Characterization and application of antimicrobials produced by *Enterococcus faecium* S6 isolated from raw camel milk

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ABSTRACT

The emergence of antimicrobial resistance in the food chain and the consumer's demand for safe food without chemical preservatives have generated much interest in natural antimicrobials. Thus, our main goal was to study the mode of action of the crude extract, the enterocins, and the organic acid produced by a bacteriocinogenic *Enterococcus faecium* strain S6 previously isolated from raw camel milk. Then, we aimed to evaluate their potential application in a food system. These antimicrobials exhibited antimicrobial activity against *Listeria monocytogenes*, *Salmonella enterica*, and *Escherichia coli*. The enterocins were synthesized as primary metabolites beginning at the lag phase, with optimal production at the exponential and stationary phases. The antimicrobials had a direct effect in extending the lag phase of *L. monocytogenes*, along with a significant inhibitory activity. The organic acid, in particular, inhibited both *L. monocytogenes* and *S. enterica* by inducing a total lysis and damage of the cell wall. The enterocins acted on disrupting the cell wall with pore formation, leading to cell death. Moreover, the crude extract revealed a combined inhibitory activity between enterocins and organic acid. Furthermore, the antimicrobials showed promising results through inhibiting *L. monocytogenes* cells in milk samples up to 1 wk at 4°C.

Key words: antimicrobial resistance, antimicrobial, camel milk, food preservation

INTRODUCTION

Several challenges have arisen in the 21st century, notably the emergence of antimicrobial resistance in the food chain and the negative effects of the consumption of food containing chemical preservatives on human health (Cotter et al., 2013; Silva et al., 2018).

Antimicrobial resistance is defined as the ability of microorganisms to withstand the effect of antimicrobials, including antibiotics (Cotter et al., 2013). Antibiotic-resistant pathogens can easily spread at any stage of the food production chain and can be transmitted to humans (da Costa et al., 2013). The administration of antibiotics in humans and animals poses selective pressure on the indigenous bacterial communities by selecting a resistant subpopulation within a susceptible bacterial population (Ferri et al., 2017). The imbalance in the human commensal microbiota, which plays a key role in the biological homeostasis, may lead to a malfunctioned digestive system. This could contribute to increased incidences of infectious and intestinal immune-mediated diseases, atopic and autoimmune diseases, and metabolic and neuropsychiatric disorders (Cotter et al., 2013; Kho and Lal, 2018).

Antimicrobial resistance has generated much interest toward the identification and characterization of natural antimicrobials to reduce the abuse of antibiotics as growth promoters. Furthermore, the ability of these antimicrobial agents to extend the shelf life and prevent spoilage of food products make them promising candidates for the food industry to fulfill consumers' demands for safer food items without chemical preservatives (Silva et al., 2018).

One of the successful alternatives to antibiotic and chemical preservatives is the use of bacteriocins, alone or in conjunction with other antimicrobials, to compensate for the inefficacy of some antibiotics. Bacteriocins are ribosomally synthesized small peptides with high specific antimicrobial activity, commonly against food-borne pathogens (Mills et al., 2011). Although bacteriocins are produced by gram-positive and gram-negative bacteria, those produced by lactic acid bacteria (LAB) have received attention because LAB and their metabolites are generally recognized as safe (Ferrer Valenzuela et al., 2015). The LAB usually exert their antimicrobial activity by producing lactic acid, bacteriocins, hydrogen peroxide, carbon dioxide, and diacetyl (Vieco-Saiz et al., 2019). Bacteriocins can be applied as a crude extract from the producer strain for use in animal feed

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or in a purified or semipurified form for food preservation in the food industry (Lagha et al., 2017; Silva et al., 2018). Many studies demonstrate the potential applications of bacteriocins into various foods, mainly dairy products, for biopreservation (Muñoz et al., 2007; Mitra et al., 2011; Dal Bello et al., 2012; Alves et al., 2016; Shi et al., 2016; Yildirim et al., 2016; Ribeiro et al., 2017). Furthermore, several in vitro studies provide evidence about the possibility of the use of bacteriocins to combat the overuse of conventional antibiotics in food-animal production, particularly poultry and swine (Lagha et al., 2017).

Similar to other bacteriocins, enterocins that are produced by enterococci display inhibitory activity against a broad spectrum of foodborne pathogens (Vimont et al., 2017). Although many enterocins have antimicrobial activity against *Listeria monocytogenes*, few have been reported to be active against gram-negative bacteria (Khan et al., 2010). Enterocin TJUQ1, a novel class II bacteriocin, showed wide antibacterial activity against *L. monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella enterica* (Qiao et al., 2020). Most enterocins are class II bacteriocins, defined as small (<10 KDa), nonmodified, and heat stable peptides (Cotter et al., 2013). Bacteriocins have distinct mechanisms of action promoting bactericidal or bacteriostatic effects (da Silva Sabo et al., 2014). Generally, the class II bacteriocins target the lipid II, an intermediate in the peptidoglycan biosynthesis machinery within the bacterial cell envelope, and inhibit peptidoglycan synthesis or bind to the pore-forming receptor mannose phosphotransferase system (Breukink and de Kruijff, 2006; Silva et al., 2018). Enterocins showed promising results when applied to dairy products. Enterocin AS-48 and enterocin KP showed bactericidal effects against staphylococci and *L. monocytogenes*, respectively, in skim milk (Muñoz et al., 2007; Yildirim et al., 2016). A recent study reported the ability of a semipurified enterocin to reduce *L. monocytogenes* to an undetectable level in cheese (Ribeiro et al., 2017). Although a large number of studies have focused on the improvement of cheese quality and safety, only a few studies have focused on the application of bacteriocins, more specifically enterocins, in milk and yogurt preservation. Camel milk and its byproducts are known for their important nutritive and health-promoting properties. The use of nonthermal preservatives, such as bacteriocins, could extend the shelf life of the milk. This approach has minimal effects on the beneficial bacteria naturally present in the milk. Furthermore, the use of bacteriocins in combination with thermal treatments could preserve the sensory, nutritional, and organoleptic properties of milk (Sobrino-López and Martín-Belloso, 2008).

In this context, a multiple bacteriocins-producer strain, *Enterococcus faecium* S6, was isolated from raw camel milk in our laboratory. The produced enterocins A, B, and P exhibit strong antilisterial activity. They were semipurified and characterized as heat-resistant and stable over a wide range of pH values, ranging from 2 to 10 (Rahmeh et al., 2018). Enterocins A, B, and P belong to class II of bacteriocins. Enterocins A and P are enterocins of the pediocin family, whereas enterocin B is a linear nonpediocin-like enterocin (Franz et al., 2007). Enterocins A and B have slightly different inhibitory spectra and act synergistically against a wide range of gram-positive bacteria (Casaus et al., 1997).

Listeria monocytogenes is a common foodborne pathogen in dairy products responsible for a disease called listeriosis. This pathogen has been associated with many foodborne outbreaks and is considered an important issue for food safety and public health (De Buyser et al., 2001). *Listeria monocytogenes* is considered a major concern for the food industry because of its growth characteristics, highly salt-tolerant qualities, and ability to grow at temperatures ranging from 1 to 45°C and at a low pH (Camargo et al., 2017).

This study aimed to screen the antibacterial activity of the crude extract, organic acid, and the enterocins produced by *E. faecium* S6 against foodborne pathogens. Additionally, the mode of action of these antimicrobials were tested on *L. monocytogenes* based on time-kill kinetics and microscopic analysis. Furthermore, the approach of biopreservation and application in milk of these antimicrobials was evaluated.

MATERIALS AND METHODS

Bacterial Cultures, Bacteriocin Purification, and Quantification

Enterococcus faecium S6, the bacteriocin-producing LAB isolated from raw camel milk, was previously identified and characterized. The produced bacteriocins were identified by PCR as enterocins A, B, and P using enterocin-specific primers as described previously by Rahmeh et al. (2018).

De Man, Rogosa and Sharpe (MRS) broth and agar medium (Thermo Fisher Scientific, Waltham, MA) were used to cultivate the *E. faecium* strain. An overnight culture of the *E. faecium* strain was used to inoculate (1% vol/vol) 300 mL of MRS broth. The culture was incubated aerobically at 37°C for 24 h. Then, the supernatant was collected after centrifugation of the overnight culture of the *E. faecium* strain at $7,000 \times g$ for 15 min at 4°C followed by filtration through a 0.22- μ m syringe filter (Merck Millipore, Burlington, MA).

This filtered supernatant will be referred to the crude extract. Then, the enterocins were partially purified as described by Rahmeh et al. (2018) using ammonium sulfate precipitation (40% saturation; Sigma-Aldrich, St. Louis, MO). The semipurified enterocins will be referred to enterocins. The organic acid was obtained by treating the crude extract with proteinase K (final concentration of 1 mg/mL). The total protein concentration of the crude extract and the enterocins was estimated according to Bradford (1976). The MIC of the crude extract and semipurified enterocins was calculated using *L. monocytogenes* as an indicator strain to be 10 µg/mL and 35 µg/mL, respectively.

Antimicrobial Spectrum of Antimicrobials Produced by *E. faecium* S6

Well-Diffusion Assay. The agar well-diffusion assay was conducted as described by Tagg and McGiven (1971) to determine the antimicrobial activity of the crude extract, the organic acid, and the enterocins against gram-positive and gram-negative foodborne pathogens. The indicator strains were *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *Shigella flexneri* ATCC 12022, *S. enterica* ATCC 13076, *L. monocytogenes* ATCC 7644, *Raoultella planticola* isolated from raw camel milk in Kuwait, and multidrug-resistant *Salmonella* sp. isolated from local chicken cecum from a previous study (Al-Zenki et al., 2007). The indicator strains were cultured aerobically in Nutrient broth (NB; Thermo Fisher Scientific) and incubated at 37°C for 24 h. Wells were made in 0.75% soft nutrient agar plates (Thermo Fisher Scientific), inoculated with the indicator strain at a final concentration of about 10^7 cfu/mL (calculated by serial dilution), and were filled with 80 µL of crude extract (10 µg/mL), enterocins (35 µg/mL), or organic acid (the same volume of the crude extract at 10 µg/mL treated with proteinase K). Sterile MRS broth (80 µL) used to cultivate *E. faecium* S6 was used as a negative control. The crude extract neutralized at pH 6 and treated with proteinase K (final concentration of 1 mg/mL) was also tested to check if the observed antimicrobial activity involved other metabolites. The plates were incubated overnight, aerobically at 37°C. Results of 3 assays were determined by measuring the diameter of the clear zone (mm) around the wells. Zones of inhibition >5 mm were considered positive. The test was done in triplicate, and the average diameter was taken.

Microplate Inhibition Assay. The antimicrobial activity of enterocins was also evaluated using the microplate inhibition assay, as described previously by Steinberg and Lehrer (1997), with modifications. Briefly, the indicator strains listed in the agar diffusion

assay were inoculated into 5 mL of tryptic soy broth (Thermo Fisher Scientific) and incubated for 18 h at 37°C. Cultures were diluted into fresh tryptic soy broth (10^5 cfu/mL). Then, 24 µL of the indicator strains were dispensed into each well of a 96-well microtiter plate with 120 µL of the enterocins and 96 µL of MRS broth. The MRS broth was used as a negative control. The 96-well plate was incubated at 37°C, and the optical density (OD) was measured at 600 nm at 0 and 18 h. This assay was done in triplicate and the mean OD values at 600 nm were calculated.

Dynamics of Growth and Enterocins Production. The *E. faecium* strain was cultivated in MRS broth. The growth of *E. faecium* S6 was measured by recording the OD hourly at 600 nm after incubation at 37°C for 48 h. Samples were retrieved hourly for checking the enterocins production. The pH was adjusted to 6 with 2 M NaOH (Sigma-Aldrich) to exclude the effects of organic acids. The neutralized crude extract was treated with catalase (1 mg/mL; Sigma-Aldrich) to eliminate the inhibitory effect of H₂O₂.

Subsequently, the antimicrobial activity of the crude enterocins against *L. monocytogenes* (the most susceptible pathogen to enterocins) was determined by the well-diffusion assay as described above. Bacteriocinogenic activity was measured following a 2-fold serial dilution of the crude extract. The titer, in arbitrary units per milliliter (AU/mL), was defined as the reciprocal of the highest 2-fold dilution providing a distinct inhibition zone (Mayr-Harting et al., 1972; Hernández et al., 2005). These assays were done in triplicate.

Mode of Action: Growth Curve and Time-Kill Kinetics

The modes of action of the crude extract, the organic acid, and the enterocins were tested on *L. monocytogenes*. This test was performed as described by Jiang et al. (2017) with some modifications. An overnight culture of *L. monocytogenes* cultured aerobically in NB at 37°C for 24 h was diluted to about 10^6 cfu/mL. The crude extract or the enterocins were added to this culture at their MIC. The organic acid was added to the culture at the same volume as the crude extract. Untreated culture of *L. monocytogenes* was used as the control. All conditions were incubated at 37°C, samples were taken hourly for 10 h, and the viable cell counts were performed on tryptic soy agar (Thermo Fisher Scientific). This assay was conducted in triplicate and the mean of the log₁₀ values were calculated.

Microscopic Analysis

Scanning Electron Microscopy and Transmission Electron Microscopy. Scanning electron mi-

croscopy was executed according to Lü et al. (2014) and Jiang et al. (2017) to examine the effects of the crude extract, organic acid, and enterocins produced by *E. faecium* S6 on *L. monocytogenes* and *S. enterica*. The indicator strains were cultured in NB at 37°C for 18 h. Then, the cells were treated with the crude extract, the enterocins at their MIC, or the organic acid (the same volume as the crude extract) and incubated at 37°C for 30 min. Untreated cells were used as the control. Cells were collected by centrifugation at $7,000 \times g$ for 15 min at 4°C, and washed twice with sterile phosphate-buffered saline (Sigma-Aldrich).

For scanning electron microscopy, the *L. monocytogenes* cells were fixed with 3.0% glutaraldehyde (Sigma-Aldrich) for 5 min and then washed twice with 500 μ L of PBS. Then, the cells were fixed by 2 to 3 drops of 1% osmium tetroxide (Sigma-Aldrich) for 30 min. The samples were dehydrated progressively with ethanol solutions (Sigma-Aldrich; 50–100%, 5 min). The cells were coated with gold and observed by scanning electron microscope (Leo Supra 50VP, Carl Zeiss, Oberkochen, Germany).

For transmission electron microscopy (TEM), *L. monocytogenes* and *S. enterica* cells were fixed in 3% glutaraldehyde for 3 h, after which they were fixed by 1% (wt/vol) osmium tetroxide. Dehydration was done by serial ethanol dilutions (30–100%, vol/vol), followed by treatment with propylene oxide and propylene oxide-resin mixtures. The samples were then embedded in Araldite 502 embedding medium. Thin sections obtained after polymerization were double stained with uranyl acetate and lead citrate. Ultrathin sections were cut and observed by TEM (JEOL 1200EX II TEM).

Application of Antimicrobials in Skim Milk.

The effect of the crude extract, the organic acid, and the enterocins on the cell viability of *L. monocytogenes* inoculated in UHT skim milk was evaluated. The milk was purchased from a local market and inoculated with *L. monocytogenes* cells at 10^6 cfu/mL. The inoculated UHT milk was treated with the crude extract, the enterocins at their MIC, or the organic acid at the same volume as the crude extract. Untreated UHT skim milk inoculated with *L. monocytogenes* cells was used as the control. All conditions were incubated at 4°C for 1 wk, and samples were taken every 24 h. The viable cell counts were performed on tryptic soy agar. This assay was conducted in triplicate, and the mean of the $\log_{10}$ values were calculated.

Statistical Analysis

All experiments were conducted in triplicate. The data are expressed as mean $\pm$ SD. Statistical differ-

ences were determined using one-way ANOVA, followed by Tukey's multiple comparison test. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Antimicrobial Activity of Antimicrobials Produced by *E. faecium* S6

The antimicrobial activity of the crude extract, the enterocins, and the organic acid produced by *E. faecium* S6 was evaluated against the most common food-borne pathogens using the agar diffusion assay. In the agar diffusion assay, the crude extract and the organic acid exerted strong inhibitory activity against all of the tested gram-positive and gram-negative pathogens, with diameters of inhibition zones ranging from 15 to 21 mm. The enterocins inhibited *L. monocytogenes* and *R. planticola* among the tested pathogens (Figure 1). As reported previously, the crude extract of other *E. faecium* strains have shown a broad antimicrobial spectrum against pathogenic bacteria (Vimont et al., 2017). It is also known that organic acids reduce the food pH values, which adversely affects the survival and proliferation of gram-positive and gram-negative pathogens (Tamanini et al., 2012).

The microplate inhibition assay was performed in parallel to the agar diffusion assay to determine the spectrum of inhibition of enterocins against pathogens. The antimicrobial activity on the target pathogen that reduces the growth by more than 50% compared with the control was considered as positive inhibitory activity (Goh and Philip, 2015). This assay revealed dif-

Figure 1. Antimicrobial activity of the crude extract, the organic acid, and the enterocins produced by *Enterococcus faecium* S6 against foodborne pathogens using agar diffusion assay. Data are expressed as mean $\pm$ SD ($n = 3$).

ferent susceptibilities of the tested pathogens against the enterocins. The enterocins showed the highest inhibitory activity against *L. monocytogenes* and *S. enterica*. In addition, they significantly inhibited *E. coli*. However, the enterocins had no significant inhibitory activity against *S. aureus*, *S. flexneri*, *R. planticola*, and multidrug-resistant *Salmonella* sp. (Figure 2).

The observed difference in the results of the inhibitory activity of enterocins using agar diffusion and microplate inhibition assays may be because the well-diffusion assay was not suitable and applicable for enterocins, but were well suited for assaying other bacteriocins. This is due to the dispersal ability (diffusion and rate of diffusion) of enterocins in particular, affecting the well-diffusion assay (Choyam et al., 2015).

In both assays, the crude extract neutralized at pH 6 and treated with proteinase K showed no inhibitory activity against all tested pathogens. These results suggested that the antimicrobial activity of the crude extract might be mediated by the production of organic acid and enterocins by *E. faecium* S6, excluding the possibility that the observed antimicrobial activity involved other metabolites.

Several studies observed that gram-negative bacteria are naturally resistant to bacteriocins due to their outer membrane. To enable these peptides to affect gram-negative bacteria, these microorganisms can be sensitized by several mild treatments or by physical treatments that destabilize their outer membrane (Prudêncio et al., 2015). In this study, the enterocins produced by *E. faecium* S6 showed promising results against gram-negative bacteria such as *E. coli* and *S. enterica* without combination with other treatments.

Dynamics of Growth of *E. faecium* S6 and the Effect of Enterocins on Indicator Strain

Enterococcus faecium S6 entered the exponential phase at 3 h and reached the stationary phase at 10 h. The antimicrobial activity of this strain, mediated by crude enterocins production, was initiated in parallel to the growth of *E. faecium* S6. The optimal production of crude enterocins was recorded at the exponential and stationary phases from 5 to 48 h. The antibacterial activity of the crude enterocins produced against *L. monocytogenes* at the lag phase and the beginning of the exponential phase was estimated at 200 to 3,200 AU/mL with a diameter of inhibition zone of 15 to 20 mm; the antibacterial activity at the exponential and stationary phases was estimated at 6,400 AU/mL with a diameter of inhibition zone of 20 to 22 mm (Figure 3). These results showed that the produced crude enterocins are ribosomally synthesized as primary metabolites during the primary phase of growth of *E. faecium* S6.

Figure 2. Antimicrobial activity of the enterocins produced by *Enterococcus faecium* S6 against foodborne pathogens using microplate inhibition assay. Data are expressed as mean $\pm$ SD ($n = 3$).

Mode of Action: Growth Curve and Time-Kill Kinetics

We evaluated the effects of the crude extract of *E. faecium* S6, the enterocins at their MIC, and the organic acid on the cell viability of *L. monocytogenes* at the lag, exponential, and stationary phases (Figure 4). The 3 treatment conditions extended the lag phase of *L. monocytogenes* (3 h) compared with the control (2 h), along with a reduction of the viable cells count (a decrease by 1.5 $\log_{10}$) compared with the control ($P < 0.05$). The extended lag phase was due to the delay in

Figure 3. Dynamics of growth of *Enterococcus faecium* S6 (optical density; OD at 600 nm) and antimicrobial activity (inhibitory zone diameter in mm and activity in arbitrary units; AU/mL) of the produced enterocins against *Listeria monocytogenes*. Data are expressed as mean $\pm$ SD ($n = 3$).

Figure 4. Effect of the crude extract, enterocins, and organic acid produced by *Enterococcus faecium* S6 on the cell viability of *Listeria monocytogenes*. Data are expressed as mean $\pm$ SD (n = 3).

the growth of *L. monocytogenes* to adapt to the new environment in response to the antimicrobial's stress.

In the exponential phase, the crude extract and the enterocins exhibited significant inhibitory effects following similar inhibition patterns. However, the inhibition activity of the crude extract was more pronounced ($\sim 3 \log_{10}$) than 1 of the enterocins ($2.5 \log_{10}$) compared with the control ($P < 0.0001$). On the contrary, the organic acid did not induce a significant effect on the cell viability (the growth of *L. monocytogenes* increased by $1 \log_{10}$ as in the control condition). The effect of the crude extract was probably due to the combined effect of enterocins and organic acid at the lag phase.

In the stationary phase, the crude extract and the enterocins exerted a bacteriostatic effect on *L. monocytogenes*. Previous studies reported the inhibitory effect of the cell-free supernatant of LAB such as *E. faecium* CM21 and *Pediococcus pentosaceus* 4I1 on the viability of foodborne pathogens (Carina Audisio et al., 2011; Bajpai et al., 2016).

Figure 5. Scanning electron microscopic analysis of *Listeria monocytogenes* cells treated with antimicrobial substances from *Enterococcus faecium* S6. (a) Control; (b) organic acid-treated cells; (c) enterocin-treated cells; (d) crude extract-treated cells. The arrows indicate the pore formation and cell wall lysis.

Microscopic Analysis

Scanning electron microscopy analysis was performed to view the mode of action of the produced antimicrobials by *E. faecium* S6 on the cell physiology and morphology of *L. monocytogenes* (Figure 5). Control bacterial cells showed regular smooth surfaces (Figure 5a), whereas those treated with organic acid showed total lysis and damage to the cell wall (Figure 5b). The bacterial cells treated with enterocins showed pore formation on their surfaces (Figure 5c). Most of the bacteriocins produced from LAB exert their antibacterial effect by targeting the cell envelope-associated mechanisms (Cotter et al., 2013; Silva et al., 2018). However, the treatment with the crude extract showed a combined effect of enterocins and organic acid, manifested by the total lysis and damage to the cell wall and formation of pores on the surface of the bacterial cells (Figure 5d). Consistent with our findings, other cell-free supernatant of LAB strains showed marked morphological alterations on the surfaces on pathogenic bacteria such as *E. coli* and *S. aureus* (Bajpai et al., 2016).

The TEM was performed to further demonstrate the effect of the antimicrobials produced by *E. faecium* S6

on the cell wall physiology and morphology of gram-positive (*L. monocytogenes*) and gram-negative (*S. enterica*) pathogens. Compared with the smooth surface of the control cells (Figure 6a), *L. monocytogenes* cells treated with the organic acid (Figure 6b) showed total lysis of the cell wall. Enterocin-treated cells (Figure 6c) showed pores on the outer surface of the cells due to the perforation activity of the enterocins on the *L. monocytogenes* cells. Similar to our findings, pore formation is a typical characteristic caused by bacteriocins (Kaya and Simsek, 2019). The cells treated with the crude extract showed no visible cells; only the organelles of the lysed cells could be visualized (Figure 6d).

The effect of antimicrobials on *S. enterica* was also evaluated. Compared with the intact surface of the control cells (Figure 7a), cells of *S. enterica* treated with the organic acid (Figure 7b, c) showed complete lysis of the cells. However, the *S. enterica* cells treated with enterocins showed a smaller number of lysed cells, and cells were emptied out due to the activity of the enterocins (Figure 7d). The cells treated with the crude extract showed complete lysis of cells (data not shown). These results are in accordance with the microplate inhibition assay results.

Figure 6. Transmission electron microscopic analysis of *Listeria monocytogenes* cells treated with antimicrobial substances from *Enterococcus faecium* S6. (a) Control; (b) organic acid-treated cells; (c) enterocin-treated cells; (d) crude extract-treated cells.

Figure 7. Transmission electron microscopic analysis of *Salmonella enterica*. (a) Control; (b and c) organic acid-treated cells; (d) enterocin-treated cells.

Application of Antimicrobials in Skim Milk

In this assay, the effects of the crude extract of *E. faecium* S6, the enterocins at their MIC, and the organic acid on the cell viability of *L. monocytogenes* inoculated in skim milk were evaluated (Figure 8). At 24 h, a significant growth inhibition effect was observed after treatment with crude extract ($1.5 \log_{10}$) compared with the control ($P < 0.0001$). After 48 h, the crude extract showed more pronounced inhibitory activity ($2.5 \log_{10}$) compared with the effects of enterocins and organic acid ($0.9 \log_{10}$ and $0.6 \log_{10}$, respectively; $P < 0.0001$). From 48 to 120 h, the organic acid showed a decrease of *L. monocytogenes* of 1.3 to $2 \log_{10}$, and the enterocins induced a decrease of 1 to $1.5 \log_{10}$ compared with the control ($P = 0.001$ and $P = 0.003$, respectively). The treatment with the crude extract demonstrated the synergistic effect of enterocins and organic acid inducing a bactericidal effect on *L. monocytogenes*, with a decrease in the viable cells ranging from 3.4 to $4.7 \log_{10}$ compared with the control ($P < 0.0001$). A limited number of semipurified enterocins have been tested in dairy products to control *L. monocytogenes*. Ribeiro et al. (2017) reported the ability of semipurified enterocins to reduce *L. monocytogenes* to undetectable

levels in cheese. Enterocin CCM 4231 reduced *L. monocytogenes* by $4 \log_{10}$ in Saint-Paulin cheese (Lauková et al., 2001). Enterocin KP showed a high antilisterial effect in skim milk (Yildirim et al., 2016). In addition, the combination of 2 bacteriocins, reuterin and enterocin AS-48, reduced the count of *L. monocytogenes* in milk below the detection limit after 4 and 24 h (Arqués et al., 2011). Our results concluded, after 1 wk of storage, the reduction in the count of *L. monocytogenes* by 1.5, 2, and $4.7 \log_{10}$ in the presence of the enterocins, the organic acid, and the crude extract, respectively, is considered a successful result for food safety (Kaya and Simsek, 2019).

CONCLUSIONS

This study revealed the mode of action of antimicrobials produced by *E. faecium* and the performance of these antimicrobials in a food system. These antimicrobials showed remarkable inhibitory activity against a spectrum of foodborne pathogens that was reflected by reduced cell viability and morphological alterations of the pathogens. Furthermore, a synergistic antimicrobial effect between the organic acid and the enterocins was revealed. These antimicrobials possessed desirable char-

Figure 8. Antimicrobial activity of the crude extract, enterocins, and organic acid produced by *Enterococcus faecium* S6 on the cell viability of *Listeria monocytogenes* inoculated in milk. Data are expressed as mean $\pm$ SD (n = 3).

acteristics for application in dairy products, especially for extending shelf life of milk, controlling foodborne pathogens in dairy products, and preserving food quality. In further studies, more detailed information about the proteomic and the organic acids composition of the crude extract will be addressed.

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