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Interaction between *Lactobacillus reuteri* and periodontopathogenic bacteria using *in vitro* and *in vivo (G. mellonella)* approaches

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Abstract

Periodontitis is a multifactorial inflammatory disease, and the major cause of tooth loss in adults. New therapies have been proposed for its treatment, including the use of probiotics such as *Lactobacillus reuteri*. The objective of this study was to evaluate the antimicrobial effects of *L. reuteri*: live, heat-killed, and culture filtrate (cell free supernatant), on periodontopathogenic bacteria (*Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans*) *in vitro,* as well as the *in vivo* survival curve, hemocyte density, and microbial recovery using *Galleria mellonella.* For *in vitro* assays*,* all preparations reduced colony forming units of *F. nucleatum*, while only live *L. reuteri* reduced the growth of *A. actinomycetemcomitans*. All treatments reduced periodontopathogenic bacteria growth *in vivo*. The treatment with the supernatant increased the survival of larvae infected with *F. nucleatum* more than the treatment with live *L. reuteri*, and none of the treatments altered the survival of *A. actinomycetemcomitans*-infected larvae*.* In addition, the treatment with *L. reuteri* preparations did not alter the hemocyte count of *F. nucleatum*- and *A. actinomycetemcomitans*-infected larvae. This study demonstrated that *L. reuteri* preparations exerted antimicrobial effects and increased the survival of *G. mellonella* infected by *F. nucleatum,* although only live *L. reuteri* was able to reduce the growth of *A. actinomycetemcomitans in vitro*.

Keywords: Periodontal diseases. Probiotics. *Fusobacterium nucleatum*. *Aggregatibacter actinomycetemcomitans*. *Lactobacillus reuteri*. *Galleria mellonella.*

INTRODUCTION

Periodontitis is a multifactorial chronic inflammatory disease, which is highly prevalent in the adult population. It is mainly caused by the interaction between microorganisms and the host, which causes destruction of the teeth-supporting tissues, leading to tooth loss (Papapanou *et al*. 2018). It is estimated that severe periodontal disease affects approximately 10% of the global population (Frencken *et al*. 2017), and treatment of the disease incurs high costs and remains a heavy burden to the public health sector (Mohd-Dom *et al*. 2014).

Gram-negative anaerobic bacteria, such as *Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans* are among the main oral microorganisms associated with periodontitis (Ezzo and Cutler 2003). Particularly, *A. actinomycetemcomitans* serotype b is strongly related to a severe form of periodontitis (Yang *et al*. 2005), which progresses rapidly (Albandar 2014).

Reduction of periodontopathogenic bacteria is the focus of the initial conventional therapy, including scaling, root planing, and instructions on oral hygiene, which may be associated with the use of antibiotics (Haffajee, Teles and Socransky 2006). However, the use of antibiotics can result in side effects such as bacterial resistance (Ardila, Granada and Guzmán 2010).

New prevention strategies and adjunctive treatments for periodontal diseases have been studied. Among them, there are the probiotics, internationally defined as living microorganisms that benefit the host's health when administered in adequate doses (Sanders 2008). Several mechanisms of action of probiotics have been proposed, such as inhibition of pathogen adhesion, stimulation, and modulation of the host immune system, as well as production of antimicrobial substances (Sánchez *et al*. 2017).

However, the use of probiotics is not totally free of adverse effects, and in individuals with impaired immune system, probiotic usage should be treated with caution (Doron and Snydman 2015). An alternative to minimize such adverse effects is to inactivate the microorganism while retaining its beneficial properties. Hence, the terms "paraprobiotic" or “ghost probiotic” have been proposed for non-viable microbial cells, whether intact or lysed, that confer health benefits when administered in adequate dosages (Taverniti and Guglielmetti 2011). Inactivated cells can trigger the same immune response as viable cells by different mechanisms of action, and most importantly, after inactivation, they potentially cause harmless effects (Taverniti and Guglielmetti 2011).

Lactobacillus reuteri is a resident lactic-acid bacterium found in the gastrointestinal tract of humans and some animals such as mouse, rat, pig, chicken, and turkey, as well as in the breast milk and urogenital tract of humans (Oh *et al*. 2010). *Lactobacillus reuteri* produces reuterin, a broad-spectrum antimicrobial substance that induces oxidative stress in pathogenic microorganisms (Schaefer *et al*. 2010). *Lactobacillus reuteri* ATCC PTA 5289 and DSM 17938 are considered important probiotic strains associated with improved periodontal health (Laleman *et al*. 2020), control of pregnancy gingivitis (Schlagenhauf *et al*. 2016), and induced shift in the oral microbiota composition (Romani Vestman *et al*. 2015). Systematic reviews and meta-analyses reports support the use of *L. reuteri* as a short-term adjuvant therapy for periodontitis, associated with scaling and root planing, especially in deep pockets (Martin-Cabezas *et al*. 2016), leading to a significant reduction in probing depth and gingival bleeding (Gruner, Paris and Schwendicke 2016).

Invertebrates, such as *Galleria mellonella*, have been widely used as alternative models of infection because of their structural and functional similarities to the mammalian immune system (Tsai, Loh and Proft 2016). Cellular immune response in *G. mellonella* is elicited by the hemocytes present in hemolymph (Browne, Heelan and Kavanagh 2013). These larvae are easy to grow and the experiments can be performed at 37 °C, which is ideal for the study of microbial infection (Grounta *et al*. 2016). Although this invertebrate model has been used to study the interaction of different probiotics with several bacteria and fungi (Rossoni *et al*. 2019), research applying *G. mellonella* to evaluate periodontopathogens is still limited (Aparecida Procópio Gomes *et al*. 2016; Dos Santos *et al*. 2017; Geraldo *et al*. 2020; Moman *et al*. 2020).

In view of the need for new therapeutic or adjunctive therapies for periodontal disease and the successful application of *L. reuteri* in some clinical trials, we hypothesized that the heat-killed *L. reuteri* and its supernatant could also be effective in the case of infection caused by periodontopathogens.

MATERIAL AND METHODS

Microorganisms and growth conditions

F. nucleatum (ATCC 25586; Microbiologics, St. Cloud, MN) and *A. actinomycetemcomitans* (ATCC 29522; Microbiologics) were grown in Brucella agar (Acumedia, Lansing, Michigan, USA), supplemented with defibrinated sheep blood, 1% hemin, and menadione solution (Sigma-Aldrich, St. Louis, Missouri, USA) for five days under anaerobic conditions. Isolated colonies were suspended in phosphate buffered saline (PBS), and cell concentrations were

standardized using a spectrophotometer (Micronal, São Paulo, São Paulo, Brazil) at 10^7, 10^8, and 10^9 cells/mL (at 550 nm for *F. nucleatum*) (Song *et al*. 2017), and at 10^7, 10^8, and 10^9 cells/mL (at 600 nm for *A. actinomycetemcomitans*) (Noh *et al*. 2016).

L. reuteri (ATCC PTA 5289 and DSM 17938, Prodentis, BioGaia, Stockholm, Sweden) was grown in De Man, Rogosa and Sharpe broth (MRS) (Acumedia) for 24 h in anaerobic conditions. To prepare live *L. reuteri,* 10^8 cells/mL was standardized at 570 nm (Geraldo *et al*. 2020). The culture filtrate (cell free supernatant) of *L. reuteri* was prepared based on the methodology proposed by Schaefer *et al*. (2010), based on the conversion of glycerol to reuterin by *L. reuteri*. After 24 h growth in MRS broth, the culture was centrifuged and washed twice using 50 mM sodium phosphate buffer. Approximately 150 mg of bacteria (wet weight) was resuspended in 15 mL of 250 mM glycerol and incubated under anaerobic conditions for 2 h. Subsequently, the suspension was centrifuged, and the supernatant was pooled and sterilized using a membrane filter of 0.22 µm pore size.

Heat-killed *L. reuteri* suspension at 10^8 cells/mL was prepared from the 24-hour culture in MRS broth, which was autoclaved at 121 °C for 20 min (Chuang *et al*. 2016). Both the heat-killed *L.reuteri* (HKL) and the supernatant were seeded onto MRS agar (Acumedia) to confirm the absence of viable cells.

In vitro antibacterial effect of _L. reuteri_ preparations

The antibacterial activity of *L. reuteri* preparations was evaluated based on the methodology proposed by Lin *et al*. (2015), with modifications. Two hundred and fifty microliters of a suspension containing 2.5 x 10^6 cells of *F.*

nucleatum or *A. actinomycetemcomitans* were added to 250 µL of a suspension containing 2.5 x 10^6 cells of live, heat-killed *L. reuteri*, or supernatant solution, in tubes containing 1.5 mL of brain heart infusion broth (BHI) (Kasvi, Roseto degli Abruzzi, Abruzzo, Italy). Control groups were performed using *F. nucleatum* or *A. actinomycetemcomitans* in PBS or glycerol. The tubes were incubated under anaerobic conditions at 37 °C for 72 h. For CFU determination, serial decimal dilutions were performed and the microorganisms were seeded in the appropriate culture agar. After the interactions involving live *L. reuteri*, MRS agar was used to evaluate the effects of periodontopathogens on the probiotic, while growth in the supplemented Brucella blood agar was observed to evaluate the effect of *L. reuteri* and preparations on periodontopathogenic bacteria. The plates containing *F. nucleatum* and *A. actinomycetemcomitans* were incubated for five days, and *L. reuteri* plates were incubated for three days. For all groups, we performed five technical replicates, and each experiment was performed independently, at least twice.

In vivo* study using *G. mellonella

Galleria mellonella larvae *(Lepidoptera: Pyralidae*), from the Laboratory of Microbiology of the Institute of Science and Technology of São José dos Campos/Unesp (São Paulo State University), with body weights between 190-230 mg, light-colored and free of dark spots and/or pigments on their cuticles, were kept in Petri dishes at 37 °C for the experiments.

To determine the concentration of the microorganisms to be used in subsequent experiments, survival curves were investigated using 10^7, 10^8 and 10^9 cells/mL of *F. nucleatum* and *A. actinomycetemcomitans*, and 10^4 to 10^8

cells/mL of *L. reuteri*. Ten microliters of the respective suspension were inoculated using a syringe (Hamilton Inc., Reno, NV) in the last left proleg of the larvae. The number of dead larvae was recorded daily for seven days.

After determining the concentration of each microorganism to be used, 10 µL of the standardized suspension of live cells, supernatant, and heat-killed *L. reuteri* were inoculated 3 h (3 h prophylaxis) and 24 h (24 h prophylaxis) prior to inoculation with *F. nucleatum*, *A. actinomycetemcomitans,* or PBS. The number of dead larvae was recorded daily for seven days. For controls, larvae were injected with glycerol or PBS.

To evaluate hemocyte density, larvae were kept in a bacteriological incubator at 37 °C for 3 h after the periodontopathogen injection. The hemolymph was then removed by puncturing the hemocoel from three larvae, pooled, and diluted using an anticoagulant solution (2% NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, and 10 mM EDTA). The density of the hemocytes was estimated using a Neubauer's chamber. Serial dilutions of the pooled hemolymph were also seeded onto Brucella blood agar supplemented with 1% hemin and menadione solution and incubated in an anaerobic chamber for microbial recovery over five days. Colonies formed were counted (CFU/mL).

A total of 12 larvae per group were used in each experiment. Each experiment was performed at least twice, and representative data from one experiment are shown in the results section.

Statistical Analysis

Survival curve was constructed by using the Kaplan-Meier method and estimation of differences in the survival experiments were performed by using

Log-rank test (Mantel-Cox). For other analyses, ANOVA (and Tukey's post-hoc test) or Student's *t*-test were applied, depending on the distribution of data. In all tests, a significance level of 5% was used. The software GraphPad Prism 6 (La Jolla, CA) was used for all statistical analyses.

RESULTS

In vitro study

This study on the effect of live, heat-killed *L. reuteri* and cell free supernatant on periodontopathogens demonstrated that all three preparations significantly reduced the growth of *F. nucleatum*, and the greatest reduction (0.499 Log10) occurred with the supernatant. However, only live *L. reuteri* significantly reduced the growth of *A. actinomycetemcomitans* (1.546 Log10) after the interaction. Glycerol at 250 mM also demonstrated significant antimicrobial activity against *A. actinomycetemcomitans* (Fig. 1). No significant effects of *F. nucleatum* (Student's *t*-test; *P*= 0.074) or *A. actinomycetemcomitans* (Student's *t*-test; *P*= 0.092) against *L. reuteri* were observed.

In vivo study

Firstly, we evaluated whether *L. reuteri* could influence *G. mellonella* survival. It was observed that 100% of larvae survived after seven days of inoculation with live *L. reuteri* at all concentrations. Moreover, heat-killed *L. reuteri* and its supernatant were not able to cause any death in *G. mellonella*.

To determine the lethal concentration of *F. nucleatum* and *A. actinomycetemcomitans,* survival curves with different concentrations of these pathogens were obtained. When the larvae were infected with *F. nucleatum*, at

concentrations of 10^7, 10^8, and 10^9 cells/mL, the mortality rates at the end of the experimental period were 33.33%, 83.33%, and 100%, respectively. However, there was no significant difference in survivals at concentrations of 10^8 and 10^9 cells/mL [Log-rank test (Mantel-Cox), *P*= 0.067]. Infection with *A. actinomycetemcomitans* at 10^7 cells/mL led to a mortality rate of 91.66% after seven days. At concentrations of 10^8 and 10^9 cells/mL, all larvae died after 144 h and 48 h, respectively, without significant statistical difference [Log-rank test (Mantel-Cox), *P*= 0.2591]. Based on these results, the concentration chosen for inoculation in *G. mellonella* was 10^8 cells/mL for both periodontopathogenic bacteria and probiotic *L. reuteri*.

Interaction between *L. reuteri* (and preparations) and *F. nucleatum* in *G. mellonella*

Survival Curve

We evaluated two conditions of prophylaxis. At 3 h of prophylaxis, the best results were observed in the groups that received glycerol (75% survival) and supernatant (66.66% survival), followed by the group that received live *L. reuteri* (50% survival), but all were statistically higher compared to the control group, which received only *F. nucleatum* (16.67% survival). Despite the HKL-treated group had a higher survival rate than the *F. nucleatum* control group, this difference was not statistically significant (Fig. 2A).

Inoculation of *L. reuteri* and preparations 24 h prior to injecting *F. nucleatum* demonstrated that only the supernatant significantly increased the survival rate of larvae compared to the *F. nucleatum* control group (Fig. 2B).

Hemocyte density and microbial recovery

We evaluated the effect of live, heat-killed *L. reuteri* and cell free supernatant on hemocyte count before analyzing the effect of periodontopathogen interactions. The prophylactic inoculation of live and heat-killed *L. reuteri* significantly reduced the number of hemocytes after 3 h, which was similar to the PBS control after 24 h (ANOVA, $P < 0.0001$, Tukey's post-hoc test). No significant difference was observed with the supernatant treatment in comparison to the PBS control group, for the 3 h or 24 h (ANOVA, $P < 0.0001$, Tukey's post-hoc) prophylaxis experiments.

Treatment using live, heat-killed *L. reuteri* and supernatant, at 3 h or 24 h prior to the inoculation of *F. nucleatum* did not alter the number of hemocytes, which was similar to the *F. nucleatum* control group (Fig. 3A and B). In addition, all treatments reduced the CFU/mL of *F. nucleatum* recovered from the hemolymph when compared to the *F. nucleatum* control group in the 3 h prophylaxis experiment (Fig. 3C). When the pre-inoculation period was 24 h, the supernatant led to the greatest reduction of *F. nucleatum* (1.876 Log10) (Fig. 3D).

Interaction between *L. reuteri* (and preparations) and *A. actinomycetemcomitans* in *G. mellonella*

Survival curve

This study demonstrated that treatment using *L. reuteri* live, heat-killed or its supernatant could increase the survival time of the larvae, but there was no statistically significant difference among the groups or when compared to the *A. actinomycetemcomitans* control group, in the 3 h and 24 h prophylaxis experiments (Fig. 4).

Hemocyte density and microbial recovery

Live, heat-killed *L. reuteri* and supernatant did not alter the number of hemocytes of the larvae after infection when compared to *A. actinomycetemcomitans* control group, in the 3 h or 24 h prophylaxis experiments (Fig. 5A and B). CFU/mL counts in all treated groups for both periods were significantly lower than those in the *A. actinomycetemcomitans* control group; however, there was no significant difference among them (Fig. 5C and D).

DISCUSSION

The possibility of treating oral diseases using a natural, noninvasive, and non-stressful method has an important appeal and could help to prevent problems arising from pharmacological-based treatments such as antibiotic resistance. Probiotics have been extensively studied as adjuvants to conventional periodontal treatment (Martin-Cabezas *et al*. 2016; Gruner, Paris and Schwendicke 2016), unlike inactivated probiotics (Iwasaki *et al*. 2016). The use of cell-free supernatant, rather than viable bacteria, is also interesting, especially to prevent a continuous antigenic load in diseases involving the immune system. Due to the established beneficial effects of live and heat-killed *L. reuteri* ATCC PTA 5289 and DSM 17938 on the survival of larvae infected with *Porphyromonas gingivalis* (Geraldo *et al*. 2020), we evaluated their influence on other important periodontopathogens.

From the *in vitro* study, we observed that live *L. reuteri* significantly decreased the growth of the periodontopathogenic bacteria tested, indicating the action of *Lactobacillus* in the oral ecosystem. The effect of other strains of *L.*

reuteri (KCTC 3594, KCTC 3678, KCTC 3679, and ATCC 55730) on *A. actinomycetemcomitans*, *F. nucleatum*, and *P. gingivalis* has already been demonstrated *in vitro* (Kang *et al*. 2011, Moman *et al*. 2020), and in general, is related to the production of organic acids, hydrogen peroxide, and bacteriocin-like substances (Kang *et al*. 2011). In patients that used two lozenges containing *L. reuteri* per day for 12 weeks, a reduction in the growth of oral *F. nucleatum* ss *vincentii*, *F. periodicum,* and *S. mutans* was also observed (Romani Vestman *et al*. 2015).

The effect of supernatant was superior than that of live or heat-killed *L. reuteri* in reducing the growth of *F. nucleatum*. In this study, supernatant of *L. reuteri* was prepared based on the methodology of Schaefer *et al*. (2010), which involved stimulating the synthesis of reuterin (3-hydroxypropionaldehyde, 3-HPA) by exposing *L. reuteri* to 250 mM glycerol for 2 h. *L. reuteri,* through an enzymatic reaction, converts glycerol into 3-HPA (Burgé *et al*. 2015). Schaefer *et al*. (2010) confirmed the production of reuterin using a colorimetric assay and the antimicrobial activity was quantified by using the minimum inhibitory concentration assay. In addition, by using HPLC, the cell-free supernatant was proven to contain only reuterin and glycerol (Schaefer *et al*. 2010). Although we have followed the same methodology as reported, which should lead to the proven production of reuterin, we did not evaluate the supernatant composition, which could be considered a limitation of this study. Therefore, the “glycerol group” was included in both *in vitro* and *in vivo* experiments, to analyze the differences in antimicrobial effect between 250 mM glycerol and the *L. reuteri* supernatant produced through glycerol bioconversion, which we speculate that contains reuterin as the major component.

The bacteriostatic effect of glycerol was observed after interaction with *A. actinomycetemcomitans*. In the literature, the bactericidal effect of glycerol on *Streptococcus mutans*, *Staphylococcus aureus*, *Enterococcus faecalis,* and *Escherichia coli* has been demonstrated (Nalawade, Bhat and Sogi 2015), besides the bacteriostatic effect of glycerol on *Enterococcus gallinarum* (Singh 2014). Such effects vary among microorganisms exposed to different types of sugars (Singh 2014). Exposure of *L. reuteri* to glycerol also leads to the production of 3-hydroxypropionic (3-HP), which could be toxic to *Lactobacillus* (Burgé *et al*. 2015). Similar to *L. reuteri*, other bacteria are able to produce 3-HP acid from glycerol (Zhou *et al*. 2013; Lindlbauer, Marx and Sauer 2017), which may contribute to glycerol bacteriostatic effect.

Interestingly, heat-killed *L. reuteri* showed antimicrobial activity against *F. nucleatum in vitro*. Some species of *Lactobacillus*, such as *L. acidophilus* JCM1132, *L. brevis* JCM1059, *L. fermentum* JCM1173, *L. gasseri* JCM1131, *L. casei* JCM1134, *L. pentosus* JCM1558 (Aiba *et al*. 2017), and *L. reuteri* DSM 17648 (Holz *et al*. 2015), may exert antimicrobial effects even when they are inactivated by co-aggregation with pathogens. Inactivated *L. johnsonii* No. 1088 reduced the growth of *H. pylori* without causing co-aggregation, and it is believed that the presence of inactivated microorganisms could affect the viability of the pathogen by unknown mechanisms, involving cell surface molecules (Aiba *et al*. 2017).

From the *in vivo* study, the supernatant had the greatest contribution in prolonging *F. nucleatum*- infected larvae survival for both periods of prophylaxis. Glycerol led to survival rates similar to that of the supernatant after 3 h prophylaxis, however, in the 24 h, only the supernatant led to a greater survival

than the control group, indicating that the benefits of glycerol do not persist over time. Despite the *in vitro* antimicrobial activity, inactivated *L. reuteri* did not significantly increase the survival of *F. nucleatum*-infected larvae. Additionally, live, heat-killed *L. reuteri* and supernatant, in the 3 h and 24 h prophylaxis prior to inoculation of *A. actinomycetemcomitans* did not significantly increase the survival of larvae. Similarly, Moman *et al*. (2020) found that *L. reuteri* ATCC 55730 had an antimicrobial effect *in vitro* against *A. actinomycetemcomitans* ATCC 33384, but detected only a 10% increase in *G. mellonella* survival when the probiotic was injected 24 h prior to pathogen. A previous study reported a contrasting result, in which live and heat-killed *L. reuteri* increased the survival of larvae infected with *P. gingivalis*, but not those treated using supernatant (Geraldo *et al*. 2020). Thus, it is observed that the action of *L. reuteri* varied according to the periodontopathogen.

The preparations studied were not able to increase the hemocyte density in *G. mellonella*. There is a close relation between hemocyte count and pathogenicity of the infectious microorganism: high pathogenicity is related to a significant decrease in the number of hemocytes, whereas less pathogenic microorganisms are related to a modulation of the larvae immune system, increasing the amount of hemocytes (Bergin, Brennan and Kavanagh 2003). Since *L. reuteri* did not significantly increase hemocyte counts, we hypothesized that the concentration used (10^8 cells/mL) was high in this *in vivo* model. The choice of using the same concentrations of probiotic and periodontopathogens, in order to not favor any of the microorganisms, was based on studies that used the same methodology (Geraldo *et al*. 2020; Ribeiro *et al*. 2017). However, reduction in the *Lactobacillus* concentration could have led to better results

(Grounta *et al*. 2016; Jorjão *et al*. 2018). Activating and maintaining the immune response incurs a high energy cost to the insects (Freitak *et al*. 2003), which may not be sustainable, and even high concentrations of nonpathogenic bacteria can induce sepsis and death in larvae (Mukherjee *et al*. 2010).

In studies evaluating the relationship between host (*G. mellonella*) and microorganisms, several conditions must be considered. The immune system of *G. mellonella* is complex, and its activation involves several components other than hemocytes, such as proteins, melanin, and antimicrobial peptides, which are important for successful elimination of pathogen. This could explain why microbial recovery was suppressed in all groups despite the absence of increased hemocyte density. In addition, in the present study, periodontopathogens were recovered only from the hemolymph, but the microorganisms could infect other body parts, including the fat body and gut. Finally, *F. nucleatum* and *A. actinomycetemcomitans* are anaerobic bacteria, and microbial count after recovery can be influenced by oxygen exposure during experimental manipulation.

The *in vitro* and *in vivo* models we used have differences that may be responsible for the *in vivo* absence of some effects found *in vitro*. The *in vitro* co-culture assay involves direct interaction between the pathogenic and probiotic bacteria in a suitable medium, but lacking numerous factors present in *in vivo* models such as the cellular and humoral immunity of the host and the pathogen's level of expression of virulence factors and protein binding (Van den Driessche *et al*. 2017; Washington 1983). As an example, in contrast to *F. nucleatum* (Oelke *et al*. 2005), *A. actinomycetemcomitans* produces leukotoxin, which could be detrimental to human defense cells (Malik *et al*. 2015). In the *G. mellonella* model, we

chose to inject the probiotic preparations prior to pathogenic bacteria in order to evaluate their protective and immunomodulatory effects besides antimicrobial effects. Discrepancies in antimicrobial activity between *in vitro* and *in vivo* models are often reported in the literature (Cutuli *et al*. 2019; Moman *et al*. 2020), warranting the importance of validating the results from *in vitro* studies using relevant *in vivo* models (Van den Driessche *et al*. 2017; Washington 1983).

In the present study, both the probiotic and the products derived therefrom presented antimicrobial effects and increased the survival of *G. mellonella* infected with *F. nucleatum*. The supernatant demonstrated the best results, both *in vitro* and *in vivo*, for *F. nucleatum* infection. Although live *L. reuteri* exerted an antimicrobial effect on *A. actinomycetemcomitans in vitro*, results obtained could not be translated as increased larval survival. Nevertheless, new studies are needed to clarify the mechanisms of action involved.

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Conflicts of Interest

None declared.

REFERENCES

Aiba Y, Ishikawa H, Tokunaga M *et al*. Anti-*Helicobacter pylori* activity of non-living, heat-killed form of lactobacilli including *Lactobacillus johnsonii* No.1088. *FEMS Microbiol Lett* 2017;**364**.

Albandar JM. Aggressive periodontitis: case definition and diagnostic criteria. *Periodontol 2000* 2014;**65**:13-26.

Aparecida Procópio Gomes L, Alves Figueiredo LM, Luiza do Rosário Palma A *et al*. *Punica granatum* L. (Pomegranate) extract: *in vivo* study of antimicrobial activity against *Porphyromonas gingivalis* in *Galleria mellonella* model. *ScientificWorldJournal* 2016;**2016**:8626987.

Ardila CM, Granada MI, Guzmán IC. Antibiotic resistance of subgingival species in chronic periodontitis patients. *J Periodontal Res* 2010;**45**:557-63.

Bergin D, Brennan M, Kavanagh K. Fluctuations in haemocyte density and microbial load may be used as indicators of fungal pathogenicity in larvae of *Galleria mellonella*. *Microbes Infect* 2003;**5**:1389-95.

Browne N, Heelan M, Kavanagh K. An analysis of the structural and functional similarities of insect hemocytes and mammalian phagocytes. *Virulence* 2013;**4**:597-603.

Burgé G, Saulou-Bérion C, Moussa M *et al*. Diversity of *Lactobacillus reuteri* strains in converting glycerol into 3-hydroxypropionic acid. *Appl Biochem Biotechnol* 2015;**177**:923–39.

Chuang CH, Tsai CC, Lin ES *et al*. Heat-Killed *Lactobacillus salivarius* and *Lactobacillus johnsonii* reduce liver injury induced by alcohol *in vitro* and *in vivo*. *Molecules* 2016;**21**:1456.

Cutuli MA, Petronio Petronio G, Vergalito F *et al*. *Galleria mellonella* as a consolidated in vivo model hosts: New developments in antibacterial strategies and novel drug testing. *Virulence* 2019;**10**:527-41.

Doron S, Snydman DR. Risk and safety of probiotics. *Clin Inf Dis* 2015;**60 Suppl 2**:S129-34.

Dos Santos JD, de Alvarenga JA, Rossoni RD *et al*. Immunomodulatory effect of photodynamic therapy in *Galleria mellonella* infected with *Porphyromonas gingivalis*. *Microb Pathog* 2017;**110**:507-11.

Ezzo PJ, Cutler CW. Microorganisms as risk indicators for periodontal disease. *Periodontol 2000* 2003;**32**:24-35.

Freitak D, Ots I, Vanatoa A *et al*. Immune response is energetically costly in white cabbage butterfly pupae. *Proc Biol Sci* 2003;**270**:S220-2.

Frencken JE, Sharma P, Stenhouse L *et al*. Global epidemiology of dental caries and severe periodontitis – a comprehensive review. *J Clin Periodontol* 2017;**44 Suppl 18**:S94-105.

Geraldo BMC, Batalha MN, Milhan NVM *et al*. Heat-killed *Lactobacillus reuteri* and cell-free culture supernatant have similar effects to viable probiotics during interaction with *Porphyromonas gingivalis*. *J Periodontal Res* 2020;**55**:215-20.

Grounta A, Harizanis P, Mylonakis E *et al*. Investigating the effect of different treatments with lactic acid bacteria on the fate of *Listeria monocytogenes* and *Staphylococcus aureus* infection in *Galleria mellonella* larvae. *PLoS One* 2016;**11**:e0161263.

Gruner D, Paris S, Schwendicke F. Probiotics for managing caries and periodontitis: systematic review and meta-analysis. *J Dent* 2016;**48**:16-25.

Haffajee AD, Teles RP, Socransky SS. The effect of periodontal therapy on the composition of the subgingival microbiota. *Periodontol 2000* 2006;**42**:219-58.

Holz C, Busjahn A, Mehling H *et al*. Significant reduction in *Helicobacter pylori* load in humans with non-viable *Lactobacillus reuteri* DSM17648: a pilot study. *Probiotics Antimicrob Proteins* 2015;**7**:91-100.

Iwasaki K, Maeda K, Hidaka K *et al*. Daily intake of heat-killed *Lactobacillus plantarum* L-137 decreases the probing depth in patients undergoing supportive periodontal therapy. *Oral Health Prev Dent* 2016;**14**:207-14.

Kang MS, Oh JS, Lee HS *et al*. Inhibitory effect of *Lactobacillus reuteri* on periodontopathic and cariogenic bacteria. *J Microbiol* 2011;**49**:193-9.

Jorjão AL, Oliveira FE, Leão MVP *et al*. Effect of *Lactobacillus rhamnosus* on the response of *Galleria mellonella* against *Staphylococcus aureus* and *Escherichia coli* infections. *Arch Microbiol* 2018;**200**:383-9.

Laleman I, Pauwels M, Quirynen M *et al*. A dual-strain Lactobacilli reuteri probiotic improves the treatment of residual pockets: A randomized controlled clinical trial. *J Clin Periodontol* 2020;**47**:43-53.

Lin X, Chen X, Chen Y *et al*. The effect of five probiotic lactobacilli strains on the growth and biofilm formation of *Streptococcus mutans*. *Oral Dis* 2015;**21**:e128-34.

Lindlbauer KA, Marx H, Sauer M. 3-Hydroxypropionaldehyde production from crude glycerol by *Lactobacillus diolivorans* with enhanced glycerol uptake.

Biotechnol Biofuels 2017;**10**:295.

Malik R, Changela R, Krishan P *et al*. Virulence factors of *Aggregatibacter actinomycetemcomitans* - A status update. *J Int Clin Dent Res Organ* 2015;**7**:137-45.

Martin-Cabezas R, Davideau JL, Tenenbaum H *et al*. Clinical efficacy of probiotics as an adjunctive therapy to non-surgical periodontal treatment of chronic periodontitis: a systematic review and meta-analysis. *J Clin Periodontol* 2016;**43**:520-30.

Mohd-Dom T, Ayob R, Mohd-Nur A *et al*. Cost analysis of periodontitis management in public sector specialist dental clinics. *BMC Oral Health* 2014;**14**:56.

Moman R, O'Neill CA, Ledder RG *et al*. Mitigation of the toxic effects of periodontal pathogens by candidate probiotics in oral keratinocytes, and in an invertebrate model. *Front Microbiol* 2020;**11**:999.

Mukherjee K, Altincicek B, Hain T *et al*. *Galleria mellonella* as a model system for studying *Listeria* pathogenesis. *Appl Environ Microbiol* 2010;**76**:310-7.

Nalawade TM, Bhat K, Sogi SH. Bactericidal activity of propylene glycol, glycerine, polyethylene glycol 400, and polyethylene glycol 1000 against selected microorganisms*. J Int Soc Prev Community Dent* 2015;**5**:114-9.

Noh EJ, Kang MJ, Jeong YJ *et al*. Withaferin A inhibits inflammatory responses induced by *Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans* in macrophages. *Mol Med Rep* 2016;**14**:983-8.

Oelke AM, Nagaraja TG, Wilkerson MJ *et al*. The leukotoxin operon of *Fusobacterium necrophorum* is not present in other species of *Fusobacterium*. *Anaerobe* 2005;**11**:123-9.

Oh PL, Benson AK, Peterson DA *et al*. Diversification of the gut symbiont *Lactobacillus reuteri* as a result of host-driven Evolution. *ISME J* 2010;**4**:377-87.

Papapanou PN, Sanz M, Buduneli N *et al*. Periodontitis: Consensus report of workgroup 2 of the 2017 World workshop on the classification of periodontal and peri-implant diseases and conditions. *J Clin Periodontol* 2018;**45**:S162-70.

Ribeiro FC, de Barros PP, Rossoni RD *et al*. *Lactobacillus rhamnosus* inhibits *Candida albicans* virulence factors in vitro and modulates immune system in *Galleria mellonella*. *J Appl Microbiol* 2017;**122**:201-11.

Romani Vestman N, Chen T, Lift Holgerson P *et al*. Oral microbiota shift after 12-week supplementation with *Lactobacillus reuteri* DSM 17938 and PTA 5289; a randomized control trial. *PLoS One* 2015;**10**:e0125812.

Rossoni RD, Ribeiro FC, Dos Santos HFS *et al*. *Galleria mellonella* as an experimental model to study human oral pathogens. *Arch oral Biol* 2019;**101**:13-22.

Sánchez B, Delgado S, Blanco-Míguez A *et al*. Probiotics, gut microbiota, and their influence on host health and disease. *Mol Nutr Food Res* 2017;**61**.

Sanders ME. Probiotics: definition, sources, selection, and uses. *Clin Infect Dis* 2008;**46 Suppl 2**:S58-61.

Schaefer L, Auchtung TA, Hermans KE *et al*. The antimicrobial compound reuterin (3-hydroxypropionaldehyde) induces oxidative stress via interaction with thiol groups. *Microbiology* 2010;**156**:1589-99.

Schlagenhauf U, Jakob L, Eigenthaler M *et al*. Regular consumption of Lactobacillus reuteri-containing lozenges reduces pregnancy gingivitis: an RCT. *J Clin Periodontol* 2016;**43**:948-54.

Singh BR. Antibacterial activity of glycerol, lactose, maltose, mannitol, raffinose and xylose. *Medicine* 2014;1-3.

Song WS, Lee JK, Park SH *et al*. Comparison of periodontitis-associated oral biofilm formation under dynamic and static conditions. *J Periodontal Implant Sci* 2017;**47**:219-30.

Taverniti V, Guglielmetti S. The immunomodulatory properties of probiotic microorganisms beyond their viability (ghost probiotics: proposal of paraprobiotic concept). *Genes Nutr* 2011;**6**:261-74.

Tsai CJ, Loh JM, Proft T. *Galleria mellonella* infection models for the study of bacterial diseases and for antimicrobial drug testing. *Virulence* 2016;**7**:214-29.

Van den Driessche F, Vanhoutte B, Brackman G *et al*. Evaluation of combination therapy for *Burkholderia cenocepacia* lung infection in different *in vitro* and *in vivo* models. *PLoS One* 2017;**12**:e0172723.

Washington JA 2nd. Discrepancies between *in vitro* activity of and *in vivo* response to antimicrobial agents. *Diagn Microbiol Infect Dis* 1983;**1**:25-31.

Yang HW, Huang YF, Chan Y *et al*. Relationship of *Actinobacillus actinomycetemcomitans* serotypes to periodontal condition: prevalence and proportions in subgingival plaque. *Eur J Oral Sci* 2005;**113**:28-33.

Zhou S, Catherine C, Rathnasingh C *et al*. Production of 3-hydroxypropionic acid from glycerol by recombinant *Pseudomonas denitrificans*. *Biotechnol Bioeng* 2013;**110**:3177-87.

Figure Legends

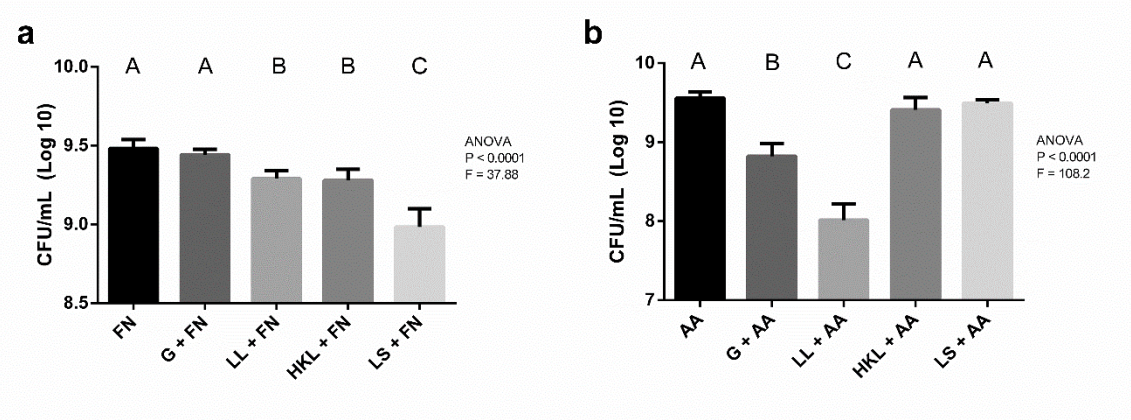


Fig. 1 Mean and standard deviation of CFU/mL data (Log10), growth obtained on Brucella blood agar supplemented with 1% hemin and menadione solution. (a) Interaction of *L. reuteri* and *F. nucleatum*. (b) Interaction of *L. reuteri* and *A. actinomycetemcomitans*. Equal letters over the bars indicate absence of statistical difference after Tukey's test.

FN, *F. nucleatum*; G, glycerol; LL, live *L. reuteri*; HKL, heat-killed *L. reuteri*; LS, *L. reuteri* supernatant; AA, *A. actinomycetemcomitans.*

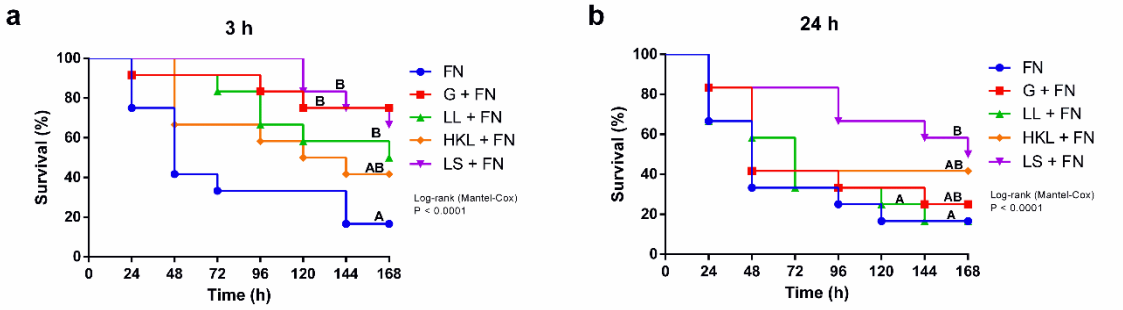


Fig. 2 Survival curve of *G. mellonella*. (a) Interaction between *L. reuteri* live, heat-killed or supernatant (3 h prophylaxis) and *F. nucleatum*. (b) Interaction between *L. reuteri* live, heat-killed or supernatant (24 h prophylaxis) and *F. nucleatum.*

Equal letters over the lines indicate absence of statistical difference after Log-rank (Mantel-Cox) test. n = 12.

FN, *F. nucleatum*; G, glycerol; LL, live *L. reuteri*; HKL, heat-killed *L. reuteri*; LS, *L. reuteri* supernatant.

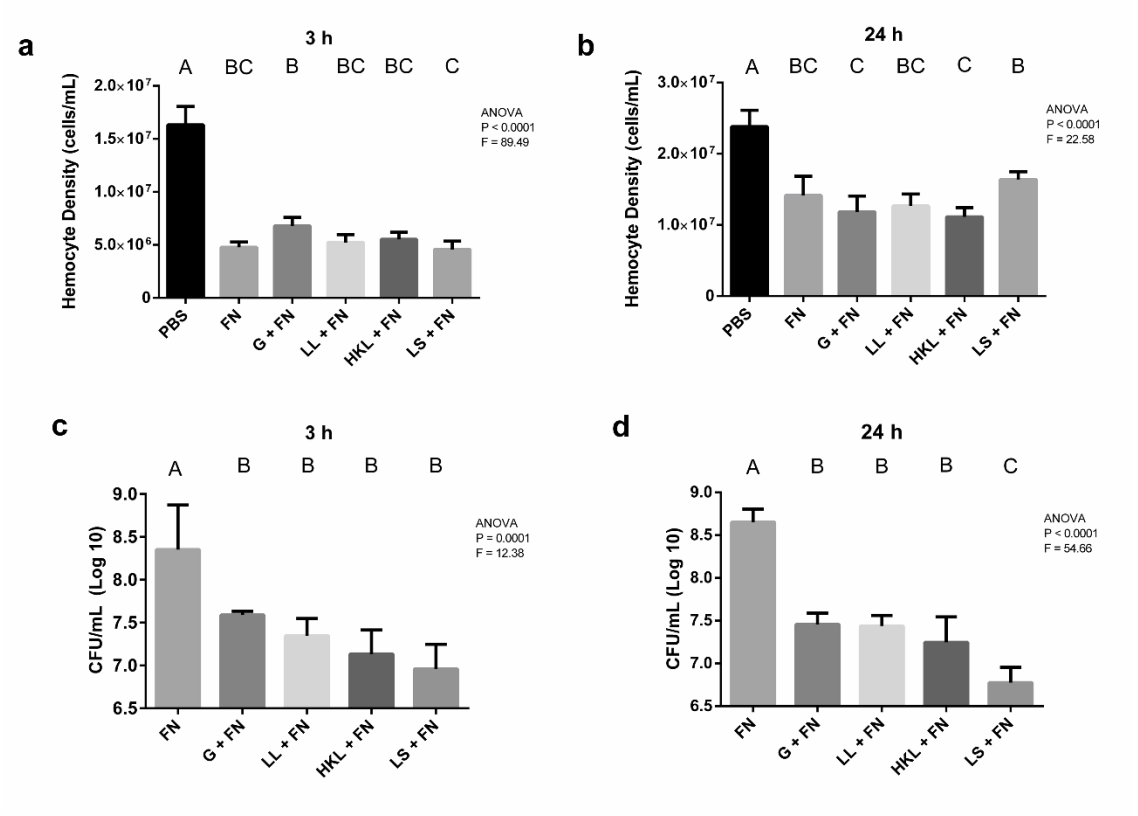


Fig. 3 Mean and standard deviation of hemocyte counts and microbial recovery after interaction between *L. reuteri* live, heat-killed or supernatant and *F. nucleatum*. (a) Hemocyte density for 3 h prophylaxis. (b) Hemocyte density for 24 h prophylaxis. (c) Microbial recovery for 3 h prophylaxis. (d) Microbial recovery for 24 h prophylaxis. Equal letters over the bars indicate absence of statistical difference after Tukey's multiple comparisons test.

PBS, phosphate buffer saline; FN, *F. nucleatum;* G, glycerol; LL, live *L. reuteri*; HKL, heat-killed *L. reuteri*; LS, *L. reuteri* supernatant.

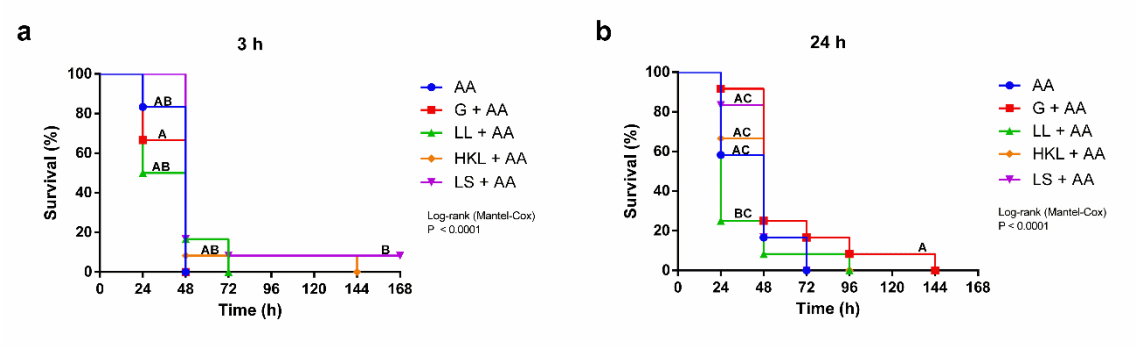


Fig. 4 Survival curve of *G. mellonella*. (a) Interaction between *L. reuteri* live, heat-killed or supernatant (3 h prophylaxis) and *A. actinomycetemcomitans*. (b) Interaction between *L. reuteri* live, heat-killed or supernatant (24 h prophylaxis) and *A. actinomycetemcomitans*. Equal letters over the lines indicate absence of statistical difference after Log-rank (Mantel-Cox) test. n = 12.

AA, *A. actinomycetemcomitans;* G, glycerol; LL, live *L. reuteri*; HKL, heat-killed *L. reuteri*; LS, *L. reuteri* supernatant.

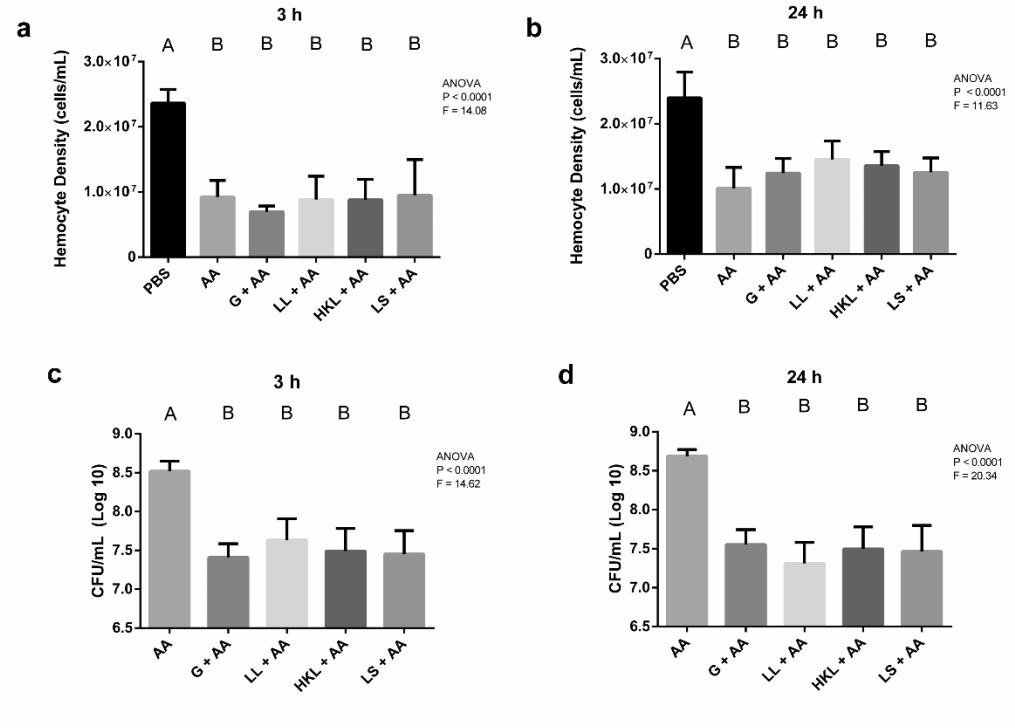
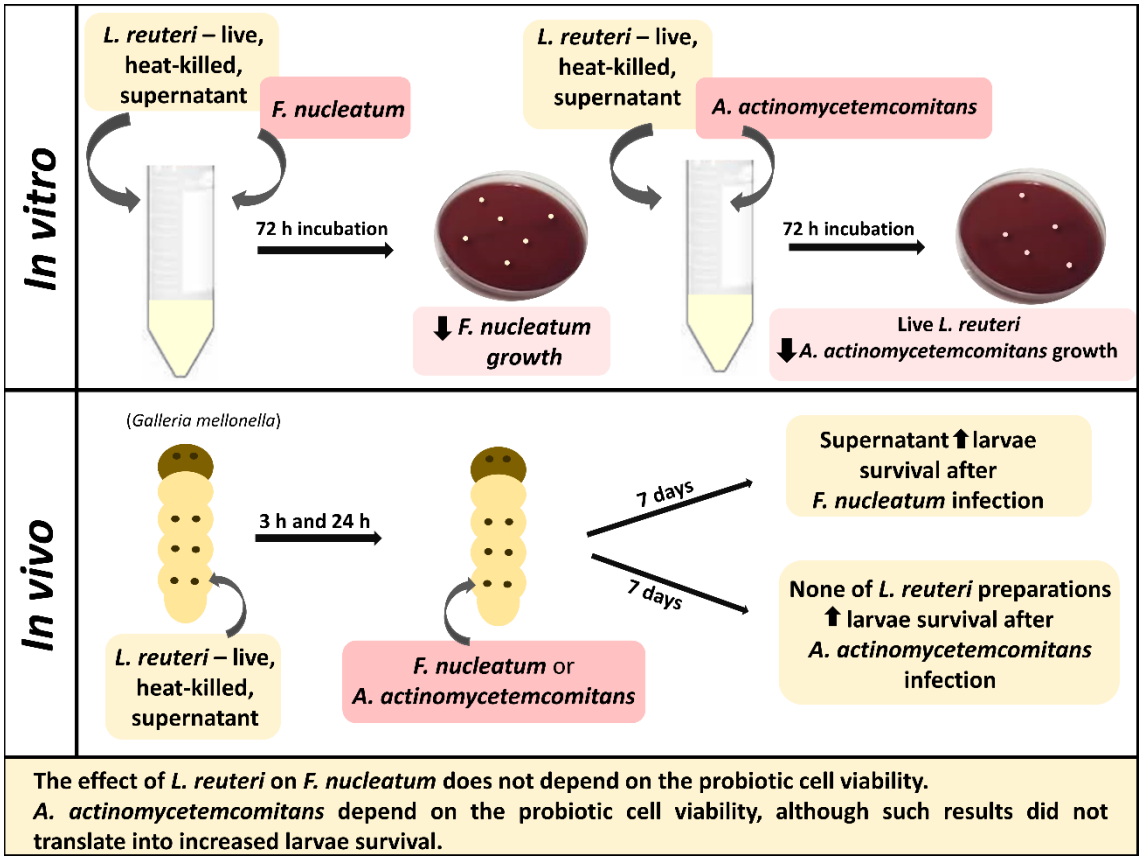


Fig. 5 Mean and standard deviation of hemocyte counts and microbial recovery after interaction between *L. reuteri* live, heat-killed or supernatant and *A. actinomycetemcomitans*. (a) Hemocyte density for 3 h prophylaxis. (b) Hemocyte density for 24 h prophylaxis. (c) Microbial recovery for 3 h prophylaxis. (d) Microbial recovery for 24 h prophylaxis. Equal letters over the bars indicate absence of statistical difference after Tukey's multiple comparisons test.

PBS, phosphate buffer saline; AA, *A. actinomycetemcomitans;* G, glycerol; LL, live *L. reuteri*; HKL, heat-killed *L. reuteri*; LS, *L. reuteri* supernatant.



The effect of *L. reuteri* on *F. nucleatum* does not depend on the probiotic cell viability. *A. actinomycetemcomitans* depend on the probiotic cell viability, although such results did not translate into increased larvae survival.

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