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ANTIMICROBIAL ACTIVITY OF *ENTEROCOCCUS FAECIUM* FAIR-E 198 AGAINST GRAM-POSITIVE PATHOGENS

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ABSTRACT

This study investigated the antimicrobial activity of *Enterococcus faecium* FAIR-E 198 against *Bacillus cereus*, *Listeria monocytogenes* and *Staphylococcus aureus*. Using the critical-dilution method, the bacteriocin produced by *E. faecium* FAIR-E 198 inhibited all *L. monocytogenes* strains evaluated (1,600 to 19,200 AU mL^{-1}). However, none of the *B. cereus* and *S. aureus* strains investigated were inhibited. The maximum activity of this bacteriocin (800 AU mL^{-1}) was observed in MRS broth, while the activity in milk was 100 AU mL^{-1}. In the co-cultivation test in milk, *B. cereus* K1-B041 was reduced to below the detection limit (1.00 log CFU mL^{-1}) after 48 h. *E. faecium* reduced the initial *L. monocytogenes* Scott A population by 1 log CFU mL^{-1} after 3 h at 35ºC. However, the pathogen regained growth, reaching 3.68 log CFU mL^{-1} after 48 h. *E. faecium* did not influence the growth of *S. aureus* ATCC 27154 during the 48 h of co-cultivation. Therefore, it can be concluded that the effectiveness of the antimicrobial activity of *E. faecium* FAIR-E 198 is strictly related to the species and strain of the target microorganism and to the culture medium.

Key words: *Enterococcus faecium,* bacteriocin, biopreservation, gram-positive pathogen

INTRODUCTION

The potential risk posed by the presence of pathogens in dairy products is a constant concern in the field of food safety for both the food processing industry and government authorities. A number of foodborne disease outbreaks have been associated with these products (19). Although most of these outbreaks were closely related with the consumption of dairy products made from raw milk (5, 7), post-processing contamination must be taken into account as an important risk factor in the manufacture of such products.

Reduction of pH due to the production of organic acids, particularly lactic acid, by fermentation of naturally occurring carbohydrates in foods is responsible for the main antagonistic effect against a series of different microorganisms. However, nutrient competition and the formation of other compounds with inhibitory activity (hydrogen peroxide, diacetyl and bacteriocin) are important antimicrobial functions of lactic-acid bacteria (17). Bacteriocins are peptides or proteins with antimicrobial activity that are synthesized by the ribosomes of lactic-acid bacteria.

Numerous reports describing bacteriocin activity in

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Enterococcus species have been published in recent years. Most of the bacteriocin producer strains have been isolated from dairy products (10, 23). Some of these species, particularly *Enterococcus faecium*, provide probiotic and technological benefits, including the ability to grow at refrigeration temperatures over a wide pH range and in the presence of up to 6.5% NaCl (4, 20), in addition to improving cheese flavor. However, there is controversy in literature regarding its pathogenic potential. Giraffa *et al*. (14) state that, like other lactic-acid bacteria, enterococci species may occasionally be involved in clinical infections, but that in spite of this, many strains are considered safe for use in foods. An example of such a strain is *E. faecium* K770, which was approved in 1996 in the United Kingdom for use in cultured dairy products (13).

E. faecium FAIR-E 198, a strain isolated from Greek Feta cheese, is a bacteriocin producer (26) and was found to be a poor acidifier in milk (25). In general, enterocins have selective antimicrobial activity against the genus *Listeria* and are not effective against lactic acid bacteria (16, 20). However, some authors observed activity of enterocins against other Gram-positive pathogens like *Bacillus cereus* (9) and *Staphylococcus aureus* (2). This suggests that bacteriocinogenic entrococci strains or their enterocins may be used to enhance the microbiological safety of fermented dairy products.

The aim of this study was to verify the viability of using *E. faecium* FAIR-E 198 as an adjunct culture in dairy products to control gram-positive pathogens. To this end, the growth and production of the bacteriocin by *E. faecium* FAIR-E 198 in MRS broth and milk was evaluated and the spectrum of antimicrobial activity was determined. In addition, the behavior of *Bacillus cereus*, *Listeria monocytogenes* and *Staphylococcus aureus* during co-cultivation with this bacteriocins-producing bacterium in milk were analyzed.

MATERIALS AND METHODS

Bacterial strains and media

Bacterioci- producing *Enterococcus faecium* FAIR-E 198 (strain ACA-DC 1618, Laboratory of Dairy Research, Agricultural University of Athens, Greece) was isolated from Feta cheese (26). This strain was stored at 4 ºC in de Man Rogosa Sharpe (MRS) broth (Difco, Becton Dickinson, Sparks, MD). The pathogenic bacteria were purchased from the Faculty of Food Engineering, Campinas State University (Campinas, Brazil) and the Bacterial Collection of the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil). *Listeria monocytogenes* strains were maintained in trypticase soy agar supplemented with 0.6% yeast extract (TSA-YE, Difco), *Staphylococcus aureus* in brain-heart infusion agar (BHI, Difco) and *Bacillus cereus* in trypticase soy agar (TSA, Difco). Before being used in the tests, the strains were grown in media and at temperature indicated for each culture.

Pathogenicity tests

Gelatinase production was determined in nutrient gelatin agar (Difco) after incubation at 37ºC for up to seven days. A positive reaction was indicated by liquefaction of the medium after incubation at 21 ºC for two hours. Thermonuclease activity was assessed using Bacto-DNAse Test agar (Difco) supplemented with 0.83% toluidine blue (Sigma-Aldrich, Saint Louis, MO.). Each well made in the agar was filled with aliquots of 50 µL of the strain that had been previously heated for 15 min at 100 ºC. The presence of a pink coloration around the wells after incubation for 24 h at 37 ºC was indicative of thermonuclease activity (3).

Determination of antimicrobial spectrum

The sensitivity of 25 *B. cereus* strains, ten *L. monocytogenes* strains and five *S. aureus* strains to the bacteriocin produced by *E. faecium* FAIR-E 198 were evaluated using an adapted critical-dilution method (18). Culture plates were prepared with TSB soft agar (0.9% agar) and TSB-YE soft agar previously inoculated with 1% (0.2 mL) of the *B. cereus, S. aureus* and *L. monocytogenes* (10^6-10^7 CFU mL^{-1}) cultures, respectively. The overnight bacteriocinogenic cultures were centrifuged at 7,500 × g for 15 min at 4 ºC (Beckman J2-21 Centrifuge, Palo Alto, Calif.). The supernatants were adjusted to pH 6.5 and sterilized by filtration

through a 0.22 µm membrane (Millipore, Carrigtwohill, Ireland). Then, 10 µL of serial two-fold dilutions of cell-free culture supernatants in 10 mM sodium phosphate buffer, pH 7.0, were spotted onto plates containing the pathogens. The plates were incubated for 24 h at 35 ºC. The bacteriocin activity was expressed in arbitrary units per milliliter (AU mL^{-1}), corresponding to the reciprocal of the highest dilution causing a clear inhibition zone on the indicator strain and was multiplied by a factor of 100.

Effect of culture media on bacteriocin production

The cell suspensions, containing approximately 10^5–10^6 CFU mL^{-1} of the bacteriocinogenic culture, were inoculated into MRS broth and into 10% reconstituted skim milk powder (RSM). The inoculated samples were incubated at 37 ºC for 48 h. Then, samples of the cultures were collected at 3 h intervals during the first 12 h and after 24 and 48 h of incubation. Microbial counts were determined by plating onto KF *Streptococcus* agar (Difco) and incubated at 37 ºC for 48 h. The pH was determined by a pH-meter (Hanna Instruments HI 9110, Singapore). Bacteriocin production was determined by the critical-dilution method using *L. monocytogenes* Scott A as an indicator microorganism.

Co-cultivation of gram-positive pathogens and FAIR-E 198 in milk

The bacteriocin-producing culture (10^6–10^7 CFU mL^{-1}) was cultivated separately with *B. cereus* K1-B041, *L. monocytogenes* Scott A and *S. aureus* ATCC 27154 (approximately 10^2 CFU mL^{-1}) in RSM and incubated at 35 ºC for 48 h (6, 24). Milk samples individually inoculated with each of the species investigated were used as control. Samples were collected after 0, 3, 6, 9, 12, 24 and 48 h of incubation. *E. faecium* counts were performed on KF *Streptococcus* plates after incubation at 37 ºC for 48 h. Modified Oxford agar (Difco) was used for *L. monocytogenes*, Baird-Parker agar (Difco) for *S. aureus*, both of which were incubated at 35 ºC for 48 h, and Mannitol-Egg-yolk-Polymyxin agar (MYP, Difco) was used for *B. cereus*, with incubation at 30 ºC for 24 h. The identity of the colonies recovered in the selective media was confirmed using biochemical tests (12). Immediately upon collection, the pH of each sample was measured by a pH meter. The bacteriocin activity against the three pathogens investigated was determined by the critical-dilution method.

Statistical analysis

Analysis of variance (ANOVA) and the Tukey test at the 5% level of significance were used to compare means among and within experimental groups.

RESULTS AND DISCUSSION

Pathogenicity potential

Before being used in food technology, all enterococci strains must have their pathogenicity factors carefully analyzed. The results of the pathogenicity tests performed showed that *E. faecium* FAIR-E 198 was negative for gelatinase and thermonuclease activities. In previous studies, this strain was non-hemolytic when tested on sheep or human blood, and exhibited no resistance to vancomycin and teicoplanin (26, 28). All the above characteristics are indicative of the absence of pathogenicity. However, more tests are necessary to confirm this, such as the identification of virulence genes.

Spectrum of activity

The use of bacteriocins or bacteriocinogenic cultures in biopreservation of foods may be considered as an additional tool to enhance the microbiological safety and reduce the risk of the development of spoilage microorganisms. In this study, the enterocin produced by *E. faecium* FAIR-E 198, like most Class IIa bacteriocins, showed activity predominantly against *L. monocytogenes*, inhibiting all ten strains evaluated (1,600 to 19,200 AU mL^{-1}) by the critical-dilution method. The differing susceptibility observed in *L. monocytogenes* strains and the absence of sensitivity shown by all the 25 *B. cereus* and five *S. aureus* strains to the bacteriocin (Table 1) can be explained by natural variation in susceptibility and the ability to develop

resistance to bacteriocins. Ennahar and Deschamps (11) observed that enterocin A produced by *E. faecium* EFM01 inhibited 13 out of a total of 14 *L. monocytogenes* strains, while none of 7 *S. aureus* strains investigated were inhibited by this bacteriocin. On the other hand, Ammor *et al*. (2) reported antimicrobial activity of enterocins produced by *E. faecium* against *S. aureus* strains.

Table 1. Antimicrobial spectrum of the bacteriocin produced by *E. faecium* FAIR-E 198.

Pathogen specie	Strain	Inhibitory activity (AU/mL)
Listeria monocytogenes	IOC[a] 1551, SCOTT A	1.600
	IOC 1324, *ATCC*[b]*19111*, ATCC 7644	3.200
	IOC 1630	4.800
	IOC 1359, IOC 1898	6.400
	ATCC 19115	12.800
	IOC 1527	19.200
Staphylococcus aureus	ATCC 8095, ATCC 13565, ATCC 19095, ATCC 27154, ATCC 27664	ND[d]
Bacillus cereus	ATCC 14579, K1[c]- B010, K1-B016, K1-B020, K1-B021, K1-B025, K1-B037, K1-B040, K1-041, K1-B043, K1-B048, K1-B050, K1-B069, K1-B070, K1-B071, K1-B072, K1-B074, K1-B097, K1-B098, K1-B128, K1-B129, K1-B130, K1-B131, K1-B132, K1-B146	ND

[a] IOC: Instituto Oswaldo Cruz (Rio de Janeiro, BR),
[b] ATCC: American Type Culture Collection (Rockville, USA),
[c] K1: Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas (Campinas, BR),
[d] Not detected.

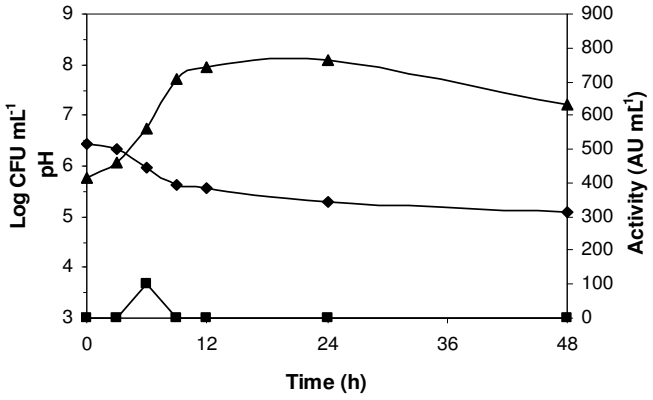
Bacteriocin production in MRS broth and milk

The production of bacteriocins by *E. faecium* FAIR-E 198 was influenced by the culture medium. In MRS broth, this strain reached a maximum population level of 7.90 log CFU mL^{-1} after 12 h, remained stable up to 24 h of incubation, but decreased by 1 log CFU mL^{-1} after 48 h. Acidification of the MRS broth began after 6 h, resulting in a pH of 3.72 after 48 h. The bacteriocin activity, measured using *L. monocytogenes* Scott A as an indicator microorganism, was first detected after 9 h of incubation (100 AU mL^{-1}), during the log-phase, and reached a maximum of 800 AU mL^{-1} after 24 h, during the stationary phase (Fig. 1A). However, several authors have reported maximum enterocin activity during or at the end of the exponential growth phase (1, 26). The pronounced decrease in bacteriocin activity (87.5%) observed after 24 h may be related to protein aggregation, proteolytic inactivation or adsorption of the bacteriocin onto the producer cell surface (8).

In milk, *E. faecium* FAIR-E 198 reached a count of 8.10 log CFU mL^{-1} after 24 h, followed by a drop of 0.90 log CFU mL^{-1} after 48 h. During bacterial growth, the pH of the milk

decreased from 6.45 to 5.08. Bacteriocin activity was observed only after 6 h (100 AU mL^{-1}) and was no longer detected up until the end of the incubation period (Fig. 1B). Sarantinopoulos *et al.* (26) evaluated this same strain and obtained a similar result using milk supplemented with casein hydrolysate (1.4%). According to Moreno *et al.* (20), the enterococci showed low proteolytic activity and consequently slower metabolism. This fact might be the reason why bacteriocin production in milk is lower than in broth. Another explanation for the low enterocin activity in milk may lie in interactions of this substance with milk components, such as fat and casein (21).

A



B

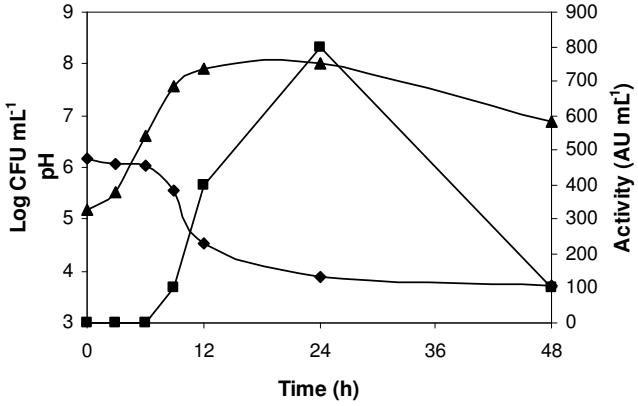


Figure 1. Growth of *Enterococcus faecium* FAIR-E 198 in MRS broth (A) and skim milk (B). Growth (▲), bacteriocin activity against *L. monocytogenes* Scott A (■) and pH (♦).

Co-cultivation with Gram-positive pathogens

The initial counts (2 log CFU mL^{-1}) of the pathogens used in these tests were based on the contamination level normally found in dairy products. *B. cereus* K1-B041 in the control sample reached a count of 8.37 log CFU mL^{-1} after a 48-h incubation (Fig. 2A). During the first 24 h of co-cultivation with the bacteriocinogenic culture, no significant reduction (<0.70 log CFU mL^{-1}) was observed in the *B. cereus* population. However, after 48 h, this pathogen population was reduced to below the detection limit (<1.00 log CFU mL^{-1}). The bacteriocinogenic culture reached viable cell counts of 8.13 log CFU mL^{-1} after 48 h. No bacteriocin activity against *B. cereus* was detected during 48 h of incubation. A significant difference ($P \leq 0.05$) was observed between the pH values of the control sample and the co-cultivation sample after 9 h. Therefore, the significant reduction of the *B. cereus* K1-B04 population observed after 48 h of co-culture with *E. faecium* FAIR-E 198 may be attributed to the reduction of the pH to lower than 4.4. Wong and Chen (29) reported that the development of *B. cereus* is impaired when the pH of the culture medium is lower than 5. According to Rossland *et al.* (24), a rapid decrease of the pH at the beginning of the logarithmic phase would be strongly related to pathogen inhibition.

The effect of the bacteriocin produced by *E. faecium* FAIR-E 198 on the cell viability of *L. monocytogenes* Scott A during growth in milk is shown in Figure 2B. The control sample, initially inoculated with 2.63 log CFU mL^{-1} of *L. monocytogenes*, reached a population of 7.26 log CFU mL^{-1} after 48 h. The bactericidal activity of *E. faecium* reduced the *L. monocytogenes* Scott A count from 2.19 to 1.15 log CFU mL^{-1} during the first 3 h of incubation, after which the remaining bacterial cells regained growth, attaining 3.68 log CFU mL^{-1} after 48 h of incubation. During the same period, the population of the bacteriocinogenic culture increased from 6.00 to 7.32 log CFU mL^{-1}. Bacteriocin activity (100 AU mL^{-1}) produced by this culture was detected only in the sample analyzed after a 6-h incubation. The pH values of the co-cultivated *E. faecium* sample were lower than the pH values of

the control sample containing only *L. monocytogenes* over the entire incubation period. After 48 h, the pH of the co-cultivated sample was 5.30, while the control sample exhibited a pH value of 6.10. The reductions of *L. monocytogenes* obtained in this study were greater than those reported by Elotmani *et al*. (9), using another *E. faecium* strain. The initial bactericidal effect of *E. faecium* FAIR-E 198 against *L. monocytogenes* Scott A and the subsequent growth recovery of the pathogen may be related to the production of amounts of bacteriocin insufficient to inhibit all the cells of the target microorganism. A similar phenomenon has previously been commented upon by Muriana (22) with respect to other bacteriocinogenic cultures.

In relation to *S. aureus* ATCC 27154, the control sample initially inoculated with 2.79 log CFU mL^{-1} exhibited a population of 8.80 log CFU mL^{-1} after 24 h (Fig. 2C). This was followed by a decline phase, and after 48 h of incubation, the *S. aureus* reached a viable cell count of 7.10 log CFU mL^{-1}. The count of the bacteriocinogenic culture increased by 2.00 log CFU mL^{-1} after 48 h. Nonetheless, no bacteriocin activity of any kind against *S. aureus* was observed throughout the incubation period. No significant difference ($P \geq 0.05$) was found between the pH of the *S. aureus* control sample and the co-cultivation sample. *E. faecium* FAIR-E 198 did not show bacteriocin activity or any other significant inhibitory effect ($P \geq 0.05$) against *S. aureus* ATCC 27154 during the 48 h incubation. According to Sutra *et al*. (27) *Staphylococcus* possess the ability to form a viscous or gelatinous polysaccharide capsule that prevents the penetration of antimicrobial substances into the bacterial cells. However, Lauková *et al*. (15) studied the effect of enterocin CCM 4231 on the growth of *S. aureus* in milk and observed a reduction in the number of viable cells of the pathogen from 10^{10} CFU mL^{-1} to 10^{2} CFU mL^{-1} after a 24-h incubation.

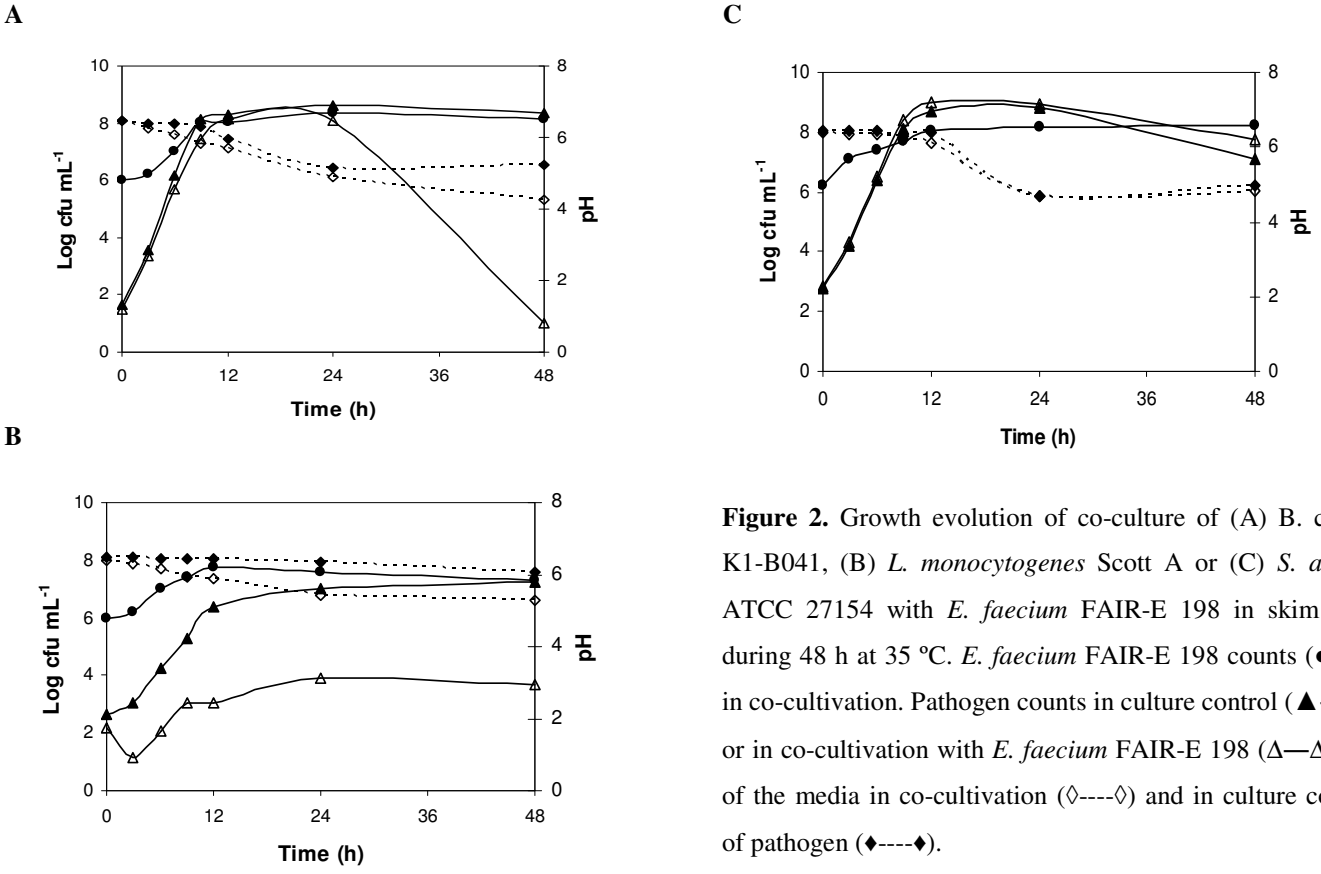


Figure 2. Growth evolution of co-culture of (A) B. cereus K1-B041, (B) *L. monocytogenes* Scott A or (C) *S. aureus* ATCC 27154 with *E. faecium* FAIR-E 198 in skim milk during 48 h at 35 ºC. *E. faecium* FAIR-E 198 counts (●—●) in co-cultivation. Pathogen counts in culture control (▲—▲) or in co-cultivation with *E. faecium* FAIR-E 198 (Δ—Δ). pH of the media in co-cultivation (◊----◊) and in culture control of pathogen (♦----♦).

CONCLUSION

The effectiveness of the antimicrobial activity of *E. faecium* FAIR-E 198 is strictly related to the species and strain of the target microorganism and to the culture medium. This lactic-acid bacterium shows interesting characteristics for use as an additional safety provision within the context of hurdle technologies to interact with other barriers for the control of foodborne Gram-positive pathogens in dairy products. However, to this end, more studies on virulence factors and antibiotic resistance are necessary.

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