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Influence of Some Growth Conditions on The Antibacterial Activities of *Lactobacillus fermentum* Against Human Fecal *Staphylococcus aureus*

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Abstract

One of the best-known and widespread bacterial pathogens that cause invasive infections as well as skin infections is *Staphylococcus aureus* to contribute to specific pneumonia and other respiratory tract infections, cardiovascular infections, infections at surgical sites, infections of prosthetic joints, and nosocomial bacteremia. Meanwhile, Lactic acid bacteria (LAB) have an effective antibacterial potential that may be used to safeguard both human and animal health, according to several research. Numerous LAB strains are side effect free and incapable of harming people and lab animals. Hence, this study was performed to investigate the optimal conditions, including MIpH, InTe, and InTi, for Lactobacillus fermentum (LAF) isolated from traditional fermented foods against human fecal *Staphylococcus aureus* (hf-SA).

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Introduction

Every year, *Staphylococcus aureus* is acknowledged as one of the well-known and pervasive bacterial pathogens that cause invasive infections and skin infections worldwide (Kleven et al., 2007; Rasigade et al., 2014). This pathogenic bacterium has been found to cause pneumonia, respiratory tract infections, cardiovascular infections, surgical site infections, prosthetic joint infections and nosocomial bacteremia (Tong et al, 2015). A review in 2012 estimated that among 100,000 bacterial cases, *S. aureus* occupied from 20 to 50 cases per year, and the death rate of these patients is 10% to 30% (van Hal et al., 2012). In 2017, in the US, the number of deaths by *S. aureus* was reported to be 20,000 (Kourtis et al., 2019). The number of deaths caused by *S. aureus* bacteremia was much more than the combination of acquired immune deficiency syndrome (AIDS), tuberculosis, and viral hepatitis (Klevens et al., 2007; van Hal et al., 2012).

Methicillin-resistant *S. aureus* (MRSA) is the most clinically significant because *S. aureus* infections are particularly difficult due to regularly occurring antibiotic resistance in *S. aureus* isolates (Turner et al, 2019). Compared to infections brought on by methicillin-sensitive *S. aureus* (MSSA), MRSA infections are associated with higher rates of death, morbidity, and hospitalization (Ippolito et al., 2010). Furthermore, methicillin-sensitive *S.*

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aureus (MSSA) strains are becoming more well-recognized for their significant clinical value, with sequence type (ST) 398 causing a deadly infection (Bonesso et al., 2016; Bouiller et al., 2020). MSSA infections are less frequently seen than MRSA infections, and newly deployed anti-MRSA measures did not result in a comparable decline in MSSA infections (Kavanagh et al, 2019).

Many studies have proved that lactic acid bacteria (LAB) have a strong antibacterial ability to protect human and animal health (Bernardeau et al., 2006; Bourdichon et al., 2012). LAB are Gram-positive and usually non-spore-forming, catalase-negative, and devoid of cytochromes. They are anaerobic or aerobic tolerant, difficult to culture, acid-tolerant, and can produce lactic acid as the principal end product of sugar fermentation (Holzapfel et al., 2001). The genus *Lactobacillus* is the largest genera of the LAB groups, including *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*, which are classified into obligate homofermentative, facultative heterofermentative, and obligate heterofermentative groups (Axelsson, 2004).

Many LAB strains are safe and incapable of causing side effects on humans and laboratory animals. LAB HM3 isolated from human milk was tested in Sprague-Dawley rats by oral supplementation at a dose of $1x10^{10}$ CFU/kg/day for 14 to 28 days. The results showed that LAB HM3 adapted to the digestive system and did not affect the growth ability, food consumption, cellular composition of blood and muscles, and essential organs of tested mice (Shokryazdan et al., 2016). LAB SJRP30 isolated from cheese did not cause hemolysis or breakdown of the mucous membrane of intestinal hair, as well as no genes related to diseases such as endocarditis, antigens, collagen adhesion, tyrosine decarboxylase, ornithine decarboxylase, tetracycline resistance, and erythromycin resistance was observed (Casarotti et al., 2017).

Milk fermented with strain LAB MTCC 5898 was administered to 16-month-old mice for two months. The results showed that the experimental mice were utterly normal in neutrophil function, interleukin, inflammation, antibody response in the intestine, liver-actant enzymes, and red blood cells (Sharma et al, 2014). LAB CECT5716 Lc40 was added to the diet of one-month-old infants at a dose of 1x109 CFU/day for those under six months of age and $7\text{-}8x10^{8}$ CFU/day for those over six months. The experiments were conducted for 12 months; the results showed that LAB CECT5716 Lc40 adapted to experimental children whose weight gains were not absolutely different from the control groups (Maldonado-Lobón et al., 2015). LAB secreted inhibitors, including bacteriocins, biosurfactants, and H_2O_2, inhibit the growth of urogenital and intestinal pathogens. LAB was clinically tested to effectively reduce pathogenic intestinal bacteria and increase the percentage of beneficial bacteria in healthy people (Kaur et al., 2013). LAB was used to produce an antibacterial compound called fermentation for food preservation and medical applications (Fuochi et al., 2017). From the benefits mentioned above, it is vital to determine the appropriate culture conditions for each strain of LAB to grow and show maximum antibacterial ability. The article provides some results of the influences of some factors, including medium initial pH, incubation temperature, and incubation time on the antibacterial abilities against human fecal *Staphylococcus aureus* of *Lactobacillus fermentum* strains (LAF1, LAF2, and LAF3). The results of the article will be able to become a helpful reference to apply LAF strains in bacteremia prevention of *S. aureus* for humans and animals.

Materials and Methods

Studied lactic acid bacteria (LAB) and pathogenic bacteria

The human fecal *Staphylococcus aureus* (hf-SA) strain in this study was obtained from human feces, and three strains of *Lactobacillus fermentum* (LAF), including LAF1, LAF2, and LAF3, originated from traditional Vietnamese fermented food. The tested bacteria and studied LAF strains were stored at the Laboratory of Microbiology and Biochemistry, Thu Dau Mot University, Binh Duong, Viet Nam.

LAF strains were activated in MRS Broth (MRSB) and stored in MRS Agar (MRSA). Meanwhile, hf-SA was activated in Nutrient Broth (NB) and stored in Nutrient Agar (NA). The antimicrobial activities of three LAF strains against hf-SA were carried out in NA. All media used in this study were purchased from Himedia, India (Balouirin et al., 2016).

Screening the antibacterial ability of LAF strains against human fecal *S. aureus* on the agar plate

The agar diffusion method determined the antibacterial activities of LAF strains against tested bacteria. The principle was based on the diffusion of the antibacterial compounds of the supernatant into the agar to inhibit the growth-tested bacteria and form an inhibiting zone. LAF strains were aerobically cultured in an MRS broth medium with different pH scales, temperatures, and incubation time values. Their biomass was centrifuged at 4500 rpm to remove the cells and collect the supernatant before neutralizing the lactic acid by 0.1N NaOH. Human fecal *S. aureus* (hf-SA) was grown in NB for 24 h at 37 °C. One hundred µl of hf-SA were placed and spread on a Petri dish containing 15 ml of NA medium using glass hockey sticks. The inoculated Petri dish with hf-SA was dug into three wells with diameters to 6 mm using a sterile cork borer. Each well on the tested dish was dropped 0.1 ml of acid-neutralized supernatant, kept the tested dish in the refrigerator at 4^{o}C for 4-8 hours before culturing at 37^{o}C for 24 hours. The antibacterial activity of LAF was evaluated by taking the difference between D (zone of inhibition diameter, mm) and d (well diameter, mm). The bigger difference of D – d (mm), the stronger the inhibitory activity of *L. fermeture (*Ouwehand & Conway, 1996; Cavalieri, 2005; Pascual et al., 2008; Balouirin et al., 2016).

Data Analysis

T-Test analyzed the data recorded in the studies for comparing means using STATGRAPHICS CENTURION XIX licensed software ($p < 0.05$).

Results and Discussion

The effect of medium initial pH (MIpH) on the antibacterial ability of LAB against hf-SA

To investigate the optimal MIpH range for antibacterial activity of three selected LAF strains against hf-SA, LAF1, LAF2, and LAF3 were cultured in MRS medium under static culture conditions at room temperature for 96 hours of incubation time (InTi) in different MIpH values including 4, 5, 6, 7 and 8. Their antibacterial abilities were tested by the method of diffusion on agar plates. The results of the experiment are presented in Table 1.

Table 1. Effect of MIpH on antibacterial activity of LAF strains against hf-SA

MIpH	LAF1 D – d Mean ± SD (mm)	LAF2 D – d Mean ± SD (mm)	LAF3 D – d Mean ± SD (mm)
4	4.67±1.53^{a}	6.67 ±1.53^{a}	5.0 ±2.0^{a}
5	6.33 ± 1.15^{a}	6.33 ±1.15^{a}	5.33±1.53^{a}
6	10.67 ±0.58^{b}	10.0 ± 1.73^{b}	10.67±0.58^{b}
7	13.33 ±1.53^{c}	11.33 ± 1.53^{b}	13.67±1.53^{c}
8	6.67 ±1.53^{a}	6.67 ± 1.53^{a}	6.0±1.0^{a}

Note: Means with the same letter in the same column are not statistically significant differences (P > 0.05)

The results showed that the MIpH of the culture medium strongly influenced the antibacterial ability of three strains of studied LAF. The studied strains weakly performed their antibacterial activities against hf-SA at different investigated pH values, of which all recorded inhibition zones were always less than 15 mm in all experimental treatments. Initially, In a turn of LAF1, LAF2, and LAF3, their ZOIs were weakly performed at 5.0 ± 2.0 mm, 6.67 ±1.53 mm, and 4.67±1.53 mm in the experimental MIpH = 4, increasing to 6.33 ±1.15 mm, 6.33 ± 1.15 mm, and 5.33 ± 1.53 mm in the experimental MIpH = 5 before being recorded the values at 10.67 ±0.58 mm, 10.0 ± 1.73 mm, and 10.67 ± 0.58 mm in the experimental MIpH = 5. Although, all three strains, LAF1, LAF2, and LAF3, showed their highest inhibition zones against hf-SA in experimental treatment with MIpH = 7, including 13.33 ± 1.53 mm, 11.33 ± 1.53 mm, and 11.7 ± 1.2 mm, respectively. In the experimental MIpH = 4, the recorded ZOIs' values initiated to reduce rapidly to 6.67 ±1.53 mm of LAF1 and LAF2 and 6.0 ± 1.0 mm of LAF3.

The effect of incubation temperature (InTe) on the antibacterial ability of LAB against hf-SA.

To reveal the effect of InTe on antibacterial activity against hf-SA**,** continuingly, LAF1, LAF2, and LAF3 were cultured in MRS medium with MIpH = 7 under static culture conditions at room temperature for 96 hours of InTi in different temperature values, including 20^{o}C, 25^{o}C, 30^{o}C, 35^{o}C, 37^{o}C, and 50^{o}C. Their antibacterial abilities were tested by the method of diffusion on agar plates. The results of the experiment are presented in Table 2.

Table 2. Effect of InTe on antibacterial activity of *LAB* strains against hf-SA

Temp (^{o}C)	LAF1 D – d Mean ± SD (mm)	LAF2 D – d Mean ± SD (mm)	LAF3 D – d Mean ± SD (mm)
20	6.67 ± 1.53^{b}	6.67 ± 1.53^{b}	6.0 ± 1.0^{b}
25	12.0 ± 1.0^{cd}	11.0 ± 1.0^{c}	11.67 ± 1.15^{c}
30	14.33 ± 1.15^{e}	13.33 ± 1.53^{d}	15.33 ± 2.08^{d}
35	13.33 ± 0.58^{de}	12.33 ± 0.58^{cd}	14.33 ± 2.08^{cd}
37	11.0 ± 1.0^{c}	10.67 ± 1.15^{c}	14.33 ± 2.08^{d}
40	2.0 ± 1.0^{a}	1.83 ± 0.76^{a}	2.67 ± 0.58^{a}

Note: Means with the same letter in the same column are not statistically significant differences (p > 0.05)

The results showed that different InTes influenced the antibacterial ability of three studied LAF strains. They started to well perform 12.0 ± 1.0 mm, 11.0 ± 1.0 mm, and 11.67 ± 1.15 mm of the zone of inhibition (ZOI) at the 25°C of InTe, hitting the highest ZOIs at 14.33 ± 1.15 mm 13.33 ± 1.53 mm and 15.33 ± 2.08 mm at the 30°C of InTe. They slightly reduced to 13.33 ± 0.58 mm, 12.33 ± 0.58 mm, and 14.33 ± 2.08 mm at 35°C of InTe, continuing to fall to 11.0 ± 1.0 mm, 10.67 ± 1.15 mm, and 14.33 ± 2.08 mm at 37°C of InTe before dramatically decreasing to the lowest values at 2.0 ± 1.0 mm, 1.83 ± 0.76 mm and 2.67 ± 0.58 mm at 40°C of InTe. In general, the antibacterial activities of studied LAF strains against hf-SA were improved in different InTes ranging from 25°C to 37°C, and the optimum InTe for the LAF strains to show their best ZOIs was 30°C.

The effect of incubation time (InTi) on the antibacterial ability of LAB against hf-SA

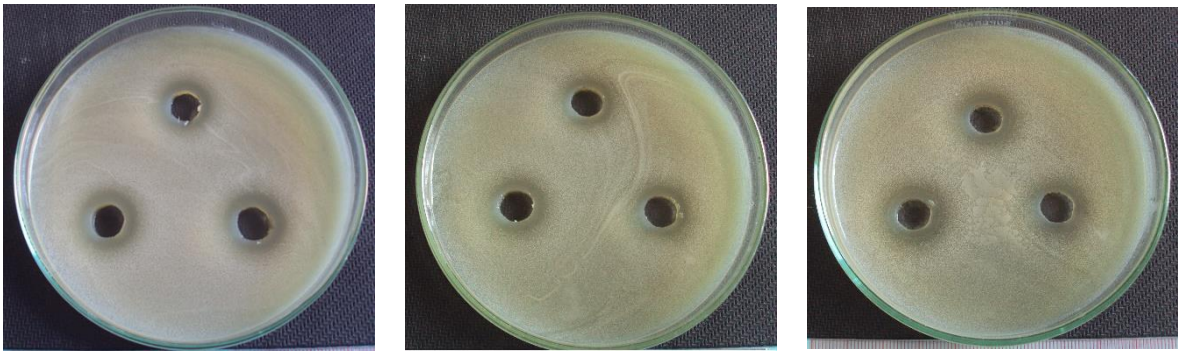
To reveal the effect of InTi on antibacterial activity against hf-SA, continuingly, LAF1, LAF2, and LAF3 were continued to culture in MRS medium with MIpH = 7, 35°C of InTe and under different periods of InTi including 24 hrs, 48 hrs, 72 hrs, 96 hrs, 120 hrs, and 144 hrs. Their antibacterial abilities were tested by the method of diffusion on agar plates. The results of the experiment are presented in Table 3.

Table 3. Effect of InTi on antibacterial activity of LAF strains against hf-SA

Time (hrs)	LAF1 D – d Mean ± SD (mm)	LAF2 D – d Mean ± SD (mm)	LAF3 D – d Mean ± SD (mm)
24	1.67 ± 0.58^{a}	1.33±0.58^{a}	1.67±0.58^{a}
48	5.33±1.15^{b}	3.0±1.0^{ab}	3.0±1.0^{ab}
72	6.33±1.53^{b}	3.67±1.15^{b}	5.0±1.0^{b}
96	14.67±0.58^{d}	13.33±0.58^{cd}	15.33±0.58^{d}
120	17.67±0.58^{e}	15.33±1.53^{d}	19.0±0.0^{e}
144	11.0±1.0^{c}	11.33±1.53^{c}	11.0±1.0^{c}

Note: Means with the same letter in the same column are not statistically significant differences (P > 0.05)

All LAF strains performed weak ZOIs lowering 6,5 mm from 24 hrs to 72 hrs of InTi. In comparison, the highest ZOI was shown by LAF1 at 6.33 ± 1.53 mm, the second one was 5.0 ± 1.0 mm of LAF3, and the third one was 3.67 ± 1.15 mm of LAF2. At 96 hrs of InTi, their ZOIs grew dramatically upto 15.33 ± 0.58 mm of LAF3, 14.67 ± 0.58 mm of LAF1, and 13.33 ± 0.58 mm of LAF2, which rapidly reached the top recorded values at 120 hrs of InTi including 19.0 ± 0.0 mm of LAF3, 17.67 ± 0.58 mm of LAF1 and 15.33 ± 1.53 mm of LAF2. After taking the highest values, the observed ZOIs of LAF strains start to gradually fall to 11.0 ± 1.0 mm, 11.33 ± 1.53 mm, and 11.0 ± 1.0 mm of LAF1, LAF2, and LAF3, respectively.



LAF1 **LAF2** **LAF3**

Figure 1. Antibacterial activity of studied LAF strains against hf-SA cultured in optimal condition medium.

Genus *Lactobacillus* has been known as the most potential source of biocontrol agents producing abundant and broad spectrum antibacterial compounds; amongst them, many *L. fermentum* strains were carefully researched in the optimal culture conditions to perform intense antibacterial against *S. aureus,* a dangerous pathogenic Gram-positive bacteria.

Lactobacillus fermentum (MTCC No. 1745) was studied to optimize the culture parameters for producing antimicrobial compounds to inhibit *S. aureus*. Regarding the MIpH effect, *L. fermentum* (MTCC No. 1745) also performed weak antibacterial activity against *S. aureus*, of which the recorded ZOIs were under 10 mm at 5, 8, and 9 of the MIpH. Besides, the study showed that *L. fermentum* (MTCC No. 1745) had also given out strongly controlled *S. aureus* under from 6 to 7 of the MIpH range. In detail, the highest ZOIs was 12 mm, observed at MIpH value six before slightly falling to 11 mm at value 7 of MIpH. *L. fermentum* (MTCC No. 1745) was also reported to optimize the InTe at 35^{o}C, of which the ZOIs were recorded at 14 mm. Meanwhile, other experimental InTes, including 20^{o}C, 25^{o}C, 30^{o}C, and 40^{o}C, were performed under 12 mm of ZOIs. Moreover, *L. fermentum* (MTCC No. 1745) strongest inhibited *S. aureus* after 72 hrs of InTi, of which the ZOIs' value was approximate at 12 mm, as well as the ZOIs of experimental 12 hrs, 24 hrs, 48 hrs, and 96 hrs were recorded under 10 mm (Talluri & Lanka, 2017). Fermencin SA715 is a novel, broad-spectrum, non-pore-forming, and cell wall-associated bacteriocin isolated from *L. fermentum* GA715 of goat milk origin against *S. aureus* RF122.

The optimal condition for the production of fermented SA715 occurred at 37 °C of InTe and MIpH 6–7, of which the recorded ZOI was 14 mm. Moreover, the accumulation of fermenting SA715 production involved batch fermentation in a bioreactor at a constant pH of 6.5 and 37^{o}C of InTe, which resulted in increasing the antibacterial activity of more than 20 mm of recorded ZOI after 24 hours of InTi (Samson & Koshy, 2018). In another study, Onwuakor et al. (2021) used the agar well diffusion assay method to isolate *Lactobacillus fermentum* strain COE20 from fermenting African oil bean seeds (*Pentaclethra macrophylla* Benth). LAF isolated was found to produce bacteriocin at optimal culture conditions of 31°C, pH 5.9 and 1.9% NaCl concentration, which inhibited *Staphylococcus aureus* ATCC 19095 at 11.75 mm of ZOI. The strongest ZOI of antimicrobial activity of *L. plantarum* against *S. aureus* in fermented chicken eggs was 6.977 ± 0.05 mm at an InTi of 37°C for 96 hours (Mangalisu et al., 2022). The antimicrobial activity of LAB strain Pr 4.3L from Peda fish has more potent inhibition against *S. aureus* at a temperature of 30°C compared to 37°C with a pH range of 6-7. The largest obtained ZOI was 17.7 ± 0.6mm in the treatment with a temperature of 30°C and a pH of 7 for 36 hours of InTi (Amarantini et al., 2020). Bacteriocin produced

by *Lactobacillus viridescence* was optimized, showing the highest production and antimicrobial activity against *Staphylococcus aureus* (NCIM 2079) in MRS broth with pH 7.0 incubated at 37°C for 48 hrs (Sure et al., 2016).

L. fermentum strains (LAF1, LAF2, and LAF3) in this study, along with reported others, had their optimal medium with different culture conditions for maximizing their antibacterial activities against *S. aureus* including MIpH, InTe, and InTi ranging values 6 – 7, 30^{o}C – 37^{o}C and 24 hrs – 120 hrs, respectively. As a result, the optimizing cultural parameters for producing antimicrobial compounds is extremely necessary.

Conclusion

Lactobacillus fermentum (LAF1, LAF2, and LAF3) obtained from traditional Vietnamese fermented foods performed their solid antibacterial activities against hf-SA. Optimum condition, including MRS-broth with MIpH=7, 35^{o}C of InTe, and 120 hrs of InTi for their antibacterial productions, was established to enhance them as natural biocontrol agents for human health preservation.

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