

# *Lactobacillus rhamnosus* versus *Staphylococcus aureus*: influence on growth and expression of virulence factors

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## Summary

*Staphylococcus aureus* is one of the main etiological agents of community and hospital infections. Due to the virulence factors of this species and the multiresistance of strains, diseases caused by *S. aureus* are difficult to treat. Therapeutic alternatives have been studied and, among them, probiotics stand out. In this context, the objective of the present work was to analyze the action of *Lactobacillus rhamnosus* on growth and virulence factors of *S. aureus*. Inhibitory activity was evaluated by the two-layer plate method. *L. rhamnosus* was seeded in MRS agar and incubated at 37°C and CO<sub>2</sub> for 24h. Subsequently, BHI agar was added to the medium, *S. aureus* was seeded, and the plates were incubated for 24, 48 and 72h. *S. aureus* strains isolated from co-culture with *L. rhamnosus* were investigated for coagulase production in horse plasma, by observation of clot formation, haemolysin production, by visualization of hemolysis in blood agar, and for biofilm formation in microliter plates. In the control group, strains of *S. aureus* were grown in the absence of *L. rhamnosus*. The results showed that there was an inhibitory activity of *L. rhamnosus* on *S. aureus*, as well as a significant reduction in the production of coagulase, in all analyzed periods. It was also observed that after 72h of co-cultivation with *L. rhamnosus*, *S. aureus* significantly reduced the biofilm formation capacity. Concludes with the present study *L. rhamnosus* is able to inhibit *S. aureus* growth and to interfere with coagulase production and biofilm formation, suggesting a possible beneficial use of these probiotic bacteria in the prevention and / or treatment of staphylococci diseases.

**Keywords:** virulence factors, probiotics, *Staphylococcus aureus*

## Introduction

*Staphylococcus aureus* is one of the most common aetiologic agents of infections acquired in the community and in hospital environments.<sup>1,2</sup> This bacteria is part of the transient microbiota of the skin and mucosa, mainly in hot and humid regions, such as the groin, axilla and nasal cavity.<sup>3</sup> In addition, *S. aureus* is considered an important opportunistic pathogen causing superficial skin infections, such as impetigo, until deep infections, as bacterial endocarditis, osteomyelitis and toxic shock.<sup>4</sup>

The virulence factors of *S. aureus* are closely related to its pathogenicity. The presence of membrane proteins, enzyme production (coagulase and hemolysin), escape from the cells of the immune system, biofilm production and antimicrobial resistance are some of the factors of virulence of *S. aureus*, favoring its colonization, infection and dissemination in the host tissues.<sup>5,6</sup>

*S. aureus* produces two types of coagulase: the classical coagulase and the binding protein Von Willibrand factor, which are able to convert fibrinogen into fibrin. *S. aureus* can also interact directly with fibrinogen to form large groups of cells, mediated by cell surface proteins. These virulence mechanisms provide better adherence to host cells, and also impair the phagocytosis by cells of the immune system.<sup>7,8</sup>

One of the main mediators of cell death induced by *S. aureus* is alpha haemolysin, a toxin that open pores in the target cell membrane.<sup>9</sup> Haemolysin is also related to invasion of host cell and adhesion on surfaces of catheters and prostheses.<sup>7</sup>

*S. aureus* is also able to form biofilms, which is an association of microbial cells surrounded by an extracellular polysaccharide matrix<sup>10</sup>. The presence of microorganisms in biofilm is common in nature and contribute to its resistance to the action of immune system cells and antimicrobial substances.<sup>11-13</sup>

Studies aiming the discovery of alternative therapies for the treatment of infections caused by multidrug-resistant strains have become increasingly frequent. For this purpose, probiotics have been studied.<sup>14-16</sup>

According to the World Health Organization (WHO),<sup>17</sup> probiotics are living microorganisms that promote health benefits to the host when used in adequate amounts. *Lactobacillus* and *Bifidobacterium* strains are among the most important bacteria.<sup>18</sup>

Probiotic strains can develop mutualism with pathogenic strains, preventing the proliferation of pathogens, as well as the development of diseases.<sup>19,20</sup> In vitro studies show that *Lactobacillus* strains are able to increase phagocytic activity of human macrophages against

extracellular pathogens such as *S. aureus*.<sup>21,22</sup> In addition, *Lactobacillus* can decrease cellular proliferation of *S. aureus* by reducing the pH, due to formation of products of fermentative activity, producing of antimicrobial substances (bacteriocins) and competing for nutrients and adhesion sites.<sup>23,24</sup>

Since the knowledge about the relationship between *Lactobacillus* and *S. aureus* can contribute to the elaboration of strategies for the prevention and/or treatment of staphylococci diseases, the present work aimed to verify the action of living cells of *Lactobacillus rhamnosus* on the growth and expression of virulence factors by *S. aureus*.

## Material and methods

### Microorganisms

*Staphylococcus aureus* (ATCC 25923) was plated on Mannitol Salt Agar (Biolog, Hayward, USA) for 24h at 37°C. Aliquots from the colony form units were added in sterile saline solution (NaCl 0.9%) until obtaining suspensions of 10<sup>6</sup> cels.ml<sup>-1</sup>, standardized in spectrophotometer at 490 nanometers (nm). *Lactobacillus rhamnosus* (ATCC 1465) was plated on Man-Rogosa-Shape agar (MRS, Himedia, Mumbai, India) for 48h at 37°C and 5% CO<sub>2</sub> and suspensions of 10<sup>7</sup> cels.ml<sup>-1</sup> were obtained in saline solution, standardized at 540 nm.

### Inhibitory activity of *L. rhamnosus* on *S. aureus*

Twenty microliters of standardized suspension of *L. rhamnosus* or 20uL of sterile physiological solution (control) were pipetted in single points on the surface of a Petri dishes containing 15 ml of MRS agar. The plates were incubated at 37°C at 5% CO<sub>2</sub> for 24h. After this period, 15 ml of BHI agar were added over the MRS agar with growth of *L. rhamnosus*. After solidification, 0,1 ml of the standard suspension of *S. aureus* were sown with the aid of Drigalski handle, and the plates were incubated at 37°C for 24h, 48 h or 72h in aerobic environment. After incubation, the presence of inhibition halos was checked. Analysis of inhibition was conducted according to the methodology proposed by Tagg et al.<sup>25</sup>

After halo measurement, aliquots of *S. aureus* from colonies, located within a radius of 5 mm close to the halo of inhibition, were collected for investigation of virulence factors.

The experiments were conducted in four independent trials (n=4) and in triplicate, totalling n=12.

### Analysis of coagulase production

The aliquots of microorganisms were seeded in Mannitol Agar and incubated at 37°C for 24h, for isolation of *S. aureus*. After that, suspensions were standardized in spectrophotometer in accordance with the methodology described previously.

Aliquots of 0,3ml of standard suspensions were transferred to sterile tubes containing 0,3 ml of horse plasma. After incubation at 36±°C for 6h, the formation of clots was verified, considering the following criteria: no clot formation (negative reaction); small and disorganized clot (+); small and organized clot (++); big and organized clot (+++); coagulation of the entire contents of the tube (++++).<sup>26</sup>

### Analysis of haemolysin production

For the test of the hemolytic activity, it was used blood agar Base added with 5% of horse blood. Aliquots of 3ul of standard suspensions of *S. aureus* were sown in this medium and after incubation for 24h at 37°C the occurrence of translucent halo around the colonies was checked, indicating positive hemolytic activity.

Enzyme activity (Pz) was evaluated by the ratio of the diameter of the colony and the diameter of the colony plus the zone of hemolysis. The smaller the value of Pz, the greater the enzyme activity. The enzyme activity was classified in: negative (Pz=1), positive (≥0.64 Pz< 1) and strongly positive (Pz<0.64).

### Formation of biofilm

In microplates of 96 wells, 100µl of the standard suspensions of *S. aureus* and 100µl of BHI broth (Brain Infusion Hearth) doubly concentrated were added. The plates were incubated at 37°C with agitation for 180 min initial adhesion of cells. After this period, the contents of the wells were removed and the wells were washed three times with 200µl of sterile saline solution for removal of not attached cells. After, 200µl BHI broth were added to the wells and the plates were incubated at 37°C for 24h with agitation, for the formation of biofilms. After incubation, the contents of the wells were removed and washed three times with 200µl sterile physiological solution. The optical densities of the biofilms formed on the bottom of the wells were verified in microplate reader at 530 nm.

Subsequently, with the aid of sterilized sticks, the bottom of each well was scraped, with movements in various directions, for 30 seconds. After that, 100µl of the contents of each well were transferred to sterile microtube containing 900µl of sterile saline solution, and homogenized for 30 seconds. From this suspensions, serial dilutions were performed and 20µl of each one were seeded in Mannitol agar plates and incubated at 37°C for 24h. After the incubation period, the Colony Forming Units per milliliter (CFU/ml) were determined.

### Statistical analysis

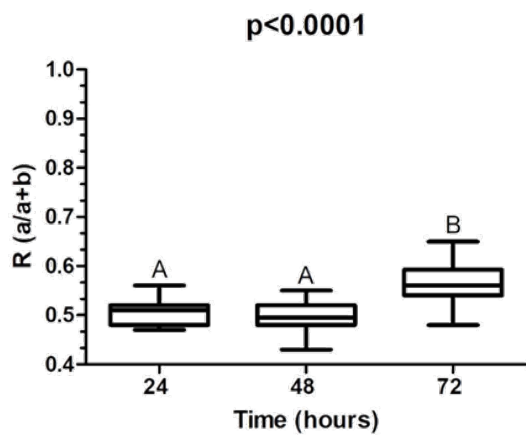
The results were analyzed for normality. The results of inhibitory activity were analysed using ANOVA and Tukey test. For the enzyme coagulase, t test was used. The other results were analysed by Kruskal Wallis test and Dunn's test. All results were analysed using the program GraphPad Prism 5.0 (GraphPad Software Inc.), considering a significance level of 5%.

## Results

### Inhibitory activity of *L. rhamnosus* on *S. aureus*

After 24, 48 and 72h of incubation, it was observed the presence of inhibition halos of *S. aureus* growth around the colony of *L. rhamnosus*.

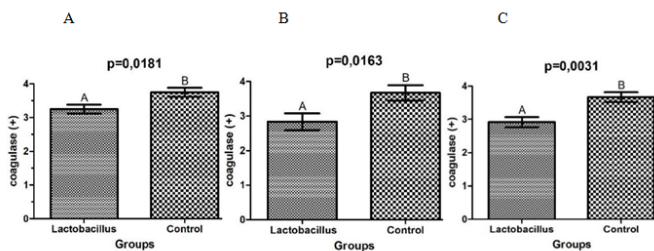
Figure 1 represents the ratio between the diameter of the colony of *L. rhamnosus* and the diameter of the colony of *L. rhamnosus* plus the halo of inhibition (a + b) of *S. aureus* after co-culture of these species. The lower the value, the higher the inhibitory activity of *L. rhamnosus*. In this way, after 24h and 48h, statistically grater inhibitory activity of *L. rhamnosus* on *S. aureus* (p<0.0001) was observed.



**Figure 1:** Inhibitory activity of *Lactobacillus rhamnosus* on *Staphylococcus aureus* growth after incubation in co-culture for 24, 48 and 72 hours. R (ratio of diameter from the colony of *L. rhamnosus* (a) and the diameter of the colony of *L. rhamnosus* plus the halo of inhibition (a + b)).

### Coagulase

There was a significant reduction in the production of coagulase by *S. aureus* isolated from co-cultures with *L. rhamnosus*, when compared to the control group (Figure 2). Comparing the percentage of reduction, with 48 h of association the lower production of the enzyme was observed (22.73%), followed by 20.45% in 72h and 13.33% in 24h.



**Figure 2:** Average of coagulase enzyme activity produced by *Staphylococcus aureus* strains isolated from co-culture with *Lactobacillus rhamnosus* during 24 (A), 48 (B) and 72 hours (C) or control strains (without contact with *L. rhamnosus*).

### Haemolysin

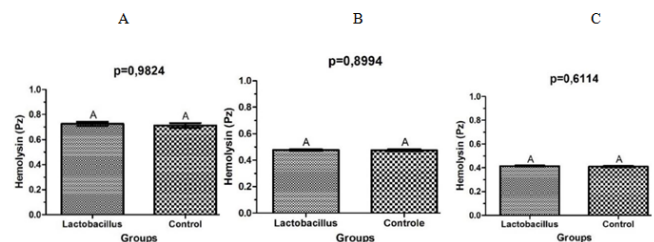
After analysis of the halos of hemolysis, it was observed that there was no statistically significant difference in the activity of hemolysin of strains of *S. aureus* cultivated in presence of *L. rhamnosus* when compared to the control group, independent of the time of interaction. Figure 3 shows the average of the values of Pz found in 24, 48 and 72h.

### Formation of biofilm

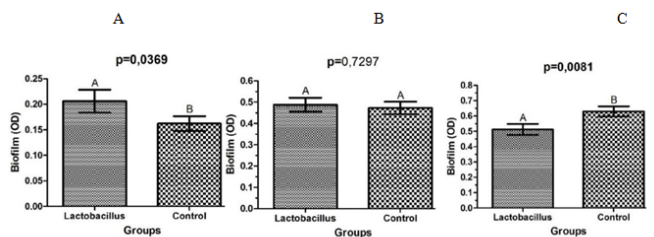
After the analysis of the optical densities of the biofilms of *S. aureus* in microplates of 96 wells, it was observed that the strains which were in the presence of *L. rhamnosus* for 24h, showed an increase in biofilm formation capacity ( $p=0.0369$ ), when compared to the control group (Figure 4A). After 48h of co-culture, there was no statistical difference between the groups ( $p=0.7297$ ) (Figure 4(B)). Instead that,

with 72h of contact with *L. rhamnosus*, *S. aureus* reduced significantly its capacity of biofilm formation ( $p=0.0081$ ) (Figure 4C).

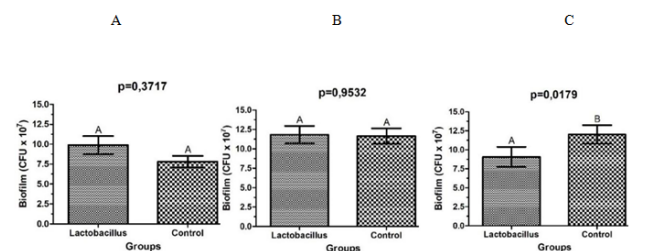
After CFU/mL counting from *S. aureus* biofilms, it was observed a reduction in the number of cells of *S. aureus* which were in contact with *L. rhamnosus* for 72h ( $p=0.0179$ ), when compared to the control group (Figure 5C). Statistically significant differences were not observed in CFU/ml counts of biofilms of *S. aureus* isolated from cultures in the presence of *L. rhamnosus* for periods of 24 ( $p=0.3717$ ) and 48 h ( $p=0.9532$ ) (Figure 5 AB).



**Figure 3:** Average of enzyme activity (Pz) of haemolysin produced by *Staphylococcus aureus* strains isolated from 24 (A), 48 (B) and 72 hours (C) plates in the presence or absence (control) of *Lactobacillus rhamnosus*.



**Figure 4:** Average of Optical Density (OD) of biofilms of *Staphylococcus aureus* isolated from cultures in the absence (control) or presence of *Lactobacillus rhamnosus* for 24 (A), 48 (B) and 72 hours (C).



**Figure 5:** Averages of Colony Forming Units per milliliter (CFU/ml) of biofilms of *Staphylococcus aureus* cultured in the absence (control) or presence of *Lactobacillus rhamnosus* for periods of 24, 48 and 72 hours (C).

### Discussion

The results obtained in this study suggest a antagonism between *L. rhamnosus* and *S. aureus*, since inhibition was observed in all tests, with greater inhibitory activity during the 24 and 48 h period of co-existence. As previously reported by other authors, *Lactobacillus* reduces the growth of *S. aureus*, through pH reduction resulted from fermentative activity, production of antimicrobial substances (bacteriocins) and competition.<sup>23,24</sup> The smaller inhibition halo

observed after 72h of co-culture probably resulted from the exhaustion of the effects of lactobacilli products which were capable to interfere with *S. aureus* growth.

Bertuccini et al. (2017)<sup>27</sup> found similar results when they studied the antimicrobial activity of *L. rhamnosus* and *L. acidophilus* on *S. aureus*, *Gardenerella vaginalis*, *Atopobium vaginae* and *Escherichia coli*, also using in vitro assays of co-culture. Glück and Gebbers<sup>28</sup> had already verified in vivo this antagonism. The authors observed that the ingestion of fermented milk containing *L. rhamnosus*, *Bifidobacterium* sp., *L. acidophilus* and *Streptococcus thermophilus* reduced significantly the occurrence of Gram-positive bacteria, including *S. aureus*, in nasal microbiota.

The antimicrobial activity of lactobacilli against *S. aureus* presented in biofilms was also demonstrated,<sup>29,30</sup> confirming that this effect is independent of staphylococci condition and of the methodology used.

The present study demonstrated that *L. rhamnosus* was also able to interfere with the production of coagulase by *S. aureus*. This enzyme was one of the first factors of virulence of *S. aureus* described and is considered one of the most important one. The coagulase linked to bacteria cell protects from the host immune system and the secreted coagulase is responsible for the formation of a fibrin clot that also protects from phagocytosis.<sup>31</sup>

Cheng et al.<sup>32</sup> using specific antibodies to neutralize the coagulase in a mouse model, demonstrated that it is a critical virulence factor for the formation of abscesses and establishment of bacteremia. The authors suggested that the inhibition of coagulase could be useful against *S. aureus* infections.

Some studies shows that the decrease in the production of alpha-haemolysin can decrease the mortality of mice with *S. aureus* pneumonia. The smaller haemolysin production also minimizes the pathological characteristics of pulmonary injury, demonstrating the importance of studies that interfere on this virulence factor as a manner to minimize the infections.<sup>33,34</sup>

Hemolysin contribute to the damage of host cells membrane, causing its lysis, and subsecente to the invasion in adjacent tissue.<sup>35</sup>

However in the presente study it was not observed significant difference regarding the formation of haemolysin by *S. aureus* when in the presence of *L. rhamnosus*. Other works investigating the interaction between these microorganisms and the production of haemolysin for *S. aureus* were not found in the literature.

The present study demonstrated that *L. rhamnosus* also interfered significantly on the ability of biofilm formation by *S. aureus*. Similarly, Andre<sup>36</sup> noted a decrease in biofilm formation, as well as interference in the composition of polysaccharides and DNA of *S. aureus*, by an antimicrobial peptide isolated from *Lactococcus lactis*. Zhou and Zang<sup>37</sup> evaluated the action of bacteriocins produced by *L. rhamnosus* on biofilms of *S. aureus* developed on rabbits knee implants. The researchers obtained significant reduction of *S. aureus* on the implants treated with the bacteriocins, when compared to the control group.

Therefore, the results of the presente study showed inhibitory action of *L. rhamnosus* on the growth of *S. aureus* and on the expression of some virulence factors, suggesting a potential beneficial use of these probiotic bacteria in the prevention and/or treatment of staphylococcal diseases. However it is important to highlight that in vitro studies have limitations, since the cells are treated outside the “normal”

environment. *In vivo* conditions include surrounding tissues, other microorganisms, blood supply, normal supply of nutrients, immune response and others. This way, further studies should be carried out, in order to confirm these possible benefits of *L. rhamnosus* and to define the best protocol for its use.

## Conclusion

In conclusion, *L. rhamnosus* was able to inhibit *S. aureus* growth, as well as reduce the production of coagulase and the capacity of biofilm formation by these microorganism.

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