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Inhibitory properties of Enterococcus spp. isolated from faeces of healthy dogs

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Inhibitory properties of *Enterococcus* spp. isolated from faeces of healthy dogs

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The aim of the present study was to evaluate the inhibitory effect by the cross-streak method of nine *Enterococcus faecium* strains isolated from faeces of healthy dogs and their treated and non-treated cell-free supernatant (CFS) by the well-diffusion test on the growth of potentially pathogenic bacteria isolated from clinical cases and aflatoxigenic *Aspergillus* section *Flavi* and the consequent aflatoxin B_1 (AFB_1) production. Results obtained from the cross-strake assay showed that *E. faecium* MF1, GJ18 and GJ40 presented the major inhibitory activity against all pathogenic strains assayed; *E. faecium* GJ40 produced the larger inhibitory zones (26–27 mm). Well-diffusion test results showed that the majority of the enterococci strains CFS had antimicrobial activity against the pathogenic microorganisms, especially on Gram negative indicators. Cell-free supernatant of *E. faecium* GJ40 was the one that produced the largest inhibition zones (14 to 21 mm) in the majority of the indicator microorganisms assayed. All supernatants treated with 10 N NaOH (pH6) showed no inhibitory effect on the indicator strain assayed. With respect to fungal inhibition, any of the CFS assayed significantly inhibited the *Aspergillus* strains growth. But, in general, all CFS reduced AFB_1 production from 8 to 87%. The results demonstrate that enterococci isolated from healthy dog feaces produce substances with the capacity to inhibit some potential pathogenic bacteria growth and the capacity of inhibiting or reducing the AFB_1 production *in vitro*.

Keywords: *Enterococcus faecium*, microbial inhibition, pathogenic bacteria, aflatoxigenic *Aspergillus* spp.

Introduction

Domestic dogs plays several important roles in modern human society.[1] Many pet owners industries are concerned about the potential risk for mycotoxins contamination in pet foods, since the pets are fed for longer periods of time and thereby may become more vulnerable to chronic exposure to toxicants, especially by aflatoxins (AFs); these toxins cause acute hepatotoxic and carcinogenic effects in dogs.[2–6] In previous works an important percentage of *Aspergillus* section *Flavi* AFs producers strains and samples containing aflatoxin B_1 (AFB_1) were detected from ready-to-eat pet foods.[7–16] Bacteria contaminating commercial dry foods for dogs can be responsible for digestive tract diseases, among other pathologies. These microorganisms are often associated with their survival to any preservation treatment, contaminating food in storage and handling by the pet's owners at home.[17] *Salmonella* spp. *Staphylococcus aureus, Clostridium perfringens* and *Escherichia coli* can be often found in dog food causing important infectious gastrointestinal disease in dogs. Other pathogens as *Enterococcus faecalis*, *Bacillus* spp. and *Bacillus cereus* have also been found in contaminated dog food.[18,19]

The reduction of spoiling pathogenic bacteria and toxigenic fungi in animal feed production is relevant due to the important economic losses that they cause worldwide, thus there is a great interest in developing efficient and safe strategies to control these losses. Biocontrol is receiving special attention since bacterial strains that possess antimicrobial activity against other bacteria and/or moulds can be found in nature.[20–24] Lactic acid bacteria (LAB) such as *Lactobacillus* spp., *Leuconostoc* spp., *Streptocccus* spp. and *Enterococcus* spp., among others, produce antimicrobial compounds that are important in the bio-preservation of foods and feeds.[25–27] LAB have been widely used in food and are "generally regarded as safe" organisms (GRAS). They are used in commercial applications to improve the shelf life of fermented products and to reduce potential health hazards associated with bacteria and mycotoxins.[24] During the last years, most of the reports on antimicrobial activity of LAB under *in vitro* conditions have been focused on antibacterial effects.[28–33] In the literature, *Aspergillus*

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section *Flavi* species growth has been described as controlled by other molds, yeasts and bacteria mainly *Bacillus* spp., propionic acid bacteria and LAB isolates.[20–24] Enterococci are used as probiotic in commercial formulation specially destined for animal feeding. These strains produce antimicrobial substances against Gram-positive and Gram-negative bacteria as well as some fungal strains.[34] In a previous work,[35] it was determined the inhibitory effect of *E. faecium* and *Lactococcus lactis* subsp. *lactis* isolated from faeces of healthy dogs on growth parameters and AFB_1 production by aflatoxigenic strains on *in vitro* assays. However, there is no information about *Enterococcus* strains and their cell-free supernatant (CFS) on AFs contamination prevention in dog food. The aim of the present study was to evaluate the inhibitory effect of (i) *E. faecium* isolated from faeces of healthy dogs on the growth of potentially pathogenic bacteria isolated from clinical cases and (ii) CFS of *E. faecium*, non-treated and treated under different conditions, on the growth of potentially pathogenic bacteria isolated from clinical cases and (iii) non-treated CFS on the growth of *Aspergillus* section *Flavi* and AFB_1 production on *in vitro* assays.

Materials and methods

Enterococcus faecium strains

Nine non-pathogenic strains (*E. faecium* MF1, MF2, MF3, MF4, MF5, GJ9, GJ17, GJ18 and GJ40)[35] previously isolated from freshly faeces of 40 adults healthy dogs of different breeds fed with different diets were used for the inhibition assays due to their recovery capacity and inhibition abilities after freezer conditions. Briefly, the samples of faeces were transferred under aseptic conditions into a sterile flask, diluted with saline solution (1:10) and mixed using orbital shaker for 2 min. After dilution, 0.1 mL each sample was plated onto De Man-Rogosa-Sharpe agar (MRS, Merck, Darmstadt, Germany). Plates were incubated at 37°C for 24–48 h in microaerophilic conditions. From each colony obtained streak plate method on MRS agar was performed in order to obtain pure cultures. Phenotypic identification was based upon physiological and biochemical characteristics; sugar fermentation profile, in the API-20 Strep CH and API-50 CH fermentation, was carried out according to the manufacturer's instructions (bioMe'rieux, Marcy l'Etoile, France). All strains were negative to virulence screening test (biofilm formation, haemolysin and gelatinase activity, antimicrobial susceptibility and slide haemaglutination test).[36–38]

Indicator strains

Ten strains of potentially pathogenic bacteria as *Salmonella* spp., *Klebsiella* spp., *Proteus* spp. P1 to P10, *Pseudomonas* spp. PS1 to PS10, *E. coli* EC1 to EC10, *S. aureus* SA1 to SA10, *Staphylococcus* spp. ST1 to ST10, *E. faecalis* EF1 to EF10, *B. cereus* BC1 to BC10 and *S. agalactiae* SA1 to SA10, isolated from animal clinical cases were used, such as conjunctivitis, otitis, diarrhea, dermatitis and mastitis.

Fungal strains

Two *Aspergillus flavus* (AF1A and AF1B) and *A. parasiticus* (NRRL 2999 and AP2A) strains were evaluated. *A. flavus* strains and *A. parasiticus* AP2A were previously isolated from commercial dry dog food in Argentina.[16] Bacteria and fungi strains used in this study were maintained in glycerol (15%) at −80°C and kept in the culture collection at the Microbiology and Immunology Department of National University of Río Cuarto, Córdoba, Argentina.

In vitro antimicrobial activity screening

Inhibitory activity of *Enterococcus* strains against target bacteria was assessed using the cross-streak procedure [39] on plates containing MRS agar medium (Britania, Buenos Aires, Argentina). A single line of each enterococci culture grown in MRS broth (adjusted to 1.5 McFarland scale equivalent to $<3.10^9$ UFC m L^{-1}) was seeded in the middle of the agar plate. The plates were cultivated for 48 h at 37°C in 5% CO_2-air atmosphere and then inactivated using chloroform vapor for 30 min. Potential pathogenic bacteria were cultured in nutritive broth (Britania) for 24 h at 37°C. The inocula of indicator strains were adjusted to 0.5 McFarland scale and seeded in duplicate perpendicular to the streak line of enterococci. The width of the zone of inhibition (mm) of target bacteria extending from the culture line of enterococci was measured, after plate incubation for 24 h at 37°C in aerobic environment. Results were interpreted as follows: inhibition (presence of inhibition zone $\geq$5 mm of indicator strains around of the main strake) and no inhibition (no inhibition zone or inhibition zone <5 mm).[40]

Inhibitory capacity of E. faecium CFS on pathogenic bacteria

This assay was performed by the well-diffusion method in order to estimate the origin of the inhibitory activity of enterococci strains. Cell-free supernatants were obtained from seven *E. faecium* strains that showed the best antimicrobial activity by the cross-streak procedure. Briefly, an enterococci culture in MRS broth grown for 18 h under microaerophilic conditions at 37°C, was centrifuged at 8.500 rpm for 20 min at 4°C. The supernatants were exposed to chloroform vapors for 30 min and then were treated under different conditions. Different portions of each supernatant were (i) boiled for 30 min, (ii) neutralized with 10 N of NaOH (Sigma Aldrich, Buenos Aires, Argentina) and (iii) treated with catalase 0.1 mg/mL (Sigma Aldrich). The indicator strains were cultured in nutritive broth for 24 h at 37°C and a suspension (adjusted to 0.5

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McFarland scale equivalent to <300 UFC/mL) was seeded into nutritive agar using sterile swabs. Then, 9-mm wells were punched, and 100 μL of the CFS was placed in each well. The plates were incubated at 37°C for 24 h under microaerophilic conditions. Each assay was done in triplicates and all the experiments were repeated three times.

Effect of the CFS of E. faecium on Aspergillus section Flavi growth and AFB_1 production

The effect of the non-treated CFS of enterococci strains on growth of *Aspergillus* section *Flavi* strains was determined according to Schillinger and Varela Villarreal.[41] Cell-free supernantants (10 μL) were spotted onto the surface of MRS agar plates overlaid with 9 mL of MEA soft agar (0.7%) which had been inoculated with 1 mL of a spore suspension (10^5 spores/mL) of each fungal strain. All the plates were incubated for 7 days at 25°C. The inhibition halos and AFB_1 levels were analyzed at 3 and 7 days of incubation, respectively. Each assay was done by triplicates and all the experiments were repeated three times. AFB_1 was extracted according to Geisen.[42] Briefly, the mycelia of the strains were collected on the agar surface with a sterile brush and transferred to microtubes. The toxin was extracted with chloroform (500 μL) and centrifuged at 896 g for 10 min. The chloroform phase was transferred to a clean microtube, evaporated to dryness and stored until AFB_1 analysis. The quantification was performed by HPLC according to the methodology proposed by Trucksess et al. [43] with some modifications [44] and the AFB_1 solutions (standards) were prepared according to AOAC.[45] The extracts were dissolved in 1 mL of mobile phase and an aliquot (200 μL) was derivatized with 700 μL trifluoroacetic acid: acetic acid: water (20:10:70, v/v). Chromatographic separations were performed on a reversed phase column (Silica gel, 150 mm · 4.6 mm (i.d.), 5-l particle size; Varian Inc., Palo Alto, CA, USA). Water: methanol: acetonitrile (4:1:1, v v^{-1}) was used as mobile phase at a flow rate 1.5 mL min^{-1}. The fluorescences of AFB_1 derivatives were recorded at excitation and emission wavelengths of 360 and 460 nm, respectively. The concentration of this toxin was quantified by correlating peak heights of sample extracts with those of standard curves. The detection limit of the analytical method was 0.1 ng g^{-1}.

Statistical analysis

Data were analyzed with an analysis of variance. Means were compared using a linear mixed model and Fisher's protected least significant difference (LSD) test to compare the inhibition zones among the treatments and AFB_1 production. The analysis was conducted using software InfoStat, 2008 version (Di Renzo, J.A., UNC., Córdoba, Argentina); group InfoStat, National University of Córdoba, Argentina.

Results

E. faecium cross-strake inhibition assay

Table 1 shows the results obtained from the cross-strake assay. *Enterococcus faecium* MF1, GJ18 and GJ40 presented the major inhibitory activity against all pathogenic strains assayed; *E. faecium* GJ40 produced the larger inhibitory zones (17 to 27 mm), followed by G18 and MF1 ($P < 0.05$). In general, *Pseudomonas* spp., *Klebsiella* spp. and *Salmonella* spp. were the most inhibited in presence of these *Enterococcus* strains, showing inhibition zones of 8 to 27 mm (Fig. 1). *Enterococcus faecium* MF5 and GJ17 did not inhibit any of the pathogenic strains assayed.

Enterococcus CFS antimicrobial activity

Table 2 and 3 show the results obtained from the well-diffusion test of CFS of *Enterococcus* strains on the potentially pathogenic Gram-negative and Gram-positive bacteria assayed. In general, the majority of the enterococci strains CFS's showed antimicrobial activity against the indicator microorganisms, observed as a formation of an inhibition zone around the wells from 10 to 21 mm. *Proteus* spp. presented the largest inhibition zones in the interaction with the non-treated enterococci CFS (19 to 21 mm). *Salmonella* spp., *Pseudomonas* spp. and *E. coli* strains presented similar inhibition zones from 10 to 13 mm (Table 2). In general, Gram-positive indicators were lesser inhibited by enterococci CFS than Gram-negative indicators. Among

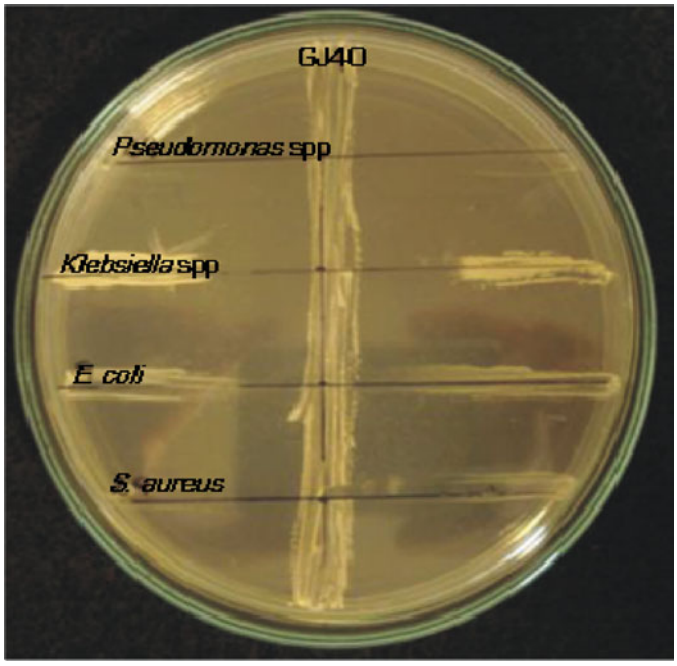

Fig. 1. Inhibition of potentially pathogenic bacteria by *Enterococcus faecium* GJ40 by the cross-strake method (color figure available online).

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Table 1. Inhibition of potentially pathogenic bacteria by *Enterococcus faecium* strains isolated from healthy dog faeces (cross-strake method).

	Enterococcus faecium strains – zone of inhibition (mm)[1] ± *SD*[2]							
Pathogenic strains[3]	*MF1*	*MF2*	*MF3*	*MF4*	*GJ9*	*GJ18*	*GJ20*	*GJ40*
S. aureus	$25–27^{abc} \pm 4$	$10–11^{lmnopq} \pm 2$	NI	$9–10^{mnpqr} \pm 1$	$8–9^{nopqrs} \pm 1$	$26–27^{ab} \pm 1$	$5–7^{rs} \pm 3$	$27–28^{a} \pm 2$
Staphylococcus spp.	$18–27^{abcdef} \pm 4$	$11–13^{klmn} \pm 3$	NI	$8^{pqrs} \pm 1$	$8–10^{mnopqrs} \pm 1$	$22–26^{bcde} \pm 2$	NI	$27–28^{a} \pm 1$
E. coli	$17–27^{abcdefg} \pm 4$	$2–10^{lmnopq} \pm 4$	$3–9^{qrs} \pm 2$	$2–12^{lmnop} \pm 1$	$10–12^{lmnop} \pm 1$	$17–27^{abcdefg} \pm 3$	$15–17^{ghij} \pm 1$	$17–27^{abcdefg} \pm 4$
E. faecalis	$13–16^{ijk} \pm 2$	$12–14^{jkl} \pm 2$	NI	$2–3^{s} \pm 1$	$12–14^{jkl} \pm 1$	$9–10^{mnpqr} \pm 0.5$	NI	NI
Salmonella spp.	$23–26^{bcd} \pm 3$	$8–10^{mnopqrs} \pm 2$	$8–10^{mnopqrs} \pm 2$	$9–10^{mnopqr} \pm 1$	$10–12^{lmnop} \pm 1$	$26–27^{ab} \pm 1$	$20–22^{cdefg} \pm 1$	$26–27^{ab} \pm 1$
Klebsiella spp.	$20–24^{bcdef} \pm 5$	$6–15^{mnopqr} \pm 3$	$8–10^{mnopqrs} \pm 1$	$8–10^{mnopqrs} \pm 1$	$9–10^{mnpqr} \pm 1$	$26–27^{ab} \pm 1$	$16–19^{ghi} \pm 3$	$26–27^{ab} \pm 2$
Pseudomonas spp.	$20–24^{bcdef} \pm 4$	NI	$9–14^{klm} \pm 2$	$12–24^{fgh} \pm 3$	$6–9^{opqrs} \pm 1$	$25–27^{abc} \pm 1$	NI	$27–28^{a} \pm 2$

[1]Range. [2]SD: Standard deviation. [3]n = 10 strains of each potentially pathogenic bacterial strains isolated from animal clinical cases. Values with a letter in common are not significantly different according to the LSD test ($P < 0.05$). NI: No inhibition (halos <2 mm).

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Table 2. Inhibition of Gram negative bacteria by non-treated and treated CFS of *E. faecium* isolated from canine faeces.

	Inhibition zones against indicator microorganisms ± SD CFS treatments															
	Salmonella spp.				*Proteus spp.*				*E. coli*				*Pseudomonas spp.*			
Strains[1]	*T1*	*T2*	*T3*	*T4*	*T1*	*T2*	*T3*	*T4*	*T1*	*T2*	*T3*	*T4*	*T1*	*T2*	*T3*	*T4*
MF1	$12.0^{b} \pm 0.2$	$12.0^{b} \pm 2.0$	NI^{a}	$10.0^{b} \pm 1.5$	$21.0^{c} \pm 1.2$	$19.0^{cb} \pm 1.5$	NI^{a}	$17.0^{b} \pm 0.4$	$12.0^{b} \pm 0.1$	$11.0^{b} \pm 0.2$	NI^{a}	$12.0^{b} \pm 2.6$	$11.0^{b} \pm 0.2$	$11.0^{b} \pm 2.1$	NI^{a}	$10.0^{b} \pm 1.5$
MF2	$12.0^{b} \pm 1.2$	$11.0^{b} \pm 0.7$	NI^{a}	$10.0^{b} \pm 1.2$	$20.0^{c} \pm 2.1$	$22.0^{c} \pm 2.1$	NI^{a}	$10.0^{b} \pm 1.2$	$12.0^{b} \pm 0.5$	$12.0^{b} \pm 0.9$	NI^{a}	$11.0^{b} \pm 1.7$	$12.0^{b} \pm 0.2$	$13.0^{b} \pm 1.6$	NI^{a}	$11.0^{b} \pm 2.3$
MF3	$12.0^{b} \pm 0.1$	$13.0^{b} \pm 1.1$	NI^{a}	$12.0^{b} \pm 0.7$	$21.0^{c} \pm 1.2$	$20.0^{c} \pm 1.6$	NI^{a}	$13.0^{b} \pm 1.4$	$14.0^{b} \pm 2.6$	$13.0^{b} \pm 0.8$	NI^{a}	NI^{a}	$12.0^{b} \pm 0.2$	$13.0^{b} \pm 2.1$	NI^{a}	$10.0^{b} \pm 1.9$
MF4	NI^{a}	NI^{a}	NI^{a}	NI^{a}	$19.0^{b} \pm 1.1$	$18.0^{b} \pm 1.4$	NI^{a}	$18.0^{b} \pm 2.3$	NI^{a}	NI^{a}	NI^{a}	NI^{a}	NI^{a}	NI^{a}	NI^{a}	NI^{a}
GJ9	$12.0^{b} \pm 2.1$	$10.0^{b} \pm 1.4$	NI^{a}	$12.0^{b} \pm 1.5$	$21.0^{d} \pm 2.3$	$18.0^{c} \pm 1.2$	NI^{a}	$13.0^{b} \pm 0.9$	$12.0^{b} \pm 0.2$	$12.0^{b} \pm 0.2$	NI^{a}	$12.0^{b} \pm 0.9$	$12.0^{b} \pm 0.8$	$12.0^{b} \pm 2.3$	NI^{a}	$12.0^{b} \pm 0.8$
GJ18	$10.0^{b} \pm 0.2$	$10.0^{b} \pm 0.8$	NI^{a}	$10.0^{b} \pm 0.4$	$19.0^{c} \pm 1.7$	$17.0^{c} \pm 1.8$	NI^{a}	$12.0^{b} \pm 2.3$	$11.0^{b} \pm 0.6$	$8.0^{b} \pm 1.2$	NI^{a}	$9.0^{b} \pm 0.5$	$10.0^{b} \pm 1.4$	$10.0^{b} \pm 1.9$	NI^{a}	$10.0^{b} \pm 1.0$
GJ40	$14.0^{b} \pm 0.6$	$13.0^{b} \pm 0.3$	NI^{a}	$12.0^{b} \pm 0.6$	$21.0^{c} \pm 1.7$	$20.0^{c} \pm 1.6$	NI^{a}	$14.0^{b} \pm 1.6$	$14.0^{b} \pm 1.3$	$13.0^{b} \pm 1.7$	NI^{a}	$12.0^{b} \pm 1.5$	$14.0^{b} \pm 0.4$	$13.0^{b} \pm 0.8$	NI^{a}	$12.0^{b} \pm 2.2$

[1]*Enterococcus faecium* strains. SD: standard deviation. CFS: cell free supernatant. n: 10 strains of each indicator microorganism isolated from animal clinical cases. NI: halos ≤2 mm. T1: non treated supernatant. T2: boiled supernatant. T3: supernatant treated with 10 N NaOH. T4: supernatant treated with catalase. Mean values with a letter in common are not significantly different according to the LSD test ($P < 0.01$). Statistical data must be red for each *Enterococcus* strain, indicator microorganism and treatment.

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Table 3. Inhibition of Gram positive bacteria by non-treated and treated CFS of *E. faecium* isolated from canine faeces.

	Inhibition zones against indicator microorganisms ± SD *CFS treatments*															
	S. aureus				*B. cereus*				*E. faecalis*				*S. agalactiae*			
Strains[1]	*T1*	*T2*	*T3*	*T4*	*T1*	*T2*	*T3*	*T4*	*T1*	*T2*	*T3*	*T4*	*T1*	*T2*	*T3*	*T4*
MF1	NI^{a}	NI^{a}	NI^{a}	NI^{a}	13.0^{b} ± 0.2	13.0^{b} ± 1.4	NI^{a}	13.0^{b} ± 0.9	NI^{a}	NI^{a}	NI^{a}	NI^{a}	11.0^{c} ± 0.1	5.0 ^{b} ± 0.5	NI^{a}	11.0^{c} ± 1.2
MF2	NI^{a}	NI^{a}	NI^{a}	NI^{a}	13.0^{b} ± 1.4	13.0^{b} ± 1.5	NI^{a}	12.0^{b} ± 1.3	15.0^{b} ± 0.4	15 ^{b} ± 2.1	NI^{a}	14.0^{b} ± 2.2	12.0^{b} ± 0.1	12.0^{b} ±1.0	NI^{a}	11.0^{b} ± 2.4
MF3	NI^{a}	NI^{a}	NI^{a}	NI^{a}	10.0^{b} ± 0.6	10.0^{b} ± 0.8	NI^{a}	13.0^{b} ± 1.9	10.0^{b} ± 1.6	NI^{a}	NI^{a}	12.0^{b} ± 2.1	NI^{a}	NI^{a}	NI^{a}	NI^{a}
MF4	NI^{a}	NI^{a}	NI^{a}	NI^{a}	14.0^{b} ± 0.3	14.0^{b} ± 1.3	NI^{a}	13^{b} ± 1.2	11.0^{b} ± 1.3	NI^{a}	NI^{a}	NI^{a}	NI^{a}	NI^{a}	NI^{a}	NI^{a}
GJ9	NI^{a}	NI^{a}	NI^{a}	NI^{a}	NI^{a}	NI^{a}	NI^{a}	NI^{a}	NI^{a}	NI^{a}	NI^{a}	NI^{a}	NI^{a}	NI^{a}	NI^{a}	NI^{a}
GJ18	NI^{a}	NI^{a}	NI^{a}	NI^{a}	NI^{a}	NI^{a}	NI^{a}	NI^{a}	10.0^{b} ± 0.6	NI^{a}	NI^{a}	NI^{a}	NI^{a}	NI^{a}	NI^{a}	NI^{a}
GJ40	NI^{a}	NI^{a}	NI^{a}	NI^{a}	14.0^{b} ± 0.2	14.0^{b} ± 1.5	NI^{a}	13.0^{b} ± 1.7	NI^{a}	NI^{a}	NI^{a}	NI^{a}	12.0^{b} ± 0.2	12.0^{b} ± 2.7	NI^{a}	10.0^{b} ± 1.6

[1]*Enterococcus faecium* strains. SD: standard deviation. CFS: cell free supernatant. n: 10 strains of each indicator microorganism isolated from animal clinical cases. NI: halos ≤ 2 mm. T1: non-treated supernatant. T2: boiled supernatant. T3: supernatant treated with 10 N NaOH. T4: supernatant treated with catalase. Mean values with a letter in common are not significantly different according to the LSD test ($P < 0.01$). Statistical data must be red for each *Enterococcus* strain, indicator microorganism and treatment.

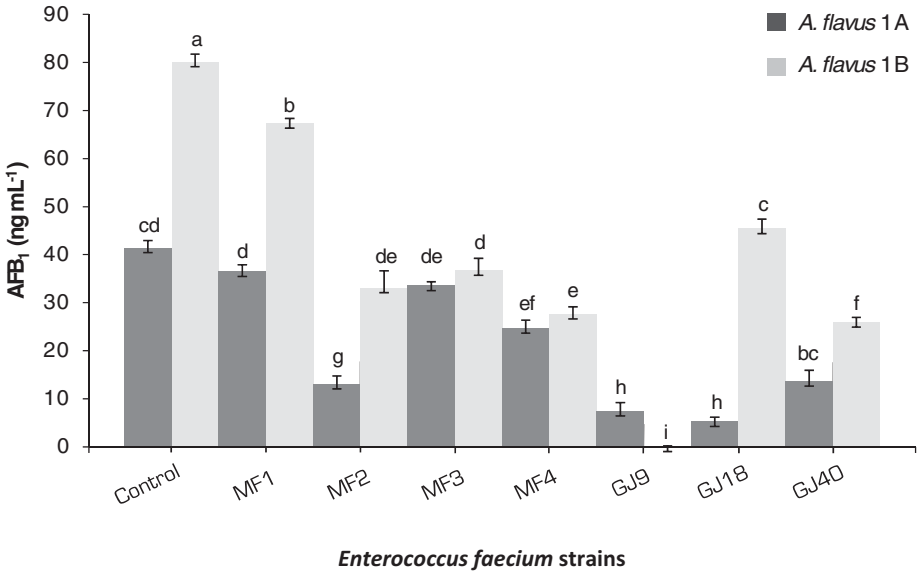

Fig. 2. Mean of AFB_1 concentration produced by *A. flavus* strains in the presence of enterococci non-treated cell-free supernatants. Mean values with a letter in common are not significantly different according to the LSD test ($P < 0.05$).

these, *Bacillus cereus* was the most inhibited, followed by *E. faecalis* (10 to 15 mm) and *S. agalactiae* (11 to 12 mm). No enterococci CFS inhibited *S. aureus* growth ($P < 0.01$) (Table 3). On the other hand, CFS of *E. faecium* GJ40 was the one that produced the largest inhibition zones (14 to 21 mm) in the majority of the indicator microorganisms assayed (*Salmonella* spp., *Proteus* spp., *E. coli*, *Pseudomonas* spp., *B. cereus* and *S. agalactiae*) followed by *E. faecium* MF3 and *E. faecium* MF2. All supernatants treated with 10 N NaOH (pH 6) did not show inhibitory effect on the indicator strain assayed. Significant differences were not observed among CFS non-treated (controls) ($P < 0.01$). Enterococci CFS treated with catalase produced inhibition zones significantly smaller than the control (non-treated CFS) in the interaction with *Proteus* spp. The CFS non-treated and treated of *E. faecium* MF4 did not inhibit the growth of none of the Gram negative and positive indicators assayed, except from *Proteus* spp. and *B. cereus* ($P < 0.01$).

Effect of the CFS of Enterococci strains on Aspergillus section Flavi growth and aflatoxin B_1 production

None of the CFS assayed significantly inhibited the *Aspergillus* strains growth. The mean levels of AFB_1 produced by *A. flavus* 1A and 1B in the control assays were 41 and 80 ng mL^{-1}, respectively. In general, all CFS reduced AFB_1 production by *A. flavus* 1A. *E. faecium* MF4, MF2, GJ9, GJ18 and GJ40 significantly reduced the production of this toxin in a 40, 68, 82, 87 and 67%, respectively ($P < 0.05$). Respecting *A. flavus* 1B, all enterococci CFSs assayed significantly reduced the production of AFB_1 among 16 - 100%. *E. faecium* GJ9 CFS totally inhibited AFB_1 production, while MF4 and GJ40 significantly reduced the production at 65 and 69% regarding control, respectively ($P < 0.05$). MF5 was the only strain that did not inhibit AFB_1 production (Fig. 2).

The mean levels of AFB_1 produced by *A. parasiticus* NRRL 2999 and *A. parasiticus* 2A in the control assays were 100 and 65 ng mL^{-1}, respectively. In the exposure assays, the levels of AFB_1 produced by *A. parasiticus* NRRL 2999 strain were significantly lower than the ones from the control assay. The inhibition percentages of AFB_1 production varied from 8 to 63%. The highest inhibition percentages of the mycotoxin were observed in the interaction with CFS of *E. faecium* MF3 (53%), MF4 (54%), GJ18 (57%) and GJ40 (63%). Respecting *A. parasiticus* 2A, AFB_1 production was significantly reduced by *E. faecium* MF3, MF4, GJ9, GJ18 and GJ40 in percentages of 55, 63, 81, 24 and 30%, respectively ($P < 0.05$; Fig. 3).

Discussion

This study was conducted to determine, *in vitro*, the inhibitory effect of *Enterococcus faecium* and its CFS on potentially pathogenic bacteria and evaluate if the last one could inhibit *Aspergillus* section *Flavi* growth and AFB_1 production.

The nature of the inhibition by LAB may be due to the production of antibacterial peptides and bacteriocins or the secretion of inhibitory compounds, such as organic and/or fatty acids and hydrogen peroxide.[46–47] In the cross-streak assay, the results showed that *E. faecium* MF1, GJ18 and GJ40 presented the highest inhibitory activity (halos ≥17 mm) against all the potential pathogens assayed.

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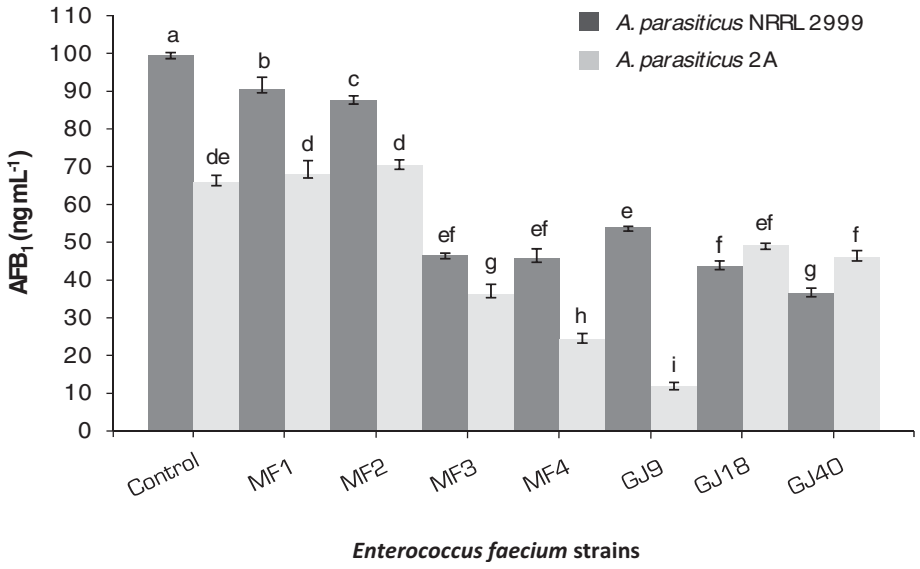

Fig. 3. Mean of AFB_1 concentration produced by *A. parasiticus* strains in the presence of enterococci non-treated cell-free supernatants. Mean values with a letter in common are not significantly different according to the LSD test ($P < 0.05$).

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These results do not agree with that previously reported by Strompfová et al.,[48,49] who informed that *S. aureus*, *E. coli, Enterobacter georgiviae, Pseudomonas* spp. and *S. enterica* sv. *Enteritidis* growth was not inhibited by *Enterococcus* spp, included *E. faecium* strains. In the present work, *S. aureus* and *Pseudomonas* spp. strains were inhibited by *E. faecium* with inhibition zones from 9 to 27 mm, as well as *Salmonella* spp. and *Klebsiella* spp. *E. faecalis* strains were also inhibited by some enterococci strains (halos from 2 to 16 mm). These results suggest that the enterococci assayed, especially *E. faecium* MF1, GJ18 and GJ40, have the ability to inhibit a wide range of potentially pathogenic microorganisms *in vitro*. The antimicrobial effects of a broad spectrum of substances from *Enterococcus* spp., especially from *E. faecium*, proved to be effective against Gram-negative and Gram-positive bacteria.[50] According to the nature of the inhibition activity, Hajikhani et al.[51] tested the antagonistic activity of the CFS of 12 enterococci strains isolated from white cheese against different bacterial strains; all enterococci assayed exhibited antagonistic activity against the most of the tested Gram-positive, except from *S. aureus*, and Gram-negative foodborne pathogens. On the other hand, from tested Gram-negative bacteria, *P. aeruginosa* and *P. vulgaris* were sensitive to CFS but, contrary to our results, *E. coli* was not sensitive to inhibitory agents produced by enterococci. These results partially agree with the results obtained in our study, due to *E. coli*, *Salmonella* spp., *Klebsiella* spp. and *Pseudomonas* spp. were significantly more sensitive to the antagonistic substances produced by *E. faecium* strains (with important inhibition zones from 10 to 21 mm). While *S. aureus*, *Staphylococcus* spp. and *E. faecalis* showed inhibition zones from 10 to 15 mm. The results obtained in the well-diffusion assays with the supernatant treated with 10 N NaOH did not show antagonistic activity. This may be due to the acid products present in the supernatants *Enterococcus* strains.

This inhibitory ability against potential pathogenic bacteria suggests that *E. faecium* strains isolated from dogs could be considered for further studies in order to formulate commercial mixtures with beneficial microorganisms as additives in dog feeding.

With respect to the *Aspergillus* section *Flavi* inhibition assay, none of the enterococci CFS inhibited the fungal growth, but promising results were found in the inhibition of AFB_1 production. These results agree with the ones reported in a previous inhibition assays using *Lactococcus* spp. and *Enterococcus* spp. cultures, not CFS.[35] Some of the *Enterococcus* strains tested, especially *E. faecium* GJ40, extended significantly the *Aspergillus* section *Flavi* lag phase, even though the growth rate was not modified. On the contrary, in the present study, none of the CFS assayed could inhibit fungal growth while AFs production was reduced. In agreement with the previous work, several strains of *E. faecium* (MF1, MF4, GJ9 and GJ40) reduced AFB_1 production as culture or CFS under *in vitro* conditions. These results suggest that *E. faecium* could produce some inhibitory metabolites of the AFB_1 production; on the other hand the competence for essential compound by AFs production must also be considered. Recently, Gerbaldo et al.[27] found that two *Lactobacillus* spp. producers of secondary active metabolites completely inhibited the fungal growth of all aflatoxigenic strains assayed; and AFB_1 production was reduced significantly (95.7 to 100%). These results partially agree with the ones of the present work because the enterococci assayed did not modify fungal growth but inhibited AFB_1 production in similar levels to *Lactobacillus* strains.

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Antifungal and antiaflatoxigenic activities in CFS of LAB have been reported by several investigators; especially *Lactobacillus* spp. have been reported to inhibit AFs production and retard *Aspergillus* growth.[22,52–54] In a previous assay, Gournama and Bullerman[20] suggested that the inhibition of AFs by *L. casei* in an inoculants mixture was suspected to be due to the low molecular weight of bacterial metabolites. Many investigators isolated antimicrobial compounds from CFS identified as lactic acid and two cyclic dipeptides as the major components responsible for this activity.[55] In addition, low concentrations of cyclic dipeptide inhibit AFs production, although higher concentrations are needed to inhibit the growth of *A. parasiticus*.[56] In an *in situ* study with contaminated corn grains, Ghonaimy et al.[57] showed that the treated corn grains with CFS of two *Lactobacillus* strains contained the minimum AFs concentration even in the occurrence of either *A. flavus* or *A. parasiticus*. Although the conditions of this work are different in the present study, the results could be compared facing the fact that even though moulds were not inhibited by enterococci CFS, AFB_1 production was reduced or inhibited. Further *in situ* assays should be performed in order to confirm the results obtained. In addition the purification, identification, chemical nature and biological characteristics of these antimicrobial and antiaflatoxigenic metabolites will be required.

Concluding, the results of this study demonstrate that enterococci isolated from healthy dog feaces produce substances with the capacity to inhibit potential pathogenic bacteria growth of *Salmonella* spp., *E. coli*, *Pseudomona* spp., *Proteus* spp., among others that are animal feed contaminants and potentially food intoxication agents, in the same way, the capacity of inhibiting or reducing the aflatoxin B_1 production *in vitro*. This effect may be due to the presence of acid products as lactic acid in the supernatants according to the well diffusion test and AFB_1 inhibition assay, but further studies must be performed in order to detect the presence of enterocins, peptides or other inhibitory substances with the aim to purify them and use them as preservative.

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