Heat-killed *Lactobacillus reuteri* and cell-free culture supernatant have similar effects to viable probiotics during interaction with *Porphyromonas gingivalis*

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ABSTRACT

Background and objective: In the last decade, numerous studies have been published to clarify the role of probiotics, especially *Lactobacillus reuteri*, as an adjunct to conventional periodontal treatment. Although the health benefits of probiotics are numerous, they are live bacteria, and the administration of live organisms is not risk-free. We evaluated the antimicrobial effect of *L. reuteri* and its cell-free culture supernatant on *Porphyromonas gingivalis,* a keystone periodontal pathogen*, in vitro.* We also evaluated the influence of this probiotic in its live, heat-killed (HKL, paraprobiotic) form and its supernatant on the *Galleria mellonella* invertebrate model after infection by *P. gingivalis.*

Methods: The interaction assay was conducted with *P. gingivalis* and *L. reuteri* preparations (live cells and supernatant preparation). For this, *P. gingivalis* and *L. reuteri* preparations were added to tubes containing Brain-Heart Infusion broth and incubated for 3 days. The suspensions were then seeded onto appropriate culture media for the calculation of colony forming units per mL (CFU/mL). An *in vivo* assay with the *G. mellonella* model was also performed. Live *L. reuteri*, HKL, or supernatant were inoculated 2 hours prior to infection with *P. gingivalis*. Survival was evaluated over 7 days, and the number of hemocytes in the hemolymph was estimated 3 h after *P. gingivalis* infection. Data were then subjected to statistical testing (α=5%).

Results: Both live *L. reuteri* and its supernatant had antimicrobial activity against *P. gingivalis* (CFU reduction up to 86%, $p<0.05$). Moreover, treatment with live and HKL had similar effects on *G. mellonella* survival (increased survival up to 46%, $p<0.05$). However, only live *L. reuteri* was able to significantly increase the hemocyte density in this invertebrate model.

Conclusion: *L. reuteri* antimicrobial activity against *P. gingivalis* and its effects on *G. mellonella* survival after infection with a periodontopathogen do not depend on cell viability. This allows the development of products without live bacterium while maintaining similar effects.

1. Introduction

In the last decade, numerous studies have been published to clarify the role of probiotics as an adjunct to conventional treatment (scaling and root planing) of periodontal diseases. In these studies, *Lactobacillus reuteri*, particularly the strains ATCC 55730 (and its substitute DSM 17938) and ATCC PTA 5289, also referred as *L. reuteri* Prodentis, was widely used. *L. reuteri* is a heterofermentative bacteria found in the gastrointestinal tract of humans and several other animal species, as well as in breast milk and the human urogenital tract.[1] In the presence of glycerol, *L. reuteri* can synthesize reuterin (3-hydroxypropionaldehyde), a small compound with antimicrobial activity for a range of pathogens, including Gram-positive and Gram-negative bacteria, fungi, and parasites.[2] Moreover, *L. reuteri* inhibits the colonization of pathogenic microbes, influences the host microbiota composition, benefits the host immune system to reduce pro-inflammatory cytokine production, and strengthens the intestinal barrier, decreasing microbial translocation from the gut lumen to the tissues.[3]

In periodontics, after the use of *L. reuteri* in patients with periodontitis or peri-implantitis, a decrease in probing depth, bleeding on probing, and decreased gingival and plaque index, or an increase in the clinical attachment level was found.[4–13] In both *in vitro* and *in vivo* studies, *L. reuteri* inhibits the growth of *Porphyromonas gingivalis.* [6,14,15] This is a Gram-negative anaerobic bacterium highly associated with periodontal disease, and is considered a keystone pathogen in chronic periodontitis.[16] In addition, systematic reviews and meta-analysis support the use of *L. reuteri* associated with conventional periodontal therapy, especially in deep pockets.[17–19]

Although the health benefits of probiotics are numerous, they are live bacteria, and the administration of live organisms is not risk-free, especially in immunocompromised patients.[20,21] Paraprobiotics or ghost probiotics are composed of intact or ruptured non-viable microbial cells, including peptideoglycans, lipopolysaccharides (LPS,) and cytoplasmic parts. These provide benefits to the consumer when administered in appropriate doses. Direct interaction of the microorganism with the host, based on the ability of human cells to recognize specific bacterial components or products, is independent of microbial viability. Receptors for various microbial components expressed on the epithelial surface and inflammatory cells appear to have an important role in intracellular signal transduction, thus reducing the production of inflammatory mediators.[22]

It is favorable to reduce the number of experimental animals used in the laboratory, as well as increasing their comfort and reducing pain. The use of the invertebrate *Galleria mellonella* has become an alternative experimental *in vivo*

infection model to traditional mammals. The larvae are easy to maintain and manipulate, and have similarities with the mammalian innate immune system, including the cellular immune response performed by hemocytes present in the insect's hemolymph. [23,24]

Treating oral diseases with a natural and non-invasive method has great appeal and can prevent problems related to pharmacological treatments, such as antibiotic resistance. Considering that conventional periodontal treatment fails to control periodontal disease progression in some patients, and that *L. reuteri* has been successfully used as an adjunct alternative in some clinical studies, we have hypothesized that heat-killed *L. reuteri* (paraprobiotic) and *L. reuteri* cell-free supernatant may be effective in cases of *P. gingivalis* infection. We evaluated the *in vitro* antibacterial effect of *L. reuteri* preparations and their effects in an invertebrate model (*G. mellonella*).

2. Materials and Methods

2.1 Microorganisms and growth conditions

L. reuteri Prodentis® (PTA 5289 associated with DSM 17938, Sunstar Americas, Chicago, Illinois, United States of America) was grown in De Man-Rogosa-Sharpe agar (MRS, Acumedia, Lensing, Michigan, USA) in an oxygen-free atmosphere (80% nitrogen, 10% carbon dioxide, and 10% hydrogen) in an anaerobic jar, at 37 °C for 3 days. Isolated colonies were then confirmed by Gram staining. To obtain live *L. reuteri* suspension, the bacteria were grown in MRS broth in anaerobiosis for 24 h. The cells were then collected by centrifugation, and cell suspensions of 10^4 to 10^8 cells/mL were standardized in the spectrophotometer (570 nm). The suspension containing 10^8 cells/mL was autoclaved at 121 °C for 15 min to prepare heat-killed lactobacillus (HKL).

For preparation of the cell-free culture supernatant, 1 mL of the suspension containing 10^8 cells/mL of live *L. reuteri* was transferred to a tube containing 6 mL of MRS broth, which was incubated for 24 h. Glycerol was then added at a concentration of 300 mM and incubated for a further 3 h. The cells were then centrifuged at 2600g for 10 minutes, and the supernatant was collected and sterilized with a membrane filter of 0.22 µm pore size. Both supernatant and HKL were seeded on MRS agar to confirm the absence of live cells.

P. gingivalis (ATCC 33277) was cultured in Fastidious Anaerobe Agar (FAA, Acumedia, Lansing, MI, USA) plates with defibrinated sheep's blood supplemented with 0.1% hemin and menadione (Sigma-Aldrich, Saint Louis, MO, USA) in anaerobiosis for

7 days. Suspensions of 10^5 to 10^9 cells/mL in PBS were standardized in the spectrophotometer (660 nm).

2.2 *In vitro* antibacterial effect of *L. reuteri* preparations

To evaluate the antibacterial activity of the *L. reuteri* preparation against *P. gingivalis*, 250 µL *P. gingivalis* (10^8 cells/mL) and 250 µL *L. reuteri* preparations (10^8 cells/mL of live cells or supernatant preparation) were added to tubes containing 1.5 mL of Brain-Heart Infusion broth (BHI, Himedia, Mumbai, India) and incubated for 3 days under anaerobic conditions.[25] Control tubes were prepared with *P. gingivalis* or *L. reuteri* associated with PBS in BHI, where the growth of both strains was confirmed. After 3 days of incubation, serial decimal dilutions of the co-cultures were performed, and the dilutions were seeded onto Petri dishes containing appropriate selective culture media (supplemented blood agar for *P. gingivalis* and MRS for *L. reuteri*). The MRS plates containing *L. reuteri* were incubated for 48 h, and those containing *P. gingivalis* (supplemented blood agar) were incubated for 7 days, both under anaerobic conditions. After this period, the colonies were counted for the calculation of CFU/mL. For all groups, n = 10 was used.

2.3 *In vivo* study using *G. mellonella*

G. mellonella (Lepidoptera: Pyralidae) from the Laboratory of Microbiology of the Institute of Science and Technology of São José dos Campos/UNESP (São Paulo State University) were maintained as described previously.[26] *G. mellonella* in their final larval stage and with a body weight of 200–300 mg was used. The insects presented no external gray markings in their cuticles, and were kept in Petri dishes in the dark at 37 ^{o}C for the experiments.

Before studying *P. gingivalis* and *L. reuteri* interaction, survival curves were performed with 10^4 to 10^8 cells/mL of live *L. reuteri* to determine the concentration to be used in further experiments. The suspensions (10 µL) were inoculated using a micro syringe (Hamilton Inc., Reno, NV, USA) into the last left proleg of 15 larvae per group. The number of dead larvae was recorded daily for 7 days. The larvae were considered dead when there was no movement upon touching with metallic forceps. A concentration of 10^8 cells/mL of *P. gingivalis* was adopted for evaluating the interaction effects.[27]

After the determination of the concentration of *L. reuteri* to be used, 10 µL of a standardized suspension of living cells, either HKL or supernatant, was inoculated in

the last right proleg of *G. mellonella* larvae. This was done 2 hours prior to inoculation of *P. gingivalis* standard suspension in the last left proleg. Controls were administered with PBS, MRS inoculation, or infection by a single microorganism in the last left proleg, and with the same volume of PBS in the last right proleg. The number of dead animals was evaluated for 7 days. Fifteen larvae in each group were used for survival evaluation.

For hemocyte density evaluation, larvae were incubated at 37 ^{o}C for 3 h after the last microorganism injection. An incision was performed on the longitudinal axis of the larvae to remove the hemolymph, which was then collected. The hemolymph of three larvae were pooled and diluted in physiological insect serum (2% NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, and 10 mM EDTA) in the proportion of 1:10. The hemocytes were identified based on cell morphology and quantified using a hemocytometer (9 larvae per group). All *in vivo* experiments were performed at least twice.

2.4 Statistical analysis

For the *G. mellonella* survival experiments, the survival curve was constructed and an estimation of the differences was performed by the Log-rank method (Mantel-Cox). For other analyses, ANOVA and Student t-tests were used to detect differences among groups. A significance level of 5% was established for all tests (GraphPad Prism 6, La Jolla, CA, USA).

3. Results

3.1 *In vitro* study: Cell-free supernatant and live *L. reuteri* have similar antibacterial effect against *P. gingivalis*

When analyzing the effects of *L. reuteri* preparations, a reduction in *P. gingivalis* CFU/mL was observed in all groups compared to the control. The interaction with supernatant and live *L. reuteri* led to a 76.81% and 86.6% CFU/mL decrease in *P. gingivalis*, respectively. This was a statistically significant reduction compared to the *P. gingivalis* control group (Figure 1).

We also evaluated whether *P. gingivalis* had any antimicrobial effect against *L. reuteri*, evaluating the growth of the *Lactobacillus* in MRS agar after interaction of both the bacteria in BHI. The periodontopathogen did not influence the probiotic growth in comparison to that in the control group (Student t-test, p=0.1525).

3.2 *In vivo* study using *G. mellonella*

After seven days of live *L. reuteri* inoculation in *G. mellonella*, the larvae presented 100% survival in all tested concentrations (10^4 to 10^8 cells/mL). The control group with only MRS broth or PBS also obtained 100% survival. The chosen concentration to study the interaction among *L. reuteri* preparations and *P. gingivalis in vivo* was 10^8 cells/mL of each microorganism, to use the same concentration for both.

3.2.1 Heat-killed and live *L. reuteri* increase *G. mellonella* survival after infection with *P. gingivalis*

After 7 days, the interaction between live *L. reuteri* or HKL with *P. gingivalis* significantly reduced the *G. mellonella* mortality rate to 46.66% and 33.33%, respectively, in comparison to the group that received only the periodontopathogen, which had a mortality rate of 100% (Log-rank Mantel Cox, p=0.0007 and p=0.0004, respectively). However, there was no difference between the groups treated with supernatant and the negative control that only received *P. gingivalis* infection (p=0.2290). In the same way, there was no significant difference between the treatment with live *L. reuteri* or HKL (p=0.5797; Figure 2).

3.2.2 Live *L. reuteri* increases the number of hemocytes in *G. mellonella* hemolymph after infection with *P. gingivalis*

We observed that the *P. gingivalis* group showed a reduction in the hemocyte numbers in relation to the PBS control group. However, when the larvae were pretreated with live *L. reuteri* before *P. gingivalis* infection, the hemocyte quantity was very similar to the values found in the PBS control group. There was a 353% increase in larval hemolymph hemocyte content in comparison to the group that received only *P. gingivalis*. HKL and supernatant pre-treatment was not enough to significantly modify the hemocyte count after periodontopathogen infection, although they are not statistically different from the PBS control group (Figure 3). These results indicate that *P. gingivalis* suppresses the hemocyte count, and that pre-treatment with live *L. reuteri* significantly increases the number of circulating hemocytes in the hemolymph, which may protect *G. mellonella* from *P. gingivalis* infection.

4. Discussion

Although probiotics are bacteria that confer benefits to the host, their use is not without risk. Adverse effects are described mainly in isolated case reports, especially in patients with immune compromise and premature infants. In contrast with the long history of use of probiotics in foods, few studies are specifically designed to assess their safety.[20,21] The use of non-viable microorganisms or microbial cell extracts has been proposed, as they could reduce the risks of microbial translocation, infection, and eliminate shelf-life problems.[22] While the recommended probiotic dosage for several conditions and diseases is well established in the literature,[28] studies about the ideal doses and adverse effects of paraprobiotics are limited, and there are not significant conclusions about the comparison of viable and non-viable bacteria beneficial and adverse effects.[29] Since there are limits to the amounts of microbial components that one can be exposed to, the use of the cell-free supernatant has its importance reinforced. We, therefore, studied the effects of *L. reuteri*, a probiotic widely studied as an adjunct treatment to periodontal disease, its paraprobiotic preparation (HKL), and the cell-free supernatant in *P. gingivalis in vitro* and *in vivo*. In addition, the study of the non-viable bacteria and their products could help us to better understand the mechanisms of *L. reuteri* associated with the improvement of periodontal disease outcome.

The major mechanisms of action of probiotics are epithelial barrier enhancement, increased adhesion to intestinal mucosa, pathogen adhesion inhibition, competitive exclusion of pathogenic microorganisms, production of anti-microorganism substances, and modulation of the immune system.[30] The inhibition of *P. gingivalis* growth by *L. reuteri* has been already reported, both *in vitro* and *in vivo.*[6,14,15] Our *in vitro* results corroborate the literature findings in regards to the effective antibacterial action of live *L. reuteri*. In addition, we have shown that *L. reuteri* cell-free supernatant was as effective in inhibiting *P. gingivalis* as living bacteria, suggesting that microbial growth reduction was mainly due to the substances produced by the probiotic. Besides reuterin, *L. reuteri* strains produce a series of other antimicrobial substances, including lactic acid, acetic acid, ethanol, and reutericyclin.[3,31,32] The literature has also shown the importance of the acidic environment in the interactions between *L. reuteri* and other microorganisms.[33] Additional well-studied metabolites such as histamine, vitamins, and exopolysaccharides contribute to *L. reuteri* probiotic action, and can be found in the culture supernatant.[3] However, *L. reuteri* metabolites are present in different concentrations in live bacterial suspension and in the supernatant, since

special conditions of time and substrate addition (glycerol) were offered to the culture to prepare the supernatant. Once we have evaluated the antimicrobial effect of the whole cell-free supernatant, further studies are necessary to identify and characterize each bioactive substance of the supernatant and its effects on the inhibition of *P. gingivalis* growth.

To elucidate the results obtained through antimicrobial activity, we investigated the probiotic action of *L. reuteri* in a *G. mellonella* model. Both live and HKL cells showed a positive effect on larvae survival, different to the cell-free supernatant. In the immunomodulation process evaluated by a survival curve, the structural features of the probiotic appeared more important than the substances produced and present in the supernatant. Several *in vitro* and animal studies have demonstrated that both live and dead probiotic cells can act as biological response modifiers. This suggests that products based on dead probiotic cells could represent an intermediate stage between an oral vaccine and a classical live probiotic[34], with great potential in the treatment of human diseases. Although probiotics are commonly studied as an adjunctive treatment of periodontal disease[4–13], the same is not true for paraprobiotics. As far as we know, the only study on non-viable probiotics and periodontal disease in humans was performed by Iwasaki et al., who administered heat-killed *Lactobacillus plantarum* L-137 to patients with periodontal disease, as an adjunct of scaling and root planing. The treatment led to a significant reduction in probing depth of periodontal pockets greater than 4 mm deep in comparison to the control group. The results were attributed to the increased IL-12 secretion induced by heat-killed *L. plantarum*, which leads to a Th1-type response through INF-y induction.[35]

The *G. mellonella* immune system comprises cellular and humoral responses. Hemocytes are the main effectors in the cellular response, and are important to phagocytosis, nodulation, and encapsulation. These cells, which are phagocytes similar to human macrophages and neutrophils, circulate freely in the hemolymph and tissues to recognize and phagocytose antigens, including invading microorganisms such as viruses and bacteria, or encapsulate and destroy larger structures.[23] In the *G. mellonella* model, infection with a highly pathogenic microorganism is related to a significant decrease in the number of hemocytes.[36] This was confirmed in the group infected only with *P. gingivalis*, in which the number of defense cells decreased significantly compared to the PBS group. On the other hand, less pathogenic microorganisms are related to a modulation of the larvae immune system, increasing the amount of hemocytes.[36] Only live *L. reuteri* led to a significant increase in hemocytes after pathogen interaction, with a statistical difference when compared to the *P. gingivalis*-infected group. Interestingly, in spite of the fact that both live

lactobacillus and HKL preparation increased survival, only live lactobacillus led to statistically increased numbers of hemocytes. In this case, an increased humoral response, involving the action of antimicrobial peptides, phenoloxidase, melanization, reactive oxygen species, and hemolymph clotting, could be suggested.[23] In addition, although the hemocyte counts from the larvae that received the HKL preparation and the cell-free supernatant were not statistically different from *P. gingivalis* control group, they were not different from the PBS control group. This suggests that the preparations (HKL preparation and the cell-free supernatant) may have some effect on hemocyte count, but not as robust as the viable *L. reuteri*.

In summary, we have demonstrated that live *Lactobacillus reuteri* and its cell-free supernatant have an antimicrobial effect against *P. gingivalis,* and that the antimicrobial and immunomodulatory effects of *L. reuteri in vivo* do not depend on cell viability. This allows the development of products without live bacteria while maintaining similar effects.

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The authors declare no conflict of interest.

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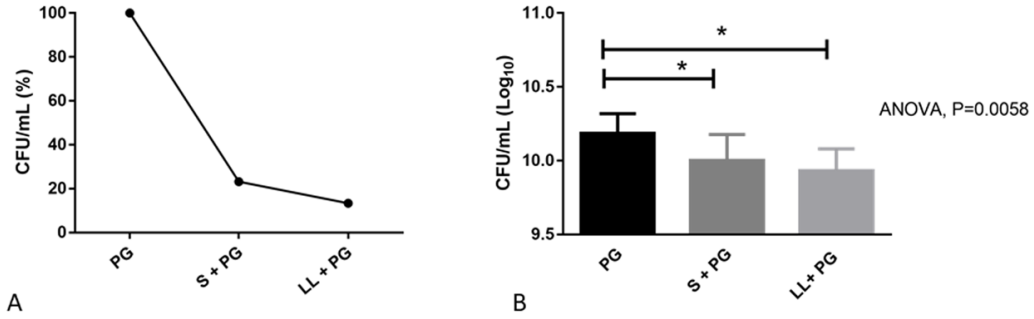

Figure 1 - A- Percentage of reduction of *P. gingivalis* grown in supplemented blood agar, after interaction with *L. reuteri* preparations. B- Mean and standard deviation of CFU/mL (log) of *P. gingivalis* growth in supplemented blood agar, after interaction with *L. reuteri* preparations. *Statistically significant difference after ANOVA and Tukey test ($p<0.05$) (PG- *P. gingivalis*, LL- live *L. reuteri,* S- Supernatant of *L. reuteri*).

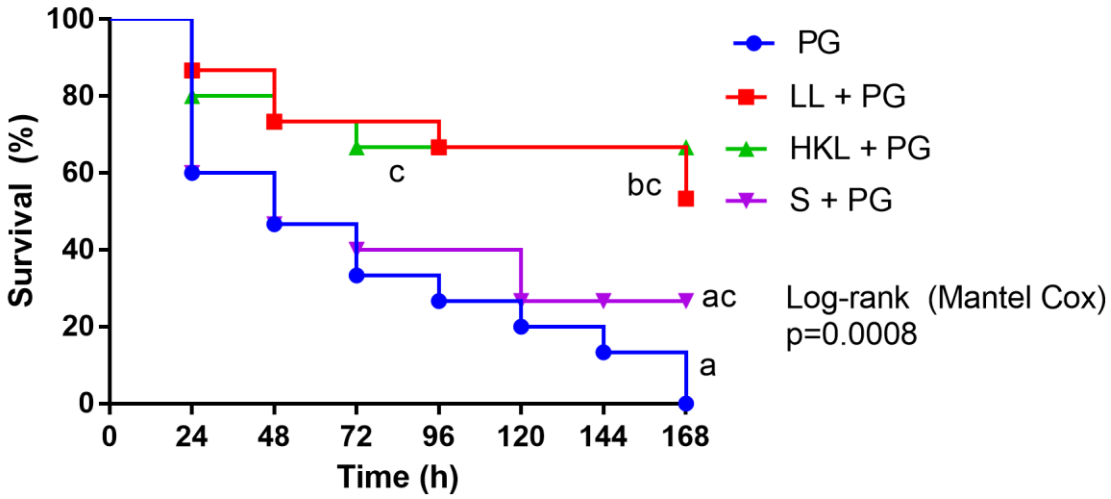

Figure 2- Survival curve of *G. mellonella* after interaction between *L. reuteri* preparations and *P. gingivalis*. Different small letters (a, b, c) close to the colored lines indicate significant statistical differences among groups after Log-rank (Mantel-Cox) test (PG- *P. gingivalis*, LL- live *L. reuteri,* HKL- heat killed *L.reuteri*, S- Supernatant of *L. reuteri*).

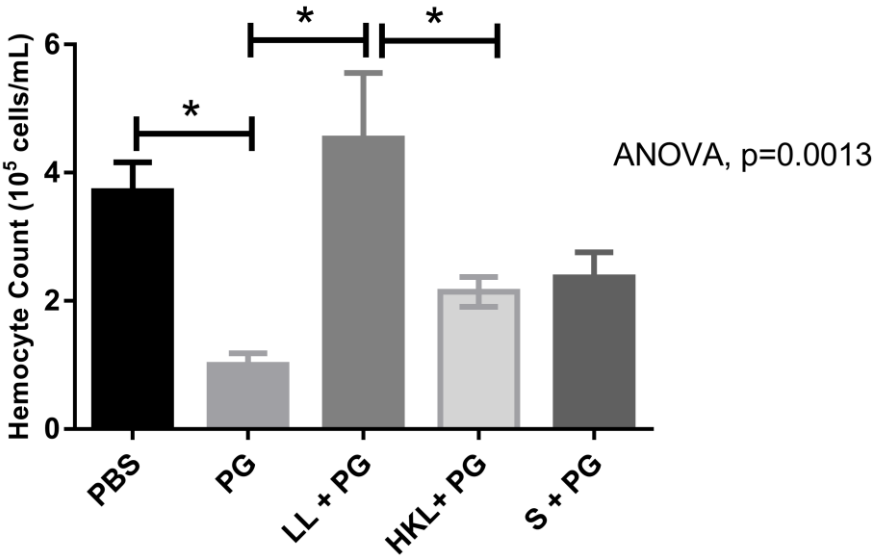

Figure 3- Hemocyte concentration. Mean and standard error of hemocyte count after the interaction between *L. reuteri* preparations and *P. gingivalis*. *Statistically significant difference after ANOVA and Tukey test ($p<0.05$) (PBS- phosphate buffered saline, PG- *P. gingivalis*, LL- live *L. reuteri,* HKL- heat killed *L. reuteri*, S- Supernatant of *L. reuteri*).