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Influence of biofilm-forming lactic acid bacteria against methicillin-resistant *Staphylococcus aureus* (MRSA S547)

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ABSTRACT

Objective: To investigate the antibacterial effect of selected lactic acid bacteria (LAB) biofilms on the planktonic and biofilm population of methicillin-resistant *Staphylococcus aureus* (MRSA) (S547).

Methods: In this study, biofilm-forming LAB were isolated from tairu and kefir. Isolate Y1 and isolate KF were selected based on their prominent inhibition against test pathogens (using spot-on-agar method and agar-well-diffusion assay) and efficient biofilm production (using tissue culture plate method). They were then identified as *Lactobacillus casei* (*L. casei*) Y1 and *Lactobacillus plantarum* (*L. plantarum*) KF, respectively using *16S rDNA* gene sequencing. The influence of incubation time, temperature and aeration on the biofilm production of *L. casei* Y1 and *L. plantarum* KF was also investigated using tissue culture plate method. The inhibitory activity of both the selected LAB biofilms was evaluated against MRSA (Institute for Medical Research code: S547) using *L. plantarum* ATCC 8014 as the reference strain.

Results: *L. casei* Y1 showed the highest reduction of MRSA biofilms, by 3.53 log at 48 h while *L. plantarum* KF records the highest reduction of 2.64 log at 36 h. In inhibiting planktonic population of MRSA (S547), both *L. casei* Y1 and *L. plantarum* KF biofilms recorded their maximum reduction of 4.13 log and 3.41 log at 24 h, respectively. Despite their inhibitory effects being time-dependent, both LAB biofilms exhibited good potential in controlling the biofilm and planktonic population of MRSA (S547).

Conclusions: The results from this study could highlight the importance of analysing biofilms of LAB to enhance their antibacterial efficacy. Preferably, these protective biofilms of LAB could also be a better alternative to control the formation of biofilms by pathogens such as MRSA.

1. Introduction

Through many advances in related studies, microbiologists worldwide are now accepting the universality of biofilms as an important phase in microbial studies. Microbial research across various fields and disciplines has embraced the biofilm-based approach instead of the conventional planktonic phase alone [1]. While this approach could be the key for tackling biofilms of pathogens, biofilms of beneficial bacteria, like lactic acid bacteria (LAB) could also be studied for their advantage as protective biofilms. Most antibacterial studies of LAB were focused on their planktonic phase and this approach is equally crucial for an early indication of potential antibacterial LAB.

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However, using the biofilm-based approach, biofilm-forming LAB could be used as protective biofilms against the persistent biofilm of pathogens. Essentially, studying the biofilm phenotype of LAB could enhance their antibacterial activity from a fresh perspective.

A fine example of biofilm-forming pathogen that is particularly difficult to eradicate is methicillin-resistant *Staphylococcus aureus* (*S. aureus*) (MRSA). The fact that *S. aureus* thrives as the normal flora of human body would definitely impact MRSA eradication. Furthermore, the genetic makeup of MRSA is highly flexible as evidenced by the vancomycin minimum inhibitory concentration (MIC) creep in its treatment [2]. This blooming pattern of MRSA infections is further exacerbated by its ability to form biofilms, which is also considered as one of its virulence mechanism [3].

In the healthcare settings, the threat of MRSA biofilms persists in chronic wound infections and indwelling medical device infections [4]. Biofilms are generally defined as surface-adhering communities of microorganisms embedded in an extracellular polymeric substance. The microbial cells within biofilms show exceptional durability against various biocides as well as the human immune response that makes their eradication much tougher [5]. Overall, the expanding rate of MRSA's resistance and their tendency to form biofilms indicate that their eradication measures should not depend solely on antibiotic discoveries [6]. Alternatively, the use of probiotic strains like LAB could be a safer eradication option as they are less likely to elevate the multi-drug resistance of MRSA [7]. In this study, the inhibitory action of selected LAB biofilms against the biofilm and planktonic population of MRSA (S547) was investigated. Also, the influence of incubation time, temperature and aeration on the biofilm production of selected LAB isolates was also evaluated.

2. Materials and methods

2.1. Isolation and identification of LAB

The LAB used in this study were isolated from tairu (traditional Indian home-made curd) and kefir. Tairu was purchased from a local wet market in Selangor, Malaysia. Kefir grains which were purchased online (kefirlady.com, Ohio, USA) were used to produce kefir in the laboratory, based on the manufacturer's instructions. Basically, 100 g of the kefir grain was placed in a sterile 500 mL beaker, before adding 500 mL of fresh cow milk (obtained from the dairy farm of University Putra Malaysia). The mixture was then incubated overnight (18 h) at room temperature. The kefir was then stirred and strained before sampling. Aseptically, 1 mL of the sample were added into 9 mL of sterile 0.85% (w/v) NaCl solution (diluent) and mixed thoroughly. Serial dilutions (10^{-1} to 10^{-7}) were performed and 0.1 mL aliquots of the appropriate dilutions were surface-plated in triplicate on de Man, Rogosa and Sharpe (MRS) agar and incubated anaerobically at 37 °C for 48 h. Bacterial colonies that developed on the plates were randomly picked and streaked on fresh MRS agar plates by dilution-streaking to obtain single colonies.

2.2. Bacterial strains and culture conditions

Lactobacillus casei (*L. casei*) Y1 and *Lactobacillus plantarum* (*L. plantarum*) KF were isolated from tairu and kefir, respectively. *L. plantarum* ATCC 8014 was used as the positive control strain. These LAB strains were cultured in MRS medium (Becton Dickinson Co., Maryland, USA). MRSA S547 was purchased from Institute for Medical Research, Malaysia and cultured in tryptic soy medium (LAB M, Lancashire, United Kingdom). Each of the isolates were inoculated in their respective agar slants for immediate use and stored as stock culture at −20 °C in 20% glycerol (Merck, Germany).

2.3. Detection of inhibitory action against MRSA (S547)

The inhibitory actions of LAB isolates against MRSA (S547) were preliminarily determined by spot-on-lawn method [8], with slight modifications. Five microlitres of 24-h grown LAB culture were spotted on the surface of MRS agar plate and incubated at 37 °C until evident growth was observed. The plates were then overlaid with MRS soft agar (0.75%) which has been seeded with MRSA (S547) (approximately, 10^6 cfu/mL). After 24 h of incubation, the plates were examined for inhibition zones.

The isolates with good inhibition against MRSA (S547) were then further tested using agar well diffusion assay (AWDA) method [9]. Cell free supernatants were prepared by centrifuging 24-h grown LAB culture (grown anaerobically) at 6 000×*g* for 10 min at 4 °C. The cell free supernatants were neutralized to pH 6.5 using 1 M NaOH and filter-sterilised using a 0.22 μm cellulose acetate filter membrane. 50 μL of cell free supernatants were then added into the pre-punched MRS agar well (diameter of 5 mm) and allowed to diffuse for 2 h at room temperature. The plates were then overlaid with MRS soft agar (0.75%) which has been seeded with MRSA (S547) (approximately, 10^6 cfu/mL). After 24 h incubation, the plates were examined for inhibition zones.

2.4. Assessment of biofilm production

All the isolates that have shown good inhibition against MRSA (S547) were then tested for their biofilm production by adherence test using modified tissue culture plate (TCP) method [10]. After 24 h of incubation, each cultures were diluted 1:100 into fresh MRS broth and grown in 96-well microtiter plates (Zhejiang Sorfa Medical Plastic Co., Ltd., Zhejiang, China) at 37 °C under aerobic conditions. Negative control wells contained MRS broth only. After 48 h, the supernatant was removed and the wells were stained with 0.1% (w/v) crystal violet. Upon incubation for 30 min at room temperature, the plates were rinsed with deionized water and the adherent cells were destained with 95% alcohol. After another 30 min, the absorbance at 595 nm was measured using microplate reader. $A_{595} > 0.1$ was designated as biofilm production, as negative controls record less than 0.1. All biofilm experiments were performed in triplicate for each isolate and repeated independently three times to minimize the variability in the absorbance measurements. Similarly, the biofilm production of MRSA (S547) was also tested.

2.5. Identification of LAB isolates

The selected LAB isolates were identified using *16S rDNA* gene sequencing. Initially, extraction of their genomic DNA was carried out. The LAB was cultured for 24 h in 5 mL of MRS broth at 37 °C and then centrifuged (12 000×*g* at 4 °C for 5 min).

After removing the supernatant, the DNA was extracted using UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA), according to the manufacturer's instructions. The amplification of the *16S rRNA* gene was done using universal primers 27F and 1429R. PCR was run using a thermocycler (Eppendorf Mastercycler Gradient Thermal Cycler, New York, USA) and the products were observed on a 1% (w/v) agarose gel in 1 × TAE buffer at 75 V for 50 min. The PCR products were then sent for purification and sequencing to NHK Bioscience. Upon manual editing, the similarity of the sequences and their match with database DNA sequences (GenBank) were carried out using the BLAST program (BLASTN) at the National Center of Biotechnology Information, NCBI. The sequences were then submitted to GenBank for accession numbers. The phylogenetic tree was constructed by the neighbour-joining method using Molecular Evolutionary Genetic Analysis 6 (MEGA version 6.0) program. *Bacillus cereus* ATCC 11778 was used as an out-group organism.

2.6. Scanning electron microscopy (SEM) analysis of LAB isolates and MRSA (S547)

Microscopy methods like SEM are usually carried out to complement the TCP assay in biofilm studies [11]. SEM was used to visualise biofilm cells of LAB isolates and MRSA (S547) based on the methods of Jalilsood *et al*. [12], with some modifications. 24-hours grown cultures were diluted to an optical density of 0.1 at 600 nm (OD_{600}) and grown in a 24-well microplate containing 12 mm diameter-glass coverslips (Deckglaser, Sondheim, Germany) at the bottom. After 48 h, the biofilm coated coverslips were fixed in 4% (w/v) glutaraldehyde for 6 h at 4 °C. Using 0.1 M sodium cacodylate buffer, the coverslips were rinsed thrice (10 min each) and then fixed again in 1% (w/v) osmium tetroxide at 4 °C. Upon washing with 0.1 M sodium cacodylate buffer for three times (10 min each), the coverslips were then dehydrated using acetone solutions [35%, 50%, 75%, and 95% (10 min each) and finally, 100% (three 15-min periods)]. After the samples were dried at critical point, and sputter-coated with gold, the biofilm images were analysed using a scanning electron microscope (JEOL, JSM-6400 Institute of Bioscience, University Putra Malaysia).

2.7. Evaluation of biofilm production at different incubation time, temperature and aeration

The influence of incubation time, temperature and aeration on biofilm production of LAB was also tested using the modified TCP method. The cultured 96-well plates were incubated at different incubation times (24 h, 48 h and 72 h), temperatures (25 °C, 30 °C, 35 °C, 40 °C and 45 °C) and aeration (aerobic, anaerobic, microaerophilic and capnophilic). The anaerobic conditions were obtained using GENbox anaer system (bioMérieux, Marcy l'Etoile, France). The microaerophilic and the capnophilic (5% of CO_2) conditions were obtained using GENbag.

2.8. Determination of inhibitory effect of LAB biofilms against MRSA (S547)

The inhibition of LAB biofilms against MRSA (S547) were investigated according to Guerrieri *et al*. [13], with slight modifications. Previously grown (24 h) LAB cultures were centrifuged at 4 000 rpm for 20 min at 4 °C. The supernatant was discarded and the remaining pellets were resuspended with fresh MRS broth. After repeating the washes for three times, the final suspensions were diluted to a concentration of approximately 10^6 CFU/mL. 24-well microtiter (Zhejiang Sorfa Medical Plastic Co., Ltd., Zhejiang, China) plates were used for the biofilm assays. Two millilitres of the LAB suspensions in MRS broth were inoculated in each well. The plates were then incubated anaerobically for 7 d at 35 °C to allow the biofilm formation and maturation to take place. Every 48 h, fresh MRS broth was used to replace 50% of the well medium. After 7 d, upon removing the suspensions, the wells were washed with 1 mL of sterile saline solution 0.85% (w/v) NaCl. Two millilitres of overnight (24 h) culture of MRSA (S547) suspension in TSB were then added to the well to yield a final bacterial count of approximately 10^6 CFU/mL and incubation was continued for 6 d.

To evaluate the viable count of planktonic bacteria, serial tenfold dilutions of the suspensions were spread on agar plates and incubated under suitable culture conditions. To evaluate the viable count of adherent bacteria in the biofilms, three wells incubated with each LAB strain were washed three times and scraped. The collected resuspension were mixed using vortex mixer before serial tenfold dilutions were spread on agar plate. The viable counts of all LAB strains during biofilm maturation and incubation with MRSA (S547) were also evaluated.

The changes in the pH of MRSA suspension when co-cultured with LAB biofilms were also recorded at regular intervals (every 24 h for 3 d). The pH reading was taken using a WalkLAB pH meter (WalkLAB Microprocessor pH meter TI9000, Bukit Merah, Singapore).

2.9. Statistical analysis

The experiments were conducted at least twice using triplicate samples to validate reproducibility. The mean values of planktonic and adherent bacteria were expressed as cfu/mL and cfu/cm^2 respectively. Differences in mean were analysed by analysis of variance (ANOVA). The statistical analysis (Duncan) was performed using SPSS Statistics 17.0 software. Statistical probability equal to or less than 0.05 was considered significant.

3. Results

3.1. Isolation and identification of LAB

Isolate Y1 and isolate KF was chosen based on their prominent inhibition against MRSA (S547) and efficient biofilm production compared to other isolates. Using *16S rDNA* gene sequencing, those two isolates were identified as *L. casei* and *L. plantarum* and designated as *L. casei* Y1 and *L. plantarum* KF, respectively. Based on the *16S rDNA* gene sequences, a phylogenetic tree was constructed using MEGA version 6.0 program. *L. casei* Y1 and *L. plantarum* KF were found to be closely related to *L. casei* ATCC 25598 and *L. plantarum* ATCC 14431 respectively (Figure 1). Both the sequences were deposited in Genbank and their assigned accession number are KR078255 (*L. casei* Y1) and KT025848 (*L. plantarum* KF), respectively.

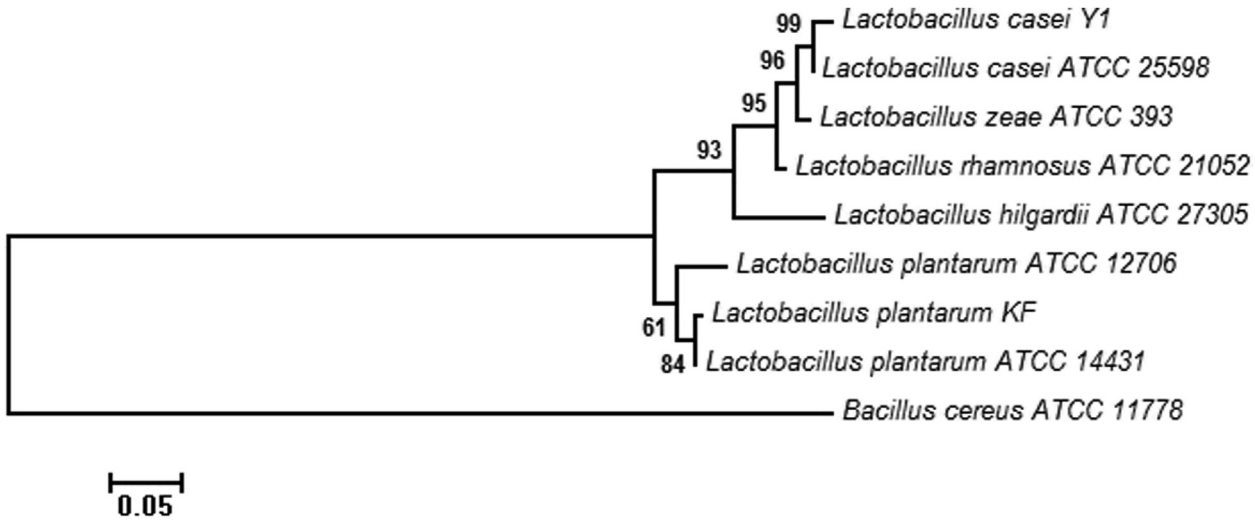

Figure 1. Phylogenetic tree constructed by using neighbour-joining method.
Bacillus cereus ATCC 11778 was used as an out-group organism. The bar indicates the number of nucleotide substitutions per site. The robustness of the tree was tested by bootstrapping with 1 000 replicates of data, and percentages are reported at the nodes (only values above 50% are reported).

3.2. Antibacterial activity against MRSA (S547)

The antibacterial activity evaluation against MRSA (S547) using the spot-on-lawn method showed that the reference strain (*L. plantarum* ATCC 8014) exhibited the largest inhibition zone [(27.0 ± 1.0) mm], compared to *L. plantarum* KF [(23.3 ± 1.5) mm] and *L. casei* Y1 [(27.0 ± 1.0) mm]. The inhibition by *L. plantarum* KF was most likely to be caused by organic acid production or hydrogen peroxide as it does not record any inhibition in the AWDA. Both reference strain [(12.0 ± 0.5) mm] and *L. casei* Y1 [(12.7 ± 1.5) mm] however, inhibited MRSA (S547) in the AWDA.

3.3. Biofilm production of LAB and MRSA (S547)

L. casei Y1 (A_{595} = 1.68 ± 0.05) recorded the highest adherence but the reading was almost similar to the control strain, *L. plantarum* ATCC 8014 (A_{595} = 1.63 ± 0.04). Following closely behind is *L. plantarum* KF (A_{595} = 1.46 ± 0.06), which was significantly lowest ($P < 0.05$). MRSA (S547) showed the highest rate of adherence (A_{595} = 1.88 ± 0.06).

3.4. SEM analysis of LAB isolates and MRSA (S547)

SEM imaging enables the biofilm structures of LAB to be observed clearly at different magnifications as shown in Figure 2a, b and c. *L. casei* Y1, *L. plantarum* KF and the reference strain showed the agglutinated biofilm rods structure. Similarly, SEM of MRSA (S547) showed their highly-agglutinated coccus structure as shown in Figure 2d.

3.5. Evaluation of biofilm production at different incubation time, temperature and aeration

Typically, both the LAB isolates tested showed similar results on all the factors tested. In the incubation time factor

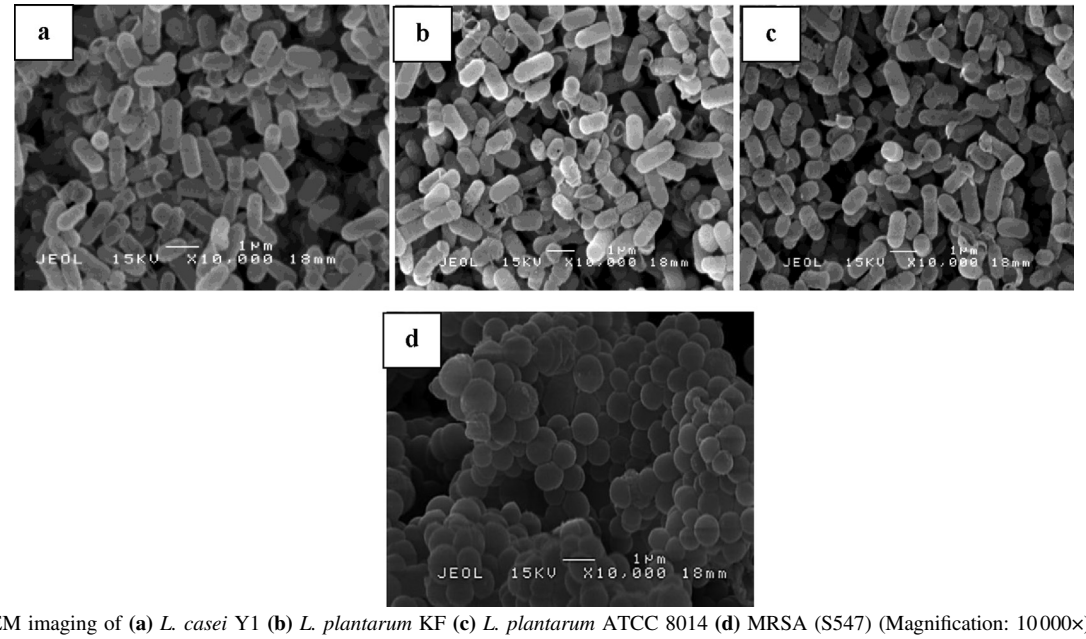

Figure 2. SEM imaging of **(a)** *L. casei* Y1 **(b)** *L. plantarum* KF **(c)** *L. plantarum* ATCC 8014 **(d)** MRSA (S547) (Magnification: 10 000×).

(Table 1) for example, it was found that biofilm production of *L. casei* Y1 (A_{595} = 1.72 ± 0.04) and *L. plantarum* KF (A_{595} = 1.51 ± 0.01) peaked at the 48th hour. Upon this, the reading taken at the 60th hour only showed a minimal difference for both the isolates (*L. casei* Y1: A_{595} = 1.89 ± 0.03; *L. plantarum* KF: A_{595} = 1.49 ± 0.02). Accordingly, statistical analysis also showed that there is no significant difference in their biofilm production at the 48th and 60th hour ($P > 0.05$). As such, further reading was ceased after the 60th hour. From the lowest biofilm production recorded on the 12th and 24th hour up to the highest on the 48th hour, a progressively increasing trend was observed between all the LAB isolates.

Both *L. casei* Y1 and *L. plantarum* KF showed quite similar results when tested on the influence of different temperatures (Table 2) on their biofilm production. The results revealed that the temperature of 35 °C is the most optimum for biofilm production of *L. casei* Y1 (A_{595} = 1.67 ± 0.06) and *L. plantarum* KF (A_{595} = 1.48 ± 0.05). In contrast, in the highest (45 °C) and lowest (25 °C) temperature, both *L. casei* Y1 and *L. plantarum* KF recorded the lowest and the second lowest biofilm production, respectively. Even though biofilm production of the isolates were reported higher at 35 °C than 30 °C, statistical analysis showed that biofilm production at these temperatures did no differ significantly ($P > 0.05$).

In terms of aeration condition (Table 3), incubation at the anaerobic atmosphere showed the largest increase in biofilm production of both *L. casei* Y1 (A_{595} = 1.62 ± 0.02) and *L. plantarum* KF (A_{595} = 1.43 ± 0.02). Coming closely as the second highest, is the microaerophilic incubation of *L. casei* Y1 (A_{595} = 1.44 ± 0.00) and *L. plantarum* KF (A_{595} = 1.28 ± 0.01). However, there was no statistically significant difference in biofilm production at these two conditions ($P > 0.05$). The least biofilm production of both the isolates was observed in the CO_2 rich condition.

Table 4
pH changes of MRSA (S547) when co-cultured with LAB.

Time	*L. casei* Y1	*L. plantarum* KF	*L. plantarum* ATCC 8014
24 h	5.04 ± 0.01[a]	5.56 ± 0.04[a]	5.18 ± 0.04[a]
48 h	4.83 ± 0.07[a]	4.16 ± 0.03[b]	4.65 ± 0.03[b]
72 h	3.63 ± 0.01[b]	4.21 ± 0.04[b]	3.37 ± 0.02[c]

Values are mean ± SD of three replicates. Means with different letters are significantly different ($P < 0.05$) from each other.

For both the isolates in general, maximum biofilm production was found to be at the 48th hour of incubation time, at the temperature of 35 °C and under anaerobic condition. From here on, all the tested LAB biofilms were cultivated under these factors to maximize their biofilm production.

3.6. Assessment of MRSA (S547) pH changes when co-cultured with LAB

At 0 h, the pH of MRSA (S547) was recorded as 5.67 ± 0.04). Upon inoculation with the 7 day-old biofilm, the pH of MRSA suspension was recorded every 24 h. Overall, *L. casei* Y1 caused a greater pH reduction in the MRSA suspension (72nd h pH: 3.63 ± 0.01) than *L. plantarum* KF (72nd h pH: 4.21 ± 0.04) and the reference strain (72nd h pH: 3.37 ± 0.02). Accordingly, statistical analysis showed significant difference in all of their pH reduction at the 72nd h ($P < 0.05$) (Table 4).

3.7. Inhibition of MRSA (S547) by LAB biofilms

The viable counts of MRSA (S547) that had been co-cultured with LAB were enumerated as planktonic (Table 5) and biofilm (Table 6) population, every 12 h for 3 d. Both the LAB biofilms

Table 1
OD_{595} at different incubation time.

LAB	12 h	24 h	36 h	48 h	60 h
L. casei Y1	0.61 ± 0.03[a]	0.78 ± 0.05[a]	1.17 ± 0.04[a]	1.72 ± 0.04[a]	1.89 ± 0.03[a]
L. plantarum KF	0.57 ± 0.06[a]	1.17 ± 0.00[b]	1.08 ± 0.03[b]	1.51 ± 0.01[b]	1.49 ± 0.02[b]
L. plantarum ATCC 8014	0.69 ± 0.01[a]	1.33 ± 0.01[b]	1.42 ± 0.04[c]	1.86 ± 0.05[a]	1.92 ± 0.03[a]

Values are mean ± SD of three replicates. Means with different letters are significantly different ($P < 0.05$) from each other.

Table 2
OD_{595} at different incubation temperatures.

LAB	25 °C	30 °C	35 °C	40 °C	45 °C
L. casei Y1	0.61 ± 0.01[a]	1.31[a] ± 0.02[a]	1.67 ± 0.06[a]	1.01 ± 0.03[a]	0.21 ± 0.03[a]
L. plantarum KF	0.58 ± 0.01[a]	1.07[b] ± 0.00[b]	1.48 ± 0.05[b]	0.85 ± 0.02[b]	0.23 ± 0.01[a]
L. plantarum ATCC 8014	0.69 ± 0.02[b]	1.33[a] ± 0.06[a]	1.77 ± 0.06[c]	1.07 ± 0.01[a]	0.19 ± 0.06[a]

Values are mean ± SD of three replicates. Means with different letters are significantly different ($P < 0.05$) from each other.

Table 3
OD_{595} at different incubation aeration condition.

LAB	Aerobic	Anaerobic	Microaerophilic	Capnophilic
L. casei Y1	1.21 ± 0.02[a]	1.62 ± 0.02[a]	1.44 ± 0.00[a]	1.11 ± 0.03[a]
L. plantarum KF	1.13 ± 0.04[b]	1.43 ± 0.02[b]	1.28 ± 0.01[b]	1.01 ± 0.00[b]
L. plantarum ATCC 8014	1.29 ± 0.00[a]	1.73 ± 0.01[a]	1.61 ± 0.01[c]	1.73 ± 0.05[b]

Values are mean ± SD of three replicates. Means with different letters are significantly different ($P < 0.05$) from each other.

Table 5
Inhibitory effect of LAB biofilms against the planktonic population of the MRSA (S547) (log10 cfu/mL).

MRSA (S547)/ LAB biofilms	0 h	12 h	24 h	36 h	48 h	60 h	72 h	84 h
MRSA (S547)	6.00 ± 0.15[a]	10.61 ± 0.35[a]	11.51 ± 0.36[a]	11.40 ± 0.32[a]	11.46 ± 0.23[a]	11.89 ± 0.63[a]	11.54 ± 0.22[a]	11.68 ± 0.26[a]
L. casei Y1	6.00 ± 0.06[a]	7.91 ± 0.16[b]	7.38 ± 0.64[b]	7.79 ± 0.15[b]	8.27 ± 0.27[b]	8.91 ± 0.11[b]	10.81 ± 0.31[a]	11.01 ± 0.31[a]
L. plantarum KF	6.00 ± 0.57[a]	8.18 ± 0.56[b]	8.10 ± 0.18[c]	8.75 ± 0.56[b]	9.20 ± 0.45[c]	9.99 ± 0.46[b]	11.38 ± 0.12[a]	11.34 ± 0.13[a]
L. plantarum ATCC 8014	6.00 ± 0.13[a]	7.53 ± 0.33[b]	8.56 ± 0.25[c]	8.88 ± 0.57[b]	9.40 ± 0.11[c]	9.53 ± 0.55[b]	10.38 ± 0.23[a]	11.35 ± 0.20[a]

Values are mean ± SD of three replicates. Means with different letters are significantly different ($P < 0.05$) from each other.

Table 6
Inhibitory effect of LAB biofilms against the biofilm population of the MRSA (S547) (log10 cfu/cm^2).

MRSA (S547)/LAB biofilms	12 h	24 h	36 h	48 h	60 h	72 h	84 h
MRSA (S547)	7.25 ± 0.36[a]	7.33 ± 0.24[a]	8.35 ± 0.38[a]	8.09 ± 0.25[a]	8.23 ± 0.42[a]	7.69 ± 0.26[a]	7.60 ± 0.15[a]
L. casei Y1	5.84 ± 0.22[b]	5.40 ± 0.31[b]	6.20 ± 0.52[b]	4.55 ± 0.23[b]	5.39 ± 0.19[b]	6.36 ± 0.52[b]	7.89 ± 0.36[a]
L. plantarum KF	6.06 ± 0.43[b]	5.34 ± 0.62[b]	5.70 ± 0.35[c]	5.77 ± 0.46[c]	6.49 ± 0.37[b]	7.00 ± 0.13[b]	7.34 ± 0.14[a]
L. plantarum ATCC 8014	5.64 ± 0.22[b]	5.16 ± 0.37[b]	5.75 ± 0.33[c]	5.79 ± 0.21[c]	5.83 ± 0.46[b]	7.05 ± 0.34[a]	8.38 ± 0.38[b]

Values are mean ± SD of three replicates. Means with different letters are statistically different ($P < 0.05$) from each other.

showed significant log reduction of the adherent and planktonic population of MRSA during the duration of 3 days. Maximum reduction of MRSA (S547) biofilm was recorded by *L. casei* Y1 and *L. plantarum* KF on the 48th hour (reduced by 3.53 log ± 0.46) and 36th hour (reduced by 2.65 log ± 0.33), respectively. *L. casei* Y1 showed slightly better inhibitory potential against MRSA (S547) at the end of 72nd h by reducing the planktonic population by 0.72 log ± 0.43 and the adherent by 1.33 log ± 0.52. In contrast, *L. plantarum* KF caused a reduction of 0.16 log ± 0.25 and 0.69 log ± 0.13 in the planktonic and adherent population, respectively.

The reference strain, *L. plantarum* ATCC 8014 also showed significant log reduction of MRSA (S547) planktonic and biofilms. The MRSA (S547) planktonic population reduction by the reference strain and *L. casei* Y1 was almost similar, although reduction at certain hours was statistically different. Their maximum reduction was found be in the early hours whereby the former recorded the highest reduction on the 12th hour (reduced by 3.07 log ± 0.33), while the latter showed maximum reduction on the 24th hour (reduced by 4.13 log ± 0.16). Similarly, *L. casei* Y1 reduced MRSA biofilms the most on the 48th hour by 3.53 log ± 0.46 compared to the latter which only showed the maximum reduction of 2.59 log ± 0.38 on the 36th hour.

Overall, the log reduction range of MRSA biofilms did not vary greatly between the LAB candidates. The reference strain, *L. plantarum* ATCC 8014, showed a steady reduction (log reduction range: 2.00 log) of MRSA biofilms compared to both *L. casei* Y1 (log reduction range: 2.20 log) and *L. plantarum* KF (log reduction range: 2.04 log). The highest reduction of MRSA biofilms was found to be on the 12th and 24th hour but there was no significant difference between these 2 h ($P > 0.05$). Similarly, there were no significant differences found in their reduction at the 60th and 72nd h (biofilm reduction) and at 72nd and 84th hour (planktonic reduction) by the LAB biofilms ($P > 0.05$). As such, the reading was discontinued after 84 h for both the MRSA (S547) phases.

4. Discussion

The antibacterial properties of LAB are widely studied and their potential benefits have been acknowledged as evidenced by their application in industry. In this study, to determine the antagonistic activities of the LAB isolates, spot-on-agar method and AWDA were employed. The antibacterial inhibition of LAB isolates against MRSA (S547) is the primary screening for potential LAB selection. All the LAB isolates that exhibited antagonistic activities against MRSA (S547) in the spot-on-agar test were then further screened using their supernatants through the AWDA. The neutralisation and anaerobic incubation steps in AWDA exclude organic acids and hydrogen peroxide as the antagonistic cause, respectively.

Generally, biofilm formation is considered as a naturally occurring phase in microbial life [14]. Ultimately, this theory could raise an important question, 'does all bacteria are capable of producing biofilms?' The answer to that question is highly debatable but as a thumb rule, it is a fact that different microbes have different biofilm-forming capacity [15]. Similarly, all the tested LAB isolates and MRSA (S547) also have showed variation in their adherence capacity. However, it can be generally concluded that they are all strong-biofilm producers as evidenced by the SEM analysis. Many studies have highlighted the fact that strain variation has an impact on their respective biofilm production [16,17]. Based on the adherence result, it is possible that this particular strain of MRSA used in this study is a good biofilm-producer. The adherence result which corresponds to the strength of the biofilm production is the second screening for potential LAB isolates. As such, based on the biofilm production and antibacterial activity against MRSA (S547), isolate Y1 and KF 1B were selected as the best representative of LAB isolates. Using *16S rDNA* gene sequencing, they were then identified and designated as *L. casei* Y1 and *L. plantarum* KF.

It is important to note here that, with a few exceptions, most studies on quantification of biofilm formation are focused on pathogens. This means the notion of studying biofilm production of beneficial bacteria like LAB as another antibacterial property is still underdeveloped. In this study, the biofilm production of LAB isolates was quantified using a modified TCP assay. Other than TCP, other common biofilm detection methods are Tube method and Congo Red Agar method. From their study, it is found the TCP assay to be more quantitative and reliable method for biofilm detection compared to Tube method and Congo Red Agar method

[18]. Furthermore, the TCP assay was recommended as a general screening procedure in laboratories for detection of biofilm producing microorganisms. Additionally, the specific use of 96-well microtiter plate in biofilm studies is also credited for their simplicity and low-cost [19].

SEM was also conducted to view the adherent cells of *L. casei* Y1 and *L. plantarum* KF. Generally in biofilm studies, microscopy methods like SEM are known to complement the TCP assay [11]. Biofilm imaging by SEM is also largely credited for their high resolution and magnification [20]. Both *L. casei* Y1 and *L. plantarum* KF showed highly agglutinated rods which were clumped upon each other, respectively. This finding is also in agreement with the shown positive control strains in the study by Jalilsood *et al*. [12], which exhibits similar stacked and adherent rods. The adherence of these biofilm cells are actually caused by their production of extracellular polymeric substances [14].

The agglutination analysis of MRSA (S547) was also conducted using SEM to verify that this particular strain of MRSA is indeed a strong biofilm producer. The finding can be compared with the work of Milanov *et al*. [21] that also used SEM to capture the microscopic images of weak and strong biofilm producers in different strains of *S. aureus*. The results revealed the highly agglutinated coccus of MRSA (S547) which is similar to the positive control strain and distinguished from the weakly adherent negative control strain [21]. The SEM analysis proved that MRSA (S547) is a strong biofilm producer and their inhibition by the tested LAB biofilms could highlight the potential inhibitory effect for other biofilm-producing MRSA strains.

According to Gomes *et al*. [19], biofilm assay using 96-well plates are well adapted for the evaluation of different biofilm parameters. Corresponding to this, several factors that could influence the biofilm production of *L. casei* Y1 and *L. plantarum* KF was also studied. The biofilm production was found to be at the peak at 48 h for both of the isolates, and no significant increase was detected upon that. This could mean that both isolates utilised maximum resources for their respective biofilm formation at 48 h. As such, the 2-day old biofilm of *L. casei* Y1 and *L. plantarum* KF could be said as a fully-matured biofilm. At this point, the biofilms cells are likely to have completed their matured development stages and subsequently shifting towards their detachment process [5].

Besides incubation time, environmental factors like temperature and O_2 could also have an effect on the development of biofilm [22]. Accordingly, it was found that the temperature of 35 °C is the most optimum for biofilm production of *L. casei* Y1 and *L. plantarum* KF. In contrast, their biofilm production was found to be substantially low at other temperatures like 25 °C and 45 °C. There are various physiological and biochemical processes (including biofilm formation) in the microbial system which is governed by enzymatic reaction, which in turn, is correlated with temperature. For example, non-optimal temperature could impact the bacterial proliferation due their lower rate of enzymatic reaction. Thus, this explains the indirect influence of temperature on the formation of biofilms by bacteria [23]. Furthermore, it is known that temperature could influence the production of extracellular polymeric substances [24]. Ultimately, this could also explain the link between temperature and their biofilm formation.

The influence of aeration condition on the biofilm production was also investigated. It was found that both biofilm production of *L. casei* Y1 and *L. plantarum* KF was more favourable in anaerobic and microaerophilic condition and there was no statistical significant difference between them. Basically, LAB are facultative anaerobic bacteria with a fermentative metabolism. The main source of their metabolic energy is provided in the form of adenosine triphosphate by substrate level phosphorylation [25]. However, despite being aerotolerant, it is said that the fermentation of LAB is highly affected by the presence of oxygen [26]. As such, it is possible that both *L. casei* Y1 and *L. plantarum* KF naturally flourish in zero to minimal oxygen concentration and form biofilms more readily than in aerobic conditions. The lack of literature in the genetic study of LAB biofilms limits the review on aeration and biofilm development. However, it is worth mentioning that there is a possibility that anaerobic conditions could promote biofilm production in certain microorganisms. A fine example would be *Staphylococcus epidermidis*. In *Staphylococcus epidermidis*, it was found that anaerobic condition activates a higher expression of genes that ultimately leads to production of enzymes involved in the synthesis of PIA, a vital component of biofilm [27].

In this study, the antagonistic activity of biofilms by *L. casei* Y1 and *L. plantarum* KF against MRSA (S547) was studied. The antibacterial studies of LAB are largely focused on the inhibition of food-borne pathogens. Even the documented interaction between LAB and *S. aureus* were reported in relation to the food industry [28,29]. In contrast, the inhibition studies of LAB against MRSA are relatively minimal. To the author's knowledge, as to date, there is no report on the use of LAB biofilms to inhibit the planktonic and biofilm population of MRSA. As such, the study of *L. casei* Y1 and *L. plantarum* KF biofilms could open up a whole new possibility in tackling the biofilms of MRSA.

Prior to MRSA inhibition, the LAB biofilms were incubated for 7 days for their adhesion and maturation to take place on the 96-well bottoms [13]. In terms of pH, *L. casei* Y1 caused the greatest pH reduction in the MRSA suspension [(3.63 ± 0.01)]. The pH range for the growth of *S. aureus* is 4.2–9.3 [30]. Organic acid production is a general trait of most LAB [31] and as such pH reduction of MRSA suspension is not uncommon.

Overall, the biofilms of *L. casei* Y1 and *L. plantarum* KF caused a significant reduction in the biofilm and planktonic population of MRSA, especially in the first 48 h. *L. casei* Y1 exhibited a slightly higher inhibition than *L. plantarum* KF and the reference strain, *L. plantarum* ATCC 8014. This could be attributed to their inhibition mechanism through organic acid production. This claim is supported by the finding that *L. casei* Y1 biofilms caused a significantly lower pH in the MRSA suspension than *L. plantarum* KF and the reference strain. Different LAB strains may utilise different metabolism pathway and their resultant metabolites could also vary [32]. As such, it is possible that *L. casei* Y1 could have produced a larger amount of organic acids than *L. plantarum* KF and the reference strain. Together with other common inhibitory metabolites, their reduction could also be the resultant effect of successful competitive-exclusion effect.

Despite earlier reduction by LAB biofilms, MRSA (S547) biofilms continued to persist as viable cell counts did not reveal any utmost reduction thereafter. Their persistent biofilm formation could be attributed to quorum sensing, a coordinated cell-to-cell communication mechanism. It is said that formation of biofilms by microbes and quorum sensing are interdependent

[33]. Specifically, the quorum sensing of *S. aureus* is associated with the accessory gene regulator (*agr*) system. Together with the *agr* phenotype, environmental factors could also affect the quorum response that could ultimately trigger biofilm production [34]. As such, it is possible that these conditions are favourable for the biofilm formation of MRSA (S547) during the conducted experiment, thus causing their reduction to be merely time-dependent. Even though the inhibition by LAB biofilms is time-dependent, their initial success in controlling the planktonic and biofilm population of MRSA (S547) could also be attributed to quorum sensing mechanism. This cell-to-cell signaling interaction could have caused the cooperative effort against the possible threats of nutrient competition and defense against MRSA [13]. Similarly, quorum sensing which also regulates antimicrobial peptides production in LAB [35] could have contributed to the successful inhibitory effect of *L. casei* Y1.

More often than not, antibacterial activities of LAB are associated with foodborne pathogen than nosocomial pathogen like MRSA. However, the interaction between LAB and MRSA is not exactly unheard-of. Specifically, Karska-Wysocki *et al*. [36] reported a high antibacterial efficiency of LAB in inhibiting MRSA. Interestingly, there is even a patent filed for the use of lactic acid bacterium strains to inhibit the growth of MRSA [37]. As mentioned before, to the author's knowledge, up to this date, there is no existing data on the use of LAB biofilms for inhibition of MRSA. Essentially, the use of LAB as protective biofilms is still new. Few studies that have used protective LAB biofilms only targeted their use against foodborne pathogens [12,13].

According to Sikorska *et al*. [38], the inhibitive interaction between LAB and *S. aureus* and/or MRSA can be attributed by two factors that could work in combination or singularly. Those factors are the competitive exclusion for nutrients and attachment sites and the production of inhibitory substances. These two factors could sum up the inhibitory actions of *L. casei* Y1, *L. plantarum* KF, and the reference strain (*L. plantarum* ATCC 8014).

Controlling MRSA infections is a daunting task that needs to be solved without adding to their growing resistance towards antibiotics [6]. In that sense, LAB comply the requirement as a potential anti-MRSA agent as they do not increase the risk of multidrug-resistance. The study was focused on the inhibition of the LAB biofilms against MRSA (S547), whereby the planktonic and biofilm population of the latter was compared on a 12-h interval for 84 h. Both *L. casei* Y1 and *L. plantarum* KF showed significant reduction of biofilm and planktonic population of MRSA (S547), with the former showing slightly better inhibitory activity. Their reduction however, was time-dependent as towards the end, MRSA (S547) continued to persist in the viable and biofilms phase, despite earlier reduction. To an extent, the reference strain (*L. plantarum* ATCC 8014) also emulated the reduction pattern of the isolated LAB. This may suggest the reproducibility potential of this study, provided that the potential LAB isolates comply with the screening procedures mentioned. The TCP method has been cited by many studies as the most relevant and reproducible method for early detection of biofilm production. Their limitations however, lie in the intensive labour work and slow detection of results. Furthermore, the results only reveal the disinfection of MRSA planktonic and biofilm up to a certain point of time, rather than total eradication. Having said that, the results could still serve as an initial cue that reflects on the capacity of the disinfectant (in this case, LAB biofilms) for further investigation that could enhance them.

Conflict of interest statement

We declare that we have no conflict of interest.

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