contain valuable nutrients for growth and health. At the same time, meat is prone to microbial contamination, leading to discoloration and off-flavor of meat, introducing significant health risks to consumers by facilitating the transmission of important foodborne pathogens, including *Listeria monocytogenes* and *Escherichia coli*.<sup>1</sup> Consumers are becoming more concerned about the safety and health of food and they are less interested in chemical additives which increase interest in employing biological preservatives and increasing information about the use of beneficial bacteria and their metabolites as antimicrobials in the food industry.<sup>2</sup> Biopreservation uses natural materials with the origin of bacteria, fungi, plants, animals, enzymes, essential oils and secondary metabolites of bacteria (postbiotics) to increase the shelf life and maintain the quality of food.<sup>3,4</sup>

Several microorganisms, especially lactic acid bacteria (LAB), produce a large variety of antibacterial compounds and many efforts have been made to use LAB as bio-preservatives.<sup>5</sup> The LAB are gram-positive and catalase-negative microorganisms which consist of different genera mainly *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Leuconostoc*, and *Bifidobacterium*.<sup>6</sup> The main antimicrobial mechanism of LAB is related to their unique activity in producing antimicrobial compounds (mostly postbiotics).<sup>7</sup> Postbiotics are an emerging area of interest in the field of food science and biopreservation which refer to the metabolic byproducts or components produced by probiotics during their fermentation process.<sup>8,9</sup> In addition to organic acids and bacteriocins, postbiotics comprise a diverse range of compounds including free fatty acids and amino acids that collectively enhance their antimicrobial and antioxidant properties.<sup>8</sup> The use of postbiotics is an inventive solution to overcome drawbacks such as low survival compatibility and limited growth of probiotic

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bacteria that are used directly in the antimicrobial coating, film and food.<sup>5</sup> The main components of postbiotics include various compounds such as enzymes, bacteriocins, bacteriocin-like inhibitory compounds, organic acids and low molecular weight metabolites with high antimicrobial properties on foodborne microorganisms.<sup>6,10</sup> The type of probiotic in which the postbiotics is prepared, the characteristics of the target bacteria and the concentration of metabolites can affect antibacterial properties of postbiotics.<sup>9</sup>

Postbiotics are available in both freeze-dried (lyophilized) and fresh (liquid) forms and there are various approaches to incorporating postbiotics into meat such as applying them directly to food or integrating them into meat formulations. In this regard, postbiotics have been recently used in meat and meat products including dipping in frankfurters,<sup>11</sup> direct addition in fresh mutton sausage,<sup>12</sup> direct addition in cooked sausage,<sup>13</sup> dipping in chicken drumsticks,<sup>14</sup> in edible film in buffalo meat patties<sup>7</sup> and direct addition in ground beef.<sup>15</sup> Additionally, they can be used by spraying onto the surfaces of fresh or ready-to-eat meat products.<sup>3,10,15</sup> Aerosolization is a novel technique in antimicrobial coating. It involves turning liquid solutions into tiny airborne particles. This method is commonly used as a disinfectant method, especially in treating respiratory ailments with pharmaceuticals. Nebulizers are commonly used devices in aerosol systems. They generate particles with a diameter of approximately 5.00 µm or larger.<sup>16</sup> Aerosolization offers various benefits such as being easy to use, effectively covering surfaces, enhancing the penetration of antimicrobial substances, and causing minimal impact on the taste and texture of food due to the development of a thin coating.<sup>17</sup> Notably, volatile compounds such as carboxylic acids, alcohols and hydrocarbons which contribute to the antimicrobial activity of postbiotics may be lost during aerosolization demanding careful consideration.<sup>18,19</sup>

*Lactiplantibacillus sakei* has received a lot of attention for its role in meat products and its ability to prevent the growth and colonization of *L. monocytogenes* in red meat products especially during cold storage.<sup>20,21</sup> The *L. sakei* postbiotics have been used in different meat in edible film and coating forms.<sup>5,14,22</sup> The *L. monocytogenes* has been thoroughly researched in relation to using postbiotics in food, owing to its significance in meat safety and sensitivity to postbiotic ingredients.<sup>5,8,10,11,13-15,22</sup> In this study, anti-*Listeria* performances of postbiotics of *L. sakei* was assessed on beef fillets by aerosolization, a method that could potentially be used for commercial purposes due to its effectiveness.

## Materials and Methods

**Preparation of postbiotics.** The *L. sakei* (PTCC 1712) was purchased from the Iranian Research Organization for

Science and Technology (Tehran, Iran). For the preparation of postbiotics, the bacteria were cultivated in de Man Rogosa and Sharpe (MRS) broth (Ibresco, Karaj, Iran) for 48 hr at 37.00 ± 1.00 °C in a CO<sub>2</sub> incubator (Sina Lab, Tehran, Iran). Then, the suspension was separated by centrifugation (Farzaneh Arman Co., Isfahan, Iran) at 4,000 g for 10min and postbiotics solution was collected, and filtered using a sterile syringe polytetrafluoroethylene filter (0.22 µm; Millipore Inc., Burlington, USA). Then, the solution was freeze-dried (Zist Farayand Tajhiz Sahand, Tabriz, Iran); freezing temperature of -40.00 °C, pump pressure of 100 mTorr, and shelf temperature of -60.00 °C; and kept at -20.00 °C.<sup>15</sup>

**Fourier transform infrared (FTIR) analysis.** The chemical structural properties and surface groups of the postbiotics were investigated at the wavelength of 4,000 - 450 cm<sup>-1</sup> using the FTIR spectroscopy (Nexus® 670; Thermo Nicolet Instrument, Madison, USA), and data processing was done on Omnic Software (version 6.0; Thermo Electron, Madison, USA).

**Antioxidant activity.** The 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was used to evaluate the total antioxidant capacity of postbiotics.<sup>23</sup> One mL of DPPH solution (4.00 mg per 100 mL<sup>-1</sup> methanol) was added to 1.00 mL of different postbiotics concentrations (0.001, 0.01, 0.50, 1.00, 2.00, and 4.00 mg mL<sup>-1</sup>). The mixture was shaken and maintained at room temperature for 45 min in the dark. The absorbance of the samples was measured at 517 nm using a spectrophotometer (Novaspec II; Pharmacia, Muttenz, Switzerland). Butylated hydroxyl toluene (Sigma, St. Louis, USA) was used as control in this assay and the percentage of free radical scavenging effect (%RS) was calculated using the following equation:

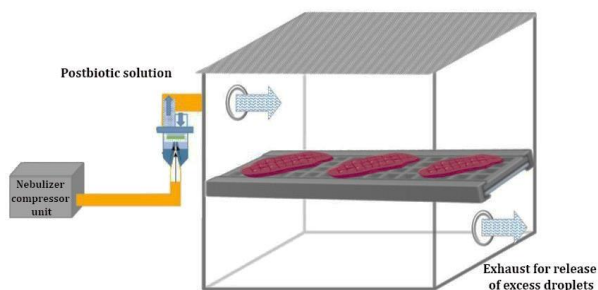
$$\%RS = ([A_{blank} - A_{sample}] / A_{blank}) \times 100$$

where,  $A_{blank}$  and  $A_{sample}$  indicate the absorption rates of the DPPH solution (control) and the postbiotics containing DPPH solution, respectively. The results were assessed as half maximal inhibitory concentration (IC<sub>50</sub>) values from the plot of the %RS concentration.

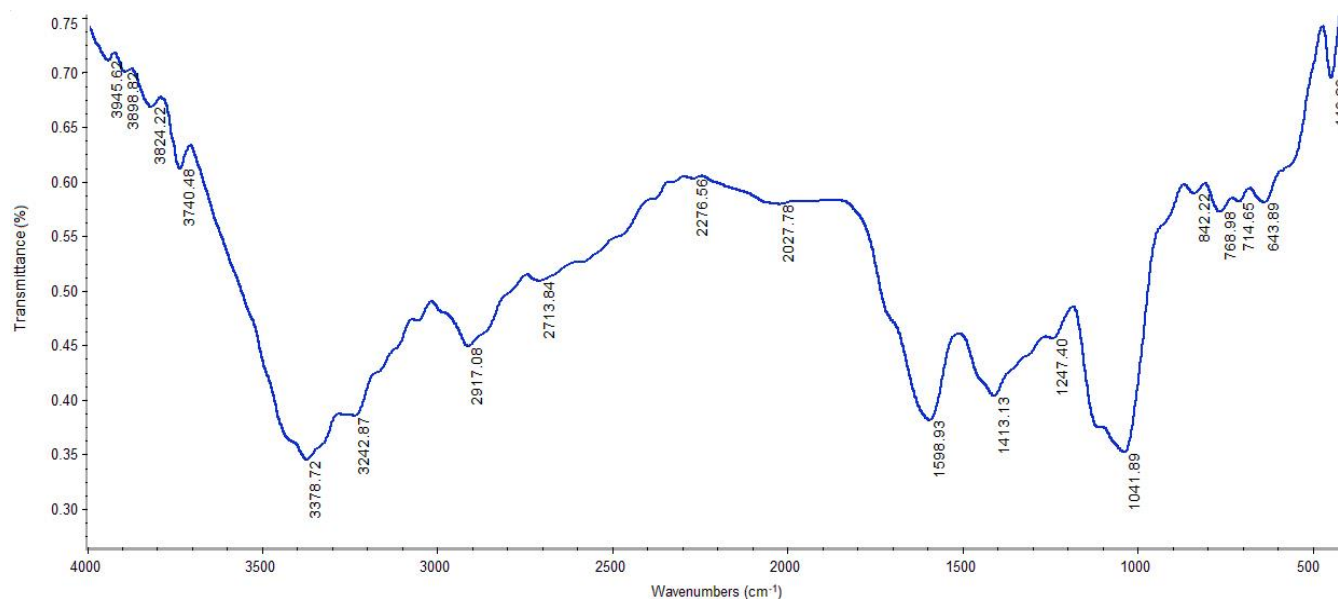
**Antimicrobial activity.** The antimicrobial activity of postbiotics was determined using the well diffusion method. An inoculum of 8.00 log<sub>10</sub> CFU mL<sup>-1</sup> of *L. monocytogenes* suspension was spread on the surface of Mueller-Hinton agar (Ibresco, Tehran, Iran) plates with a sterilized swab. Wells (8.00 mm diameter) were punched into the agar and filled with 100 µL of 40.00 % concentration of postbiotics powder. The plates were then incubated for 24 hr at 37.00 ± 1.00 °C. The diameter of the clear zone of inhibition (ZOI in mm) around the wells was measured with a digital caliper (Mitutoyo Corp., Kawasaki, Japan) in triplicate.<sup>24</sup>

**Anti-*Listeria* performance of postbiotics on beef fillet.** Fresh beef fillets were purchased directly from a butchery (Urmia, Iran) and transferred to the laboratory of

the Faculty of Veterinary Medicine, Urmia University under cold and hygienic conditions. Under an aseptic condition, fillet beef was cut into slices (~ 5.00 g) and was inoculated by immersion into *L. monocytogenes* suspension (~ 5.00 log<sub>10</sub> CFU mL<sup>-1</sup>) for 1 min and drained for 15 min.<sup>24</sup> The fillet slices were aerosolized with 10.50 mL of postbiotics solution (20.00 and 40.00%) for 30 min at a rate of 0.35 mL per min by atomizer nebulizer (3A Health Care, Lonato del Garda, Italy; Fig. 1) and packaged in sterile polyethylene box and stored at 4.00 ± 1.00 °C for 15 days.<sup>25</sup> Inoculated fillets without postbiotics were applied as controls. In the interval of days 0.00, 3.00, 6.00, 9.00, 12.00 and 15.00, 50.00 g of beef fillet was homogenized (Cole-Parmer Canada, Quebec, Canada) with 450 mL of peptone water (0.10% w/v) using a stomacher (Seward Medical Ltd., London, UK) at 260 rpm for 4 min. Then, serial dilutions of suspensions were prepared and spread on PALCAM Listeria selective agar (Oxford Agar; Merck, Darmstadt, Germany). Colony count was performed after 48hr incubation at 35.00 ± 1.00 °C and calculated as log<sub>10</sub> CFU per g.<sup>24,26</sup>



**Fig. 1.** Schematic of fabricated chamber for aerosolization of postbiotics on beef fillet.



**Fig. 2.** Fourier transform infrared spectrum of postbiotics of *L. sakei*.

**Statistical analysis.** Analysis of variance (ANOVA) was used to examine data using GraphPad Prism (version 8.01; GraphPad Software Inc., San Diego, USA). Tukey's multiple-range test was also performed to determine whether there were any significant differences ( $p < 0.05$ ) between the samples. All experiments were performed in triplicates.

## Results

**FTIR analysis.** To characterize the various functional groups of the compounds in *L. sakei* postbiotics, the mid-infrared spectrum of the lyophilized sample was applied (Fig. 2). As identified from the spectrum, the absorption peaks at 3,378, 3,242, 2,917, 1,598, 1,413, 1,247, and 1,041 cm<sup>-1</sup> were observed.

**Antioxidant and antibacterial activity.** The DPPH radical scavenging activity is commonly reported as IC<sub>50</sub>. In this research, IC<sub>50</sub> was 0.82 mg mL<sup>-1</sup> (Fig. 3). Additionally, postbiotics revealed antimicrobial activity on *L. monocytogenes* which was ZOI of 27.00 ± 1.20 mm.

**Anti-Listeria performance of postbiotics on beef fillet.** Figure 4 shows the inhibitory effects of two concentrations of postbiotics (20.00 and 40.00%) on *L. monocytogenes* in beef fillets during 15 days of storage at 4.00 °C. Accordingly, during storage, *L. monocytogenes* in all treatments was increased and 40.00% postbiotics were the most effective Anti-Listeria treatments reducing the number of bacteria from 6.20 log<sub>10</sub> CFU g<sup>-1</sup> in the control group to 3.30 log<sub>10</sub> CFU g<sup>-1</sup> ( $p < 0.05$ ) after 15 days. However, the 20.00% postbiotic treatment showed an insignificant decrease in bacterial counts compared to the control group.

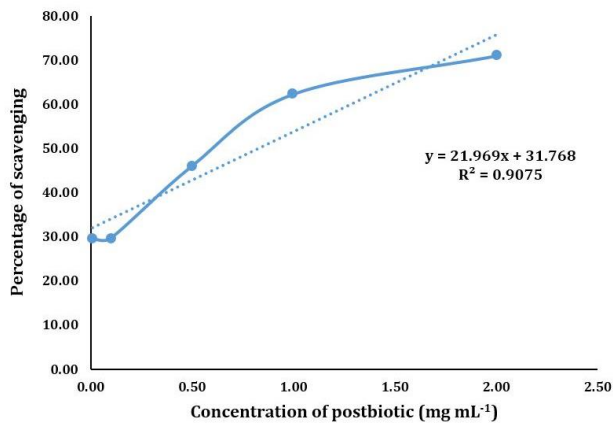


Fig. 3. The antioxidant activity 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) of postbiotics of *Lactiplantibacillus sakei*.

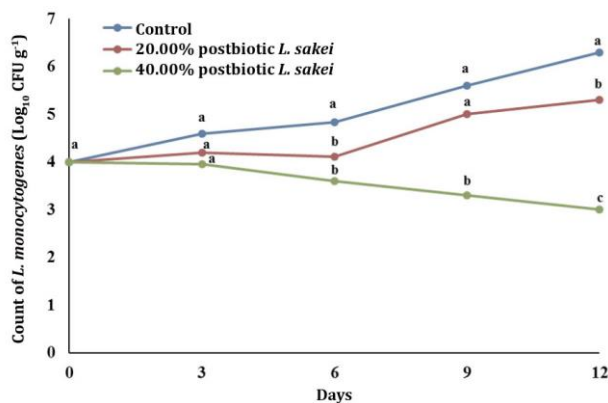


Fig. 4. The effect of different concentration of postbiotics of *Lactiplantibacillus sakei* on *Listeria monocytogenes* on beef fillet. On each sampling day, values with different letters are significantly different ( $p \leq 0.05$ ).

## Discussion

The FTIR has been recommended for obtaining qualitative information about the metabolites of postbiotics.<sup>27</sup> In this sense, Shafipour Yordshahi *et al.* used FTIR to investigate lyophilized postbiotics from *Lactobacillus plantarum*.<sup>26</sup> The FTIR technique was also used to characterize the biosurfactants and production of exopolysaccharides during the growth of LAB.<sup>28</sup> The obtained FTIR spectrum revealed that the functional molecules including biopolymers such as organic acids, polysaccharides and some others are in the postbiotics. Despite the fact that the chemical composition of postbiotics is still not completely defined, it has been found that *L. sakei* may produce several metabolites such as phosphoric acid, citric acid, butanoic acid, propionic acid and specific sugars in culture conditions.<sup>5</sup> Variations in the postbiotic composition can be attributed to the specific microbial strains employed, the culturing conditions and the composition of the culture media which collectively exert the most significant influence on the types of metabolites produced.<sup>29,30</sup> As identified from the spectrum,

the absorption bands at 3,378 and 3,242  $\text{cm}^{-1}$  can be assigned to the bonded/non-bonded O-H groups and N-H stretching vibration, respectively, and the peak centered at 2,917  $\text{cm}^{-1}$  was attributed to  $\text{sp}^3$  C-H stretching vibrations.<sup>5</sup> The spectral band found at 1598  $\text{cm}^{-1}$  can be related to the stretching of the carbonyl bond (C=O) with a look at carboxylic acids. Moreover, the peaks at 1,413 and 1,247  $\text{cm}^{-1}$  correspond to the aromatic ring vibration,  $\text{CH}_2$  scissoring vibration/COOH bending, and the sharp peak at 1041  $\text{cm}^{-1}$  is characterized as sugars index bands (C-H bending vibration).<sup>31</sup>

The  $\text{IC}_{50}$  is defined as the antioxidant concentration required to reduce 50.00% of the initial DPPH concentration.<sup>32</sup> In this study, the  $\text{IC}_{50}$  of *L. sakei* postbiotics was 0.82  $\text{mg mL}^{-1}$ . To date, there have been no reports on the DPPH activity of *L. sakei* postbiotics. Studies have proven that the various strains of bacteria and growth media can induce differences in the composition, levels of the compounds and properties of postbiotics.<sup>33</sup> Hamad *et al.* reported the antioxidant activity as 37.45  $\mu\text{g mL}^{-1}$  for the postbiotics of *Pediococcus acidilactici*,<sup>34</sup> while, İncili *et al.* reported the DPPH activity of *P. acidilactici* postbiotics to 1,291.02  $\text{mg L}^{-1}$ .<sup>33</sup> Antioxidant activity of postbiotics of LAB is linked to the type and concentration of phenolic compounds including gallic acid.<sup>11</sup>

The antimicrobial activities of LAB are related to various major compounds such as lactic acid, bacteriocin and hydrogen peroxide as well as minor compounds (*i.e.*, bacteriocin-like compounds, cyclopentane, benzoic acid, and pyrrolo [1, 2-a] pyrazine-1, 4-dione) that are secreted during the growth of bacteria.<sup>5</sup> In addition, LAB can produce various organic acids and a significant antimicrobial mechanism of postbiotics is the alteration of the bacterial cell membrane caused by organic acids. The synthesis of cell walls by prokaryotes is affected by organic acids and they can also disrupt the production of proteins.<sup>15</sup> It seems that some postbiotic metabolites such as fatty acids may facilitate organic acids to enter the cell membranes. This causes the release of cellular substances and ions endangering the survival of bacterial cells especially gram-positive bacteria.<sup>5,35</sup> Given that *L. sakei* postbiotics consist of several fatty acids, it is expected to have a great antibacterial effect.<sup>36</sup> The ZOI of *L. sakei* postbiotics on *L. monocytogenes* was  $27.00 \pm 1.20$  mm. Similar results have been reported in other studies, for example, Arrijoja-Bretón *et al.* reported ZOI of 20.00 mm for *L. sakei* postbiotics.<sup>24</sup> In another study, Bajpai *et al.* tested the antibacterial activities of lyophilized postbiotics of *Pediococcus pentosaceus* 411 on *L. monocytogenes* and reported ZOI around 18.00 mm.<sup>37</sup> Our research showed significant inhibitory activity against *L. monocytogenes* by postbiotics of *L. sakei*.

Aerosolization of 40.00% postbiotics of *L. sakei* on beef fillet caused a significant decrease in *L. monocytogenes* counts ( $p < 0.05$ ) by 3.30  $\log_{10}$  compared to the control

group during the storage period, however, 20.00% postbiotics concentration did not significantly decrease the count of *L. monocytogenes*. Based on NACMCF studies, reductions of 2.00 log<sub>10</sub> in the bacterial population in food inactivation studies have practical applications.<sup>38</sup> Several studies have reported the anti-bacterial activity of *L. sakei* postbiotics. The postbiotics of *Lactocaseibacillus casei* incorporated into whey protein films were found to be effective in controlling pathogens and reducing 1.40 log<sub>10</sub> of *L. monocytogenes* in fresh beef in 5 days.<sup>22</sup> In a study, the anti-microbial activity of postbiotics of *L. sakei*, which were incorporated in bacterial nanocellulose, was examined in buffalo meat. Results showed 2.30 - 2.60 log CFU g<sup>-1</sup> reduction in the *L. monocytogenes* count during storage for 9 days at 8.00 °C.<sup>4</sup> Hartmann *et al.* found that the 10.00% concentration of postbiotics produced by different LAB strains a significant decrease in the *L. monocytogenes* counts as 3.30 log<sub>10</sub> in ground beef.<sup>39</sup> İncili *et al.* showed that 10.00% and 50.00% postbiotics of *L. plantarum* concentrations caused a significant decrease as 1.00 log<sub>10</sub> and 2.10 log<sub>10</sub>, respectively, in the count of *L. monocytogenes*, compared to the control group.<sup>11</sup> In another study, Ünlü *et al.* used the bacteriocin-containing freeze-dried postbiotics powders obtained from different LAB strains treatment onto the hot-dog surface and showed a reduction as 2.00 - 3.00 log<sub>10</sub> in *L. monocytogenes* counts within 4 weeks.<sup>40</sup> In addition, Arrijoja-Bretón *et al.* found that the marinade of beef samples containing ten-fold concentrated postbiotics decreased the *L. monocytogenes* counts to 3.14 log<sub>10</sub> at refrigeration temperature during 14 hr.<sup>24</sup> The possible reasons for these variations among the results of studies were due to the use of different strains of LAB, the type and method of application of postbiotics and differences in food matrices.

In conclusion, this study was conducted to prepare postbiotics of *L. sakei* and evaluate their antibacterial performance in meat by aerosolization technique. Based on the results, these postbiotics had effective antioxidant capacity and implementing the aerosolization technique improved the anti-*Listeria* performance of postbiotics. We found that 40.00% postbiotics treatment effectively showed antibacterial activity and reduced *L. monocytogenes* counts by more than 3.00 log<sub>10</sub> CFU mL<sup>-1</sup> on beef fillets during storage at 4.00 °C for 15 days. It was concluded that the aerosolization technique could be successfully applied for the antimicrobial packaging of meat in which a lower amount of antimicrobials is used.

### Acknowledgments

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### Conflict of interest

The authors declare no conflict of interest regarding this study.

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