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Relationship between the probiotic *Lactobacillus rhamnosus* and *Enterococcus faecalis* during the biofilm formation

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One of the factors that make the treatment of *Enterococcus faecalis* infections difficult is their ability to form biofilm, as well as their natural and acquired resistance to antibiotics which does not have specific drugs for their inhibition. This fact makes essential the search for alternative treatments, as the use of probiotics strains of *Lactobacillus rhamnosus* has been effective in the treatment of some diseases. In this investigation, the relationship between the probiotic strain of *L. rhamnosus* and *E. faecalis* during the biofilm formation was analyzed. Standardized suspensions used in biofilm development and treatment in different stages of the biofilm formation were prepared. The *L. rhamnosus* suspension was placed in contact for 90 min with *E. faecalis* freshly created biofilms (initial adherence) in the 24 h biofilms. The same was made with *E. faecalis* suspension on *L. rhamnosus* biofilms. *L. rhamnosus* showed no inhibitory effects on *E. faecalis* biofilms formation, with an increase in the counting of colony forming units in the treated groups (p=0.0047, p=0.0060). About the *L. rhamnosus* biofilms, there was no significant difference for both treatment stages. The probiotic strain interfered *in vitro* with the *E. faecalis* biofilm formation, thereby intensifying the growth of *E. faecalis* biofilm.

Key words: *Enterococcus faecalis*, *Lactobacillus rhamnosus*, biofilm, virulence factor, probiotic.

INTRODUCTION

Enterococcus faecalis is the main cause agents of nosocomial infections and even being present on human intestinal microbiota, has been related to many cases of infections in imunossupressed individuals and or/those treated by broad-spectrum antibiotics. This bacterium, is known by its natural resistance to some antibiotics; large

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Table 1. *E. faecalis* and *L. rhamnosus* groups divided according to the time of biofilm formation (90 min or 24 h) and interaction solution (*L. rhamnosus* or *E. faecalis* or control – saline solution).

First Suspension	Time of biofilm formation		Interaction solution
	90 min.	24 h	90 min.
Enterococcus faecalis	G1	-	Control - Saline solution
	G2	-	*L. rhamnosus* suspension
	-	G3	Control - Saline solution
	-	G4	*L. rhamnosus* suspension
Lactobacillus rhamnosus	G5	-	Control - Saline solution
	G6	-	*E. faecalis* suspension
	-	G7	Control - Saline solution
	-	G8	*E. faecalis* suspension

capacity of genetic shares between the microbial cells, this potentiates its resistance to some antimicrobial agents such as vancomycin (Arias and Murray, 2012; Heintz et al., 2010; Sartelli, 2010).

The biogenesis and biofilm formation ability also contribute to the treatment of infections caused by *E. faecalis*. A matrix of exopolysaccharides surrounding offers protection against the action of antibiotics and cells of immune system (Aparna and Yadava, 2008). It stimulates the persistence of bacterial infections and supports the cells of this community (Jefferson, 2004; Mohamed and Huang, 2007; Paganelli et al., 2012; Rabin et al., 2015).

With the dissemination of resistant bacterial strains, the development of new drugs and also the search for alternative treatments, such as phytotherapy (Bhardwaj et al., 2013; Castilho et al., 2013; Sponchiado et al., 2014) and phagoterapy (Khalifa et al., 2015). There is also the use of probiotics strains with the intention of colonization and/or growth inhibition (Chapman et al., 2014).

The term probiotic was defined by the World Health Organization (WHO), in 2002, as “the use of live microorganisms administrated in adjusted amounts to promote positive physiological effects in the host”. The use is more frequent as biotherapeutics agents, especially in the preventive medicine. The most used bacteria as probiotic are those belonging to the lactic acid bacteria group, where the genus *Lactobacillus* is enclosed (Bubnov et al., 2015). This can intervene with the colonization and proliferation of pathogenic microorganisms, by the production of antimicrobials substances (Fukuda et al., 2011, Oelschlaeger, 2010; Todorov et al., 2011), or by means of immunomodulatory effects (Remus et al., 2011; Suzuki et al., 2008).

Currently, the specie of *Lactobacillus* most studied is *Lactobacillus rhamnosus* because it has good characteristics of growth and adhesion in gut epithelium and this helps in competing with pathogenic microorganisms on the gastrointestinal tract and intervening in immune system, intensifying the IgA production, stimulating the local release of interferons facilitating the antigenic transport to the lymphoid cells, thus, serving to increase the presentation of these to the Plate of Peyer (Vandenplas et al., 2015; Segers and Leeber, 2014, Gupta and Garg, 2009). In this investigation, the relation between probiotic strain of *L. rhamnosus* and *E. faecalis* during biofilm formation was analyzed.

MATERIALS AND METHODS

E. faecalis (ATCC 29212) and *L. rhamnosus* (ATCC 1465) were cultivated, respectively, on Brain Heart Infusion broth (BHI, Kasvi, Roseto degli Abruzzi, Italy) and MRS *Lactobacillus* (Hymedia, Mumbai, India), and later incubated at 37°C for 24 h, with tension of (5%) of CO_2.

Each 24 h culture was centrifuged (Centribio TDL80-2B) at 843 g for 10 min, and the supernatant was discarded. The pellet was resuspended in sterilized saline solution (NaCl 0.9%) and centrifuged again, with the supernatant discarded at another time. This procedure was repeated three times to remove the culture way residues. From the last deposit was prepared standardized suspensions for spectrophotometry (Femto 432C, São Paulo, Brazil) in wave length of 530 nm, adjusted in 10^7 cells/mL for *E. faecalis* and 10^8 cells/mL for *L. rhamnosus* (absorbance at 0.020 and 0.600 respectively).

These suspensions were used in different biofilm assays, divided in groups according to Table 1.

To the wells of 96-well microtitration plates had been added 200 µL of *E. faecalis* and *L. rhamnosus* suspensions. The plates were incubated at 37°C under agitation (75 rpm, multi-functional agitator Biomixer TS-2000) per 90 min. After this time, the wells were washed three times with sterilized saline solution to remove the cells not adhered. The groups of 90 min experiment (G1, G2, G5 and G6) immediately received the interaction solution. The groups pertaining to the 24-hours experiment (G3, G4, G7 and G8) received 200 µL of BHI broth, was incubated for more than 24 h in 37°C, and was washed three times and then, received the interaction solution.

The interaction solution was 200 µL of *L. rhamnosus* suspension (G2 and G4) or 200 µL of *E. faecalis* suspension (G6 and G8), and the control groups received 200 µL of sterilized saline solution (G1, G3, G5 and G7). The plates were placed under agitation on 75 rpm at 37°C per 90 min. A new laundering was done with sterilized saline solution, for three times, to remove the cells not-adhered. After that, 200 µl of BHI broth was added to each well. The groups G1, G2, G5 and G6 (90 min) were incubated at 37°C for more 48 h (with broth renovation after 24 h), and the groups G3, G4, G7 and

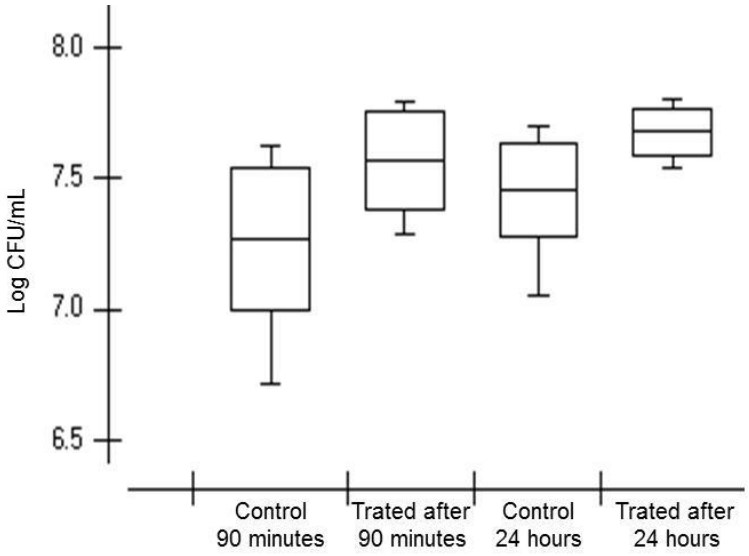

Figure 1. Average and shunting line standard of colonies units' formation for milliliters (in logarithms in base 10) after 90 min of *E. faecalis* biofilm contact (initial stage of biofilm formation and after 24 h of formation) with saline solution (control) or *L. rhamnosus*.

G8 (24h) were incubated for only 24 h. The wells were later washed by sterilized saline solution, three times, to remove the cells not-adhered. Then, 200 µL of sterilized saline was added. The loosed bacterial biofilms were carried by means of friction and homogenization for each well with sterilized tips.

Dilution series (10^{-2} up to 10^{-8}) was prepared for the loosen cells suspension and plating for the drop method (Herigstad et al., 2001), on triplicate, in *Enterococci* agar (Vetec, Rio De Janeiro, Brazil) for groups 1, 2, 3 and 4 and agar *Lactobacillus* MRS (Hymédia, Mumbai, India) for the groups 5, 6, 7 and 8. These were incubated for 24 h in 37°C with CO_2 tension of (5%), for *L. rhamnosus* groups.

The reading was carried out by counting and calculating the number of colony forming units per milliliter (CFU/mL). The number of CFU/mL was transformed into logarithms to base 10 and after analysis of normality, the data was analyzed by Wilcoxon test (program Bioestat 5.3) considering the level of significance of 5%.

RESULTS AND DISCUSSION

After 90 min of exposition, there was a significant positive interference of *L. rhamnosus* probiotic strain, with increase in the CFU/mL counts of *E. faecalis* in biofilms of 90 min (p=0.0047) and of 24 h (p=0.0060) of formation (Figure 1). The average increase was 85% in biofilms of 90 min and 58% within the 24 h biofilm, when compared with the counting in the control groups. There was no significant interference of *E. faecalis* on biofilm formation by *L. rhamnosus* (90 min, p=0.5751 and 24 h, p=0.2300) (Figure 2).

L. rhamnosus has been of the most studied probiotic strain, and its use is considered safe (Vandenplas et al., 2015), however, the interaction with different micro-organism, pathogenic or not, is still unclear. Thus, the present study is considered to evaluate if the *L. rhamnosus* probiotic strain would be capable to interfere with the growth of *E. faecalis* biofilms hindering its formation or reducing the number of cells, as well as if *E. faecalis* could interfere with the biofilm formation by *L. rhamnosus*.

The suspension contained 10^8 CFU/mL of *L. rhamnosus* opted for the use by reason of, the majority of the lyophilized *Lactobacillus*, commercialized in pharmacies, contains enters 10^8 and 10^{11} CFU/g in each dose and in microbial ecology, it is considered that a microorganism influences in the ecosystem where it only meets when its population is equal or superior to 10^7 CFU/g or mL (Stefe et al., 2008).

The choice of the species was because, in case of probiotic consumption, *E. faecalis* and *L. rhamnosus* would interact in the gastrointestinal mucosa, forming biofilms. *E. faecalis* biofilm formation ability is a key-factor in the persistence of bacterial infections and difficulty of treatments (Hoiby et al., 2011; Zoletti et al., 2011). The extracellular polymeric matrix prevents the host cells defenses or restraint to the penetrations of antimicrobials agents (Donlan and Costerton, 2002). *L. rhamnosus* and *E. faecalis* occupy inverse extremities in the current microbiological scene, *E. faecalis* is responsible for innumerable cases of infection in imunossupressed individuals, with strains resistant to antibiotics of broad spectrum, while *L. rhamnosus* is commonly used in probiotic therapy (Vandenplas et al., 2015; Rabin et al., 2015).

Thus, as it has been stated that *L. rhamnosus* presents an ample antimicrobial potential (Dubourg et al., 2015), it

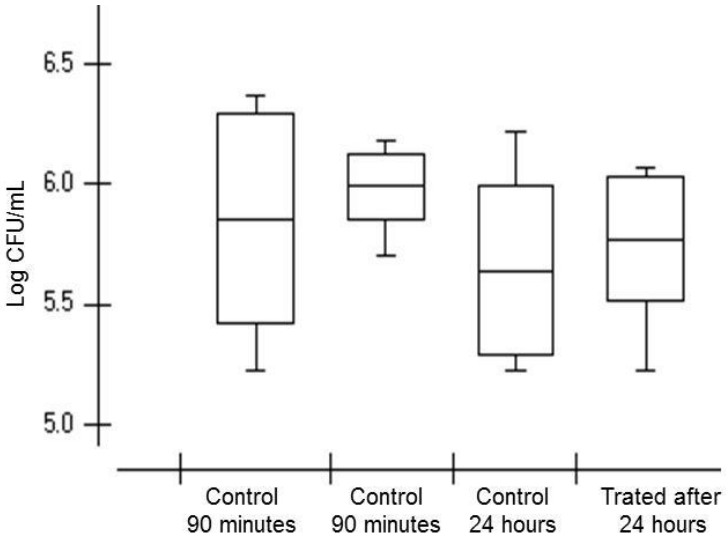

Figure 2. Average and shunting line standard of colonies units' formation for milliliters (in logarithms in base 10) after 90 min of *L. rhamnosus* biofilm contact (initial stage of biofilm formation and after 24 h of formation) with saline solution (control) or *E. faecalis*.

was expected that, when interacting with *E. faecalis*, *L. rhamnosus* would interfere with its growth and harmed the biofilm formation. However, with the methodology used in this research, *L. rhamnosus* not only did not inhibit the growth of *E. faecalis* in biofilm, but also enhanced its growth.

The metabolic and structural features of *E. faecalis* allow its adaptation (modification) in accordance to the ambient and nutritional environmental conditions (Stuart et al., 2006). It is known that in conditions with low glucose availability, the biogenesis of *E. faecalis* biofilm decay, and mechanisms, as increase of the hydrophobicity of the cell surface, increase for the maintenance of its viability (Ran et al., 2015).

Thus, the results of the *in vitro* interaction of these microorganisms must be considered as the existence of an intraspecific competition between *E. faecalis* and *L. rhamnosus*, and the availability of nutritional resources as determinative for the increase in the counting of microorganisms in the experiment.

Factors like pH and temperature act directly in cell generation time and metabolic taxes of *E. faecalis*. When leaving a favorable environment, pH 6.5 and 37°C, the time of generation cellular is extended, however, this fact is compensated by the increase of the metabolic activity (Morandi et al., 2005). The use of BHI media, which pH is around 7.2 ± 0.2, created an initial favor to the growth of *E. faecalis*, and even the possible posterior production of metabolites, as ascetic and latic acids, by L. rhamnosus was not enough to inhibit its overgrowth.

In vivo tests with administration of *L. rhamnosus* probiotic strain in children colonized by *E. faecalis* strain resistant to vancomycin (VRE), is a significant elimination of the carrier state and increase in gastrointestinal counting of colonies of *Lactobacillus* spp. was observed (Szachta et al., 2011). *In vivo,* conditions are totally different from our experimental condition, where other microorganisms strains are present besides host epithelial and immune cells.

Therefore, although the probiotic strain of *L. rhamnosus* did not present inhibitory effects on *E. faecalis* biofilm *in vitro*, it must be considered its immunomodulatory effect in the host, and does not discard it a prophylactic measure.

Thus, from the methodology used in the present research, it can be concluded that the probiotic strain of *L. rhamnosus* intervened on *E. faecalis* biofilm and intensified its growth.

Conflict of interest

The authors declare that they have no competing interests.

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