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Individual and Collective Effect of Lactic Acid Bacteria on *Staphylococcus aureus*

Abstract

Staphylococcus aureus, as a foodborne pathogen causing significant harm worldwide, was studied to assess the effectiveness of probiotic strains *Lactobacillus casei, Lactobacillus plantarum,* and *Bifidobacterium bifidum* individually and collectively (as consortia) in controlling its growth. The growth patterns of *S. aureus* were observed when co-cultured with each probiotic strain and a consortium of all three strains over 72 hours. Additionally, the antimicrobial activity of probiotic cell-free supernatants (CFS) against *S. aureus* was tested using the agar well diffusion method. This study underscores the potential of *L. plantarum* and a consortium of *L. casei, L. plantarum*, and *B. bifidum* in controlling *S. aureus* growth.

Keywords: Foodborne pathogen, *Lactobacillus casei, Lactobacillus plantarum, Bifidobacterium bifidum, Staphylococcus aureus,* probiotic

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Introduction

Staphylococcus aureus is a foodborne, disease-causing bacteria which is a major cause of mortality and morbidity in the world.[1] It is gram positive, non-spore forming bacteria that can contaminate food product during food processing and food preparation.[2] Besides being commonly found in environmental sites such as marine and freshwater, soil surfaces, plant surfaces, dust and air, they commonly colonize the mucous membrane, skin and skin glands of the human body.[3] It can also form complex 3 dimensional structures called biofilms on food contact surfaces in food processing facilities, further increasing the likelihood of their contamination in food,[4] the reason for their occurrence in all categories of food (the raw, semi and the processed).[5] *S. aureus* produces a large variety of enterotoxins which when consumed along with food can cause intoxication. *S. aureus* produces enterotoxins, releasing them in the medium (food) when it reaches a quorum, which is to the concentration of 10000-100000 cfu/gm of food.[6] About 20 types of enterotoxins are produced by *S. aureus* that is pyrogenic exotoxins.[7] The concentration of the staphylococcal enterotoxin has to reach a concentration of 10-20 ng to be able to cause foodborne intoxication.[6] It is evident therefore that containing the growth of *S. aureus* in food is vital to reducing the incidence of staphylococcal intoxication and concurring concerns of food intoxication. Lactic Acid Bacteria (LAB) have been known to have inhibitory and antagonistic behavior against foodborne pathogens, including *S. aureus*.[8] Also, given the emerging concerns of antimicrobial resistance and increasing demand for an alternative solution to control pathogens,[9] it is imperative that possible bio-control agents are explored to serve this purpose.[10] Probiotic strains of Lactic Acid Bacteria can be beneficial and worthy of application in this context.

Lactic Acid Bacteria are living organisms that have several health benefits.[11,12] In the food industry, reducing the growth of foodborne bacteria can be brought about LAB. Among LAB, *Lactobacilli* and *Bifidobacteria* are the most common probiotic strains.[13] *Lactobacillus casei*, *Lactobacillus plantarum* and *Bifidobacterium bifidum* have been studied for their capacity to competitively inhibit or exclude pathogens.[14,15] The objective of this study was to evaluate the efficacy of *Lactobacillus casei, Lactobacillus plantarum* and *Bifidobacterium bifidum* in controlling *Staphylococcus aureus*.

Materials and methods

Bacterial strains and media

Lactobacillus casei (ATCC 12116) and *Staphylococcus aureus* (ATCC 5345) were procured from National Collection of Industrial Microorganisms (NCIM) Pune, India and *Bifidobacterium bifidum* (NRRL /ATCC 29521) And *Lactobacillus plantarum* (NRRL / ATCC 8014) were procured from Northern Regional Research Laboratory (NRRL), Agriculture Research Services (ARS) United State Department of Agriculture (USDA), USA. Mannitol Salt Agar (Himedia M118-500G), Lactobacillus MRS Agar Media (Himedia M614-500G), Soyabean Casein Digestive Medium (SCDM) (Himedia M011- 500G), and Muller Hinton Agar (Himedia M173-100G) were used in this study.

Microbial analysis of S. aureus with LAB

Inhibition of *S. aureus* [SA] in co-culture conditions with *Lactobacillus casei* [LC], *Lactobacillus plantarum* [LP] and *Bifidobacterium bifidum* [BB] in broth were studied. *S. aureus* as pure culture was used as control. The cell concentration of *S. aureus* was estimated at specific time intervals 0, 24, 48 and 72 hours, 0 hours being the initial concentration of the culture at the start of the experiment. 1 ml of *S. aureus* culture was inoculated into 100ml of 5 Soyabean Casei Digestive Medium broths each, labelled as SA, SA+LC, SA+LP, SA+BB, SA+ Consortium (Table 1). Except for the pure culture broths labelled as SA, the rest were inoculated with the 1ml of respective cultures of LC, LP and BB accordingly. In the last broth, besides SA all other cultures (LC, LP & BB) were inoculated 1ml each. All 5 culture broths were incubated at 37°C. The concentration of SA was estimated at the start of the experiment (0hrs) and thereafter at specified time intervals (24, 48 and 72hrs) from each of the culture conditions (SA, SA+LC, SA+LP and SA+BB) by standard plating technique.

Antimicrobial activity assay

The Antimicrobial activity assay was performed based on agar well diffusion by Kirby-Bauer method as discussed in this section. Culture conditions were similar to the earlier study (SA, SA+LC, SA+LP, SA+BB and SA+ Consortium, for convenience hereafter referred to as T1, T2, T3 & T4). Pure culture and co-cultures were cultured in

SCDM broth and incubated at 37°C for 24 hrs. Post the incubation, the cultures were centrifuged at 5000 rpm for 20 minutes to sediment bacterial cells and cell free supernatant (CFS) was collected. The volume of CFS was 10ml. CFS was filtered with syringe filter (0.45µm in pore size). The CFS filtrate was lyophilized[16] and the lyophilized CFS was further used for antimicrobial activity assay.The lyophilized CFS was rehydrated to a volume of 2ml. Antimicrobial activity of the lyophilized CFS of LAB culture against SA was determined by agar-well diffusion method on Mueller-Hinton Agar media (M173; HiMedia). The volume of rehydrated CFS (rCFS) and the standard antibiotics (as control) used for the assay were 200µl per well. The standard antibiotics used were Penicillin G, Oxacillin, Cephalothin, Amoxycycline, Clindamycin and Erythromycin (HiMedia HX001-1PK). After 24 hours, the plates were observed for zone of inhibition (mm) around the well.

Table 1 Experimental design of the study

	0hrs, 24hrs, 48hrs, 72hrs
Set 1	SA (Control) SA+LC (Test 1)
Set 2	SA (Control) SA+LP (Test 2)
Set 3	SA (Control) SA+BB (Test 3)
Set 4	SA (Control) SA+ Consortium (Test 4)

SA, *Staphylococcous aureus*; LC, *Lactobacillus plantarum*; LP, *Lactobacillus plantarum*; BB, *Bifidobacterium bifidum*; Consortium

Result

Growth pattern of *S. aureus* with *L. casei*

Log 10 cell concentrations of *S. aureus* in control (SA) and co-culture test (SA+ LC) at fixed time interval is as shown in Figure-1.0. While the cell concentration of SA at the start of the experiment (0 hrs) was 5.46, the cell concentration after 24hrs of incubation was estimated as 7.7 in Control and 7.32 in co-culture condition after 24 hours of incubation. Similar growth pattern of SA was observed at 48 and 72 hours, when grown with *L. casei*. The cell concentrations of SA were enumerated to be 7.57, 6.7 in control, and 7.01, 6.38 in co-cultured test at 48 and 72hrs respectively (Figure 1).

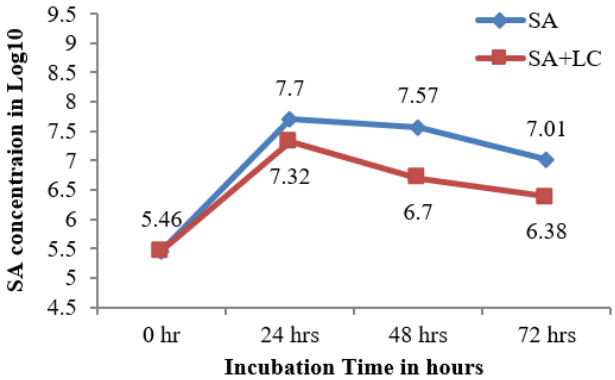

Figure 1 Growth Pattern of *S. aureus* as control and in the presence of *L .casei*

Growth pattern of *S. aureus* with *L. plantarum*

The growth pattern of *S. aureus* in control (SA) and co-culture test with *L. casei* (SA+ LC) at fixed time interval is as shown in Figure-2.0. While the cell concentration (Log10) of SA was 7.05 at the start of the experiment (0 hrs), the cell concentration of SA as pure culture (control) was estimated as 8.98 (SA) and 7.07 in co-culture with LP (SA+LP) after 24 hours of incubation. Similar change in Log 10 cell concentration of SA was observed at 48 and 72 hours when grown with *L. plantarum*. The cell concentrations of SA was estimated to be 8.2 and 7.2 in control, and 7.18 and 5.86 in co-cultured test at 48 and 72 hours respectively (Figure 2).

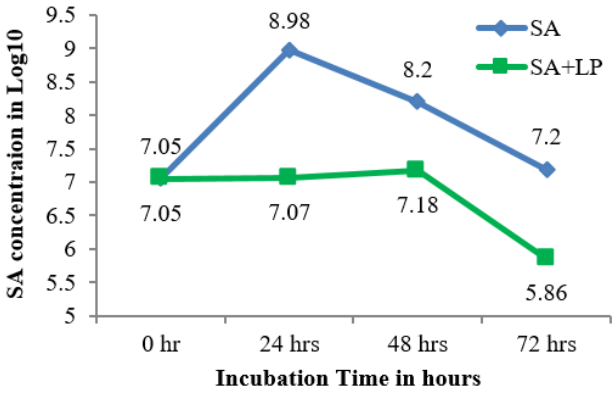

Figure 2 Growth Pattern of *S. aureus* as control and in the presence of *L. plantarum*

Growth pattern of *S. aureus* with *B. bifidum*

The growth pattern of *S. aureus* in control (SA) and co-culture test (SA+ BB) at fixed time interval is shown in Figure 3. While the cell concentration of SA at 0hrs was estimated as 9.93 (log 10). The cell concentration of SA was estimated as 8.08 (Control) and 7.85(co-culture test) after 24 hours of incubation. The log 10 cell concentration of SA was observed at 48 and 72hrs when grown with *B. bifidum* The cell concentration of SA was estimated 7.56 and 7.17 in control (SA), 7.17 and 6.82 in co-cultured (SA+BB) at 48 and 72 hours respectively (Figure 3).

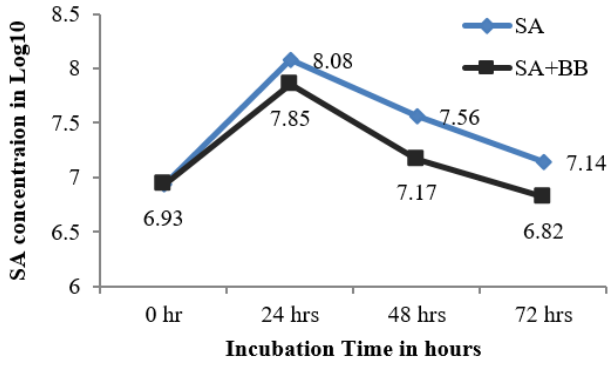

Figure 3 Growth Pattern of *S. aureus* as control and in the presence of *B.bifidum.*

Growth pattern of *S. aureus* with consortium

Growth pattern of *S. aureus* in control (SA) and co-culture test with a consortium of LC+ LP+BB was observed. The log10 concentration of SA at 0 hrs was 7.28. The culture concentration of SA as pure culture was 8.18, 7.32 and 7.20, while the concentration of SA in the presence of consortium of cultures were 7.30, 6.0and 5.8 at 24, 48 and 72 hours respectively (Figure 4).

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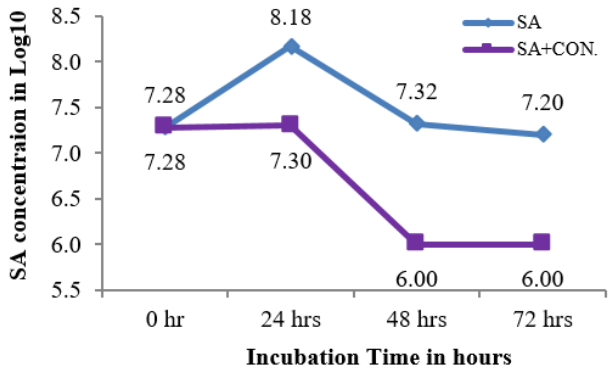

Figure 4 Growth Pattern of *S. aureus* as control and in the presence of Consortia of cultures.

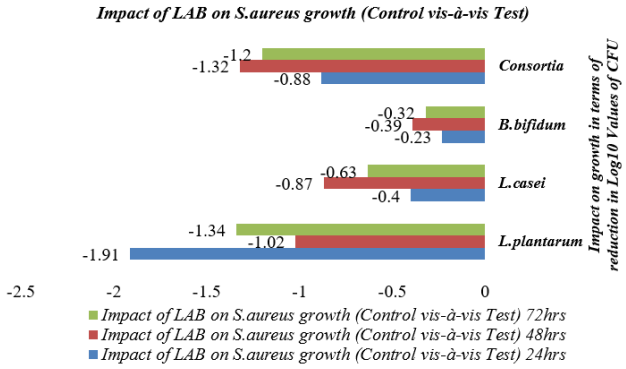

Figure 5 Effect of individual probiotic cultures and as consortia.

Result of antimicrobial activity assay

The antimicrobial activity of CFS of LAB was evaluated against *S. aureus* using the agar well diffusion method. The selected species of LAB namely *L.casei, L.plantarum* and *B.bifidum* were each inoculated individually and as consortium to grow in SCDM broth along with *S.aureus.* The objective was to screen for expression of antibiosis by the strains of LAB against *S.aureus* and experiments based on Kirby-bauer method as discussed in section 2.3 were performed. As discussed the CFS collected from each of the tests (1 to 4) were screened for antibiosis against *S.aureus.* As control, antibiotics recommended sensitive for Gram positive bacteria, namely penicillin G, oxacillin, cephalothin and amoxycycline are inhibitors of cell wall synthesis[17] and other clindamycin and erythromycin are inhibitors of protein synthesis[18] were used. While the result showed that the strain of *S.aureus* was sensitive to all the antibiotics used as control in the form of Zone of inhibition or no growth around the wells carrying the control antibiotics, no inhibition was observed around the wells with Cell Free supernatants from any of tests 1 to 4.

Discussion

Staphylococcal food poisoning is among the most prevalent foodborne intoxication in world.[19] Control and prevention of *S. aureus* therefore is a crucial public health concern.[20] Probiotics as live microorganisms that confer benefits to human health have also been studied for their potency to control pathogens through competition or inhibition via antibiosis.[21] The objective of the study was to screen for possible control of *S. aureus* using probiotic species namely *L. casei*, *L. plantarum* and *B. bifidum* by understanding their effect on the growth and proliferation of *S.aureus.*

It was observed that among the tests (1 to 4) wherein, *S.aureus* was incubated individually with each of the selected probiotic strains (*L. casei*[LC], *L. plantarum*[LP] and *B. bifidum*[BB]) and together as consortium, the growth of *S.aureus* was maximum affected when grown with *L. plantarum.* Although individually the effect of *L.casei* and *B. bifidum* on the growth of *S. aureus* was marginal, the effect of *L. casei, L. plantarum* and *B. bifidum* as consortia had a significant effect on the growth of *S. aureus.* It is also noteworthy that the growth pattern of *S. aureus* both with *L. plantarum* individually and with consortia did not show log scale rise as observed in the control. From 0hrs to 24hrs in the presence of *L. plantarum* and consortia the concentration of *S. aureus* (in Log10 value) remained close to constant. This gives us to infer that the growth rate of *S. aureus,* equated their numbers to remain at close to constant as concentration was contained by *L. plantarum* individually and also by the consortia of all the 03 probiotic strains. The variation in concentration as Log10 values/ml of *S .aureus* in control to test was -1.91 at 24hrs, -1.02 at 48hrs and -1.34 at 72hrs, in the presence of *L. plantarum.* It has been reported earlier that *L. plantarum* strains can produce variety of antimicrobial compounds like diacetyl, hydrogen peroxide, organic acid and also bacteriocins and antimicrobial peptides[22] and these probably could affect the growth *S. aureus*. Also, according to other previous studies, antagonistic behaviour via expression of phenyllactic acid and lactic acid that show antimicrobial activity have been reported.[23,24] These organic acids have been shown to have adverse effect on microorganisms sharing the same niche. It is also possible that the containing effect of the probiotic strain namely *L. plantarum* in this case could be due to competition between LAB and pathogens for limited resources and space as reported in earlier studies.[25]

Similarly, the consortia of all the 03 strains collectively contained the growth of *S. aureus* and the variation in concentration as Log10 values of CFU/ml from control to test was -0.88 at 24hrs, -1.32 at 48hrs and -1.2 at 72hrs. The probable mechanism of inhibition of *S. aureus* could be attributed to the reasons as cited above. At the same time, it is also be noted that when in the same niche as consortia the probiotic strains could be competing and or exhibiting antibiosis against each other as well. Nevertheless, the possibility of cooperation among the denizens of a niche is also a possibility which is beyond the scope of this study. Our observations that *L. casei* did not show significant effect on the growth of *S. aureus* is contrary to the previous reports. According to previous reports *L. casei* showed strong antimicrobial activity and was inferred to be probably due to production of bacteriocin, competition for resources or production of organic acid such as lactic acid which decreases the pH levels.[24] The variation in concentration as Log10 values of CFU/ml from control to test was -0.4, -0.87 and -0.63 at 24, 48 and 72 hrs respectively.

Similarly no significant effect on *S. aureus* growth was observed with *B. bifidum* in our study. However, earlier reports have indicated that *B. bifidum* could inhibit the growth of *S .aureus* and that *B. bifidum* produced bacteriocins, besides organic acids and short chain fatty acids that create unfavorable condition for pathogens.[26,27] Also, possible mechanism of competition for nutrients and space by *B. bifidum* that is inhibitory to other species in co-culture has been reported,[28,29] further emphasizing the inhibitory effect of *B. bifidum* on co-habitants. But results from our experiments indicated that as compared to *L. casei* and *L. plantarum*, *B. bifidum* was less inhibitory to *S. aureus*. The variation in concentration as Log10 values of CFU/ml from control to test was -0.23, -0.39 and -0.32 at 24, 48 and 72 hrs respectively.

Given the results that clearly indicated inhibition of *S. aureus* by the selected strains of probiotic, significant inhibition in case of *L.*

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plantarum and the consortia of *L. casei*, *L. plantarum* and *B. bifidum*, it was compelling to probe for possible antibiosis by the strains against *S. aureus*. Experiments based on the Kirby-bauer method using the CFS from each of the culture conditions (LP, LC, BB and Consortia) that were concentrated by lyophilization and thereafter rehydrated, were performed. The results however did not show any marked zone of inhibition of the *S.aureus* growth, which was otherwise explicit with antibiotics (Pencillin G, Oxacillin, Cephalothin, Amoxycycline, Clindamycin and Erthyromycin) used as reference control.

Based on the results of the study and previous reports on the subject it could be inferred that inhibition of *S. aureus* by Lactic Acid Bacteria could be strain dependent, and also that the inhibition could be more by competition for space and nutrients, and less by expression of antibiosis. Nevertheless further probing into possible expression of Quorum Quenching molecules and or bacteriocin types of molecules cannot be negated.

Conclusion

S. aureus, owing to its rapid and progressive evolution to multi-drug resistant forms, has emerged to be of major concern. LAB that have been reported to competitively exclude, and exhibit antimicrobial properties are optimal candidates for containing *S. aureus*. The inhibitory action of lactic acid bacteria against other microorganisms cannot be attributed to the production of various metabolites and antimicrobial compounds. It is important to note that the inhibitory action of LAB can vary depending on the specific strain of bacteria, type of food matrix and the environmental conditions. Further study is needed to understand the mode of action of LAB, especially to evaluate the potential use of LAB in biocontrol of foodborne pathogens and as a substitute in bio-preservation.

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Conflicts of interest

The author declares no conflicts of interest.

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