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Antimicrobial and physicochemical characterization of *Lactobacillus brevis* biofilms as biopreservative agents

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ABSTRACT

Aims: Lactic acid bacteria (LAB) biofilms constitute one of the most remarkable breakthroughs in the field of food biopreservatives and can be employed to prevent foodborne disease. The purposes of this study were to investigate the efficacy of inhibitory LAB biofilms against foodborne pathogens and evaluate their tolerance to acidic pH and bile salts, as well as their physicochemical properties.
Methodology and results: Four strains of *Lactobacillus brevis* biofilms isolated from kimchi showed antipathogenic activity to the bacteria *Staphylococcus aureus* FNCC 0049 and *Escherichia coli* FNCC 0091. These biofilms were also tolerant to pH 2.5, 0.3% bile salt and strong adhesion. Two of the four *L. brevis* biofilms (*L. brevis* biofilm KA2 and KB1) produced the highest inhibitory activity against both pathogenic bacterial indicators, tolerance to acidic pH and bile salts, and the strongest adhesion. In addition, based on Scanning Electron Microscope-Energy Dispersion X-ray Spectroscopy (SEM-EDS) analysis, both biofilm strains had a smooth surface texture; the cell morphology was rod-shaped and consisted of several elements such as carbon, oxygen and nitrogen, which was built up of extracellular polymeric substances (EPS).
Conclusion, significance and impact of study: The presence of EPS as a constituent of LAB biofilms influenced their survival abilities in an acidic pH and bile salt environment. As a result, the characteristics of *L. brevis* biofilm KA2 and KB1 made them excellent candidates for use as antimicrobial packaging systems in food biopreservative applications.

Keywords: Foodborne disease, pathogenic bacteria, adhesion, SEM-EDS, rod-shaped

INTRODUCTION

Foods contaminated with pathogenic microbes have recently caused many cases of foodborne disease in Indonesia. Based on a Badan Pengawas Obat dan Makanan (BPOM) report (2019), approximately 61% of the microbes detected in several food products were pathogenic. Two bacterial species, *Escherichia coli* and *Staphylococcus aureus* have been confirmed as the dominant pathogenic microorganisms in food products (Bundidamorn *et al*., 2018; Incili *et al*., 2019; Qi *et al*., 2021; Wang *et al*., 2021b). These infections cause several poisoning symptoms, such as diarrhea, fever, nausea, vomiting and occasionally even death. These symptoms are associated with virulence factors in both pathogenic bacterial species.

Escherichia coli comprises five virulence classes, including enterohemorrhagic *E. coli*, enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC) and enteroinvasive *E. coli* (EIEC) (Mirhoseini *et al*., 2018; Ali *et al*., 2020), whereas *S. aureus* encompasses only three, including leucocidin, toxic shock syndrome toxin-1 (TSST-1) and Staphylococcal enterotoxins (SEA, SEB, SEC, SED, SEE, SEG, SHE, SEI). Due to the different modes of virulence, the two pathogens cause numerous foodborne disease symptoms (Fang *et al*., 2020; Zhao *et al.*, 2020).

Chemical preservatives can be applied to lower the risk of foodborne disease; however, such preservatives cause major health problems such as allergy, asthma, hypersensitivity, hyperactivity, neurological damage and cancer (Abd-Elhakim *et al*., 2020; Balram *et al*., 2021; Huang *et al*., 2021). Therefore, biopreservatives based on lactic acid bacteria (LAB) biofilms have gotten a lot of interest in the last few years.

In general, LAB Biofilms are recognized as safe by the US-FDA and are considered an alternative biopreservative with beneficial properties. These biofilms

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are not toxic to eukaryotic cells and only inhibit spoilage microbes and food pathogens in small concentrations. In addition, they can be used as probiotics that are tolerant to acidic pH and bile salts; consequently, they can colonize the guts of animals and humans (Mohamad *et al*., 2020; Ashaolu and Fernández-Tomé, 2021). The use of LAB biofilms as food preservatives also does not alter food quality (Ghanbari *et al*., 2013; Johansen and Jespersen, 2017).

Biofilms are microorganism communities that are spatially structured in an extracellular polymeric substance (EPS) that is mostly made up of exopolysaccharides, lipids, proteins and extracellular DNA (eDNA) (Muruzović *et al*., 2018). The formation of biofilms can be seen as a mode of growth that protects cells, thus enabling them to thrive in unfavorable surroundings such as the presence of antagonistic microorganisms (Aoudia *et al*., 2016; Retnaningrum and Wilopo, 2016; Retnaningrum and Wilopo, 2017; Sun *et al*., 2020). Hence, most LAB biofilms exhibit high inhibitory effects against both spoilage and pathogenic microorganisms (Kumar *et al*., 2017; Sasikumar *et al*., 2017; Trabelsi *et al*., 2017; Rani *et al*., 2018).

The application of LAB biofilms in food preservatives is highly dependent on their physicochemical characteristics, which are affected by the type of LAB, availability of nutrients and environmental conditions, including pH and temperature (Margalho *et al*., 2021). Previous research has isolated and identified four strains of *Lactobacillus brevis* from local kimchi in Indonesia, which produced biofilm in de Man Rogosa Sharpe (MRS) broth medium (Sapalina and Retnaningrum, 2020). Therefore, to develop a green food biopreservative, it is important to further evaluate the inhibitory effect of biofilms against foodborne pathogens, to analyze their tolerance to acidic pH and bile salt and to investigate their physicochemical characteristics.

MATERIALS AND METHODS

LAB biofilms, pathogenic bacteria indicator and medium

Four *L. brevis* strains (*L. brevis* KA2, *L. brevis* KA5, L. *brevis* KB1 and *L. brevis* KC4) used in this research were isolated from local kimchi which purchased from an Indonesian market (Sapalina and Retnaningrum, 2020). Routinely, these strains were cultured in de MRS broth medium (Himedia) at pH 6.5, 37 °C. The formation of biofilms was investigated by culturing the bacteria in nutrient broth (NB) medium (Himedia) at 37 °C, pH 6.5 containing zeolites for biofilm attachment. *Staphylococcus aureus* FNCC 0047 and *Escherichia coli* FNCC 0091 as a foodborne disease indicator (Food and Nutrition Culture Collection, 2020) were cultivated at 37 °C in NB medium (Himedia) at pH 6.5.

Lactobacillus brevis strains and growth conditions

The accession numbers for the 16S rDNA sequences of four *L. brevis* strains (*L. brevis* KA2, *L. brevis* KA5, *L. brevis* KB1 and *L. brevis* KC4) are OK083725, OK086760, OK083727 and OK086762, respectively (https://www.ncbi.nlm.nih.gov). On a phylogenetic tree constructed with several comparative sequences of biofilm-producing LAB species, the four isolates were grouped and closely related to strains of *L. brevis* (Sapalina and Retnaningrum, 2020). These strains were cultivated in MRS broth and incubated for 24 h at 37 °C.

Measurement of the inhibitory effect of *L. brevis* biofilms against pathogenic bacteria

The biofilms of four LAB were evaluated for their ability to stop the spread of two pathogenic bacteria (*S. aureus* FNCC 0047 and *E. coli* FNCC 0091) by following the method of Gómez *et al*. (2016) with minor modifications. A 1 µL bacterial biofilm with a number of 10^7-10^8 CFU/mL, which was equivalent to a bacterial turbidity of 0.25 ± 0.05 was added to a 96-well microplate (Iwaki AGC Techno Glass Co., Ltd., Japan) containing 100 µL MRS broth and cultured at 30 °C for 48 h. After formation of biofilms at the bottom of the wells, the plate was washed with 0.85% NaCl (w/v) to remove planktonic cells. A 100 µL aliquot of a pathogenic bacterial suspension with a number of 10^7-10^8 CFU/mL, which was equivalent to a bacterial turbidity of 0.25 ± 0.05 was placed into the well and incubated for 72 h at 30 °C. Every 24 h, half of the medium in the well was replaced with new medium. To evaluate the number of viable cells of pathogenic bacteria that survived inhibition by LAB biofilm, their pathogenic bacterial culture were transferred into a sterile microtube, then serially diluted tenfold and poured on an agar plate containing nutrient agar (NA). After incubation for 24 h at 30 °C, the pathogenic bacterial colonies were observed and their number determined and expressed as CFU/mL.

Assay for tolerance of *L. brevis* biofilms to acidic pH and bile salts

Tolerance of *L. brevis* biofilms to acidic pH and bile salts was determined by measuring their survival at pH 2.5 and 0.3% (w/v) bile salt when incubated for 4 h at 37 °C (Tokatlı *et al*., 2015). Previously, four strains of *L. brevis* biofilms were cultured in MRS broth and incubated for 24 h at 37 °C. The cell pellet was obtained by centrifuging the culture samples at 6000× *g* for 15 min, suspended in sterile phosphate buffered saline containing (g/L) 9 $Na_2HPO_4 \cdot 2H_2O$, 9 NaCl and 1.5 KH_2PO_4, and diluted to a concentration of 10^9 CFU/mL, which was determined using the plate count method. To assay the tolerance of *L. brevis* biofilms to acidic pH, the suspensions were incubated at pH 2.5 and 37 °C for 4 h, whereas to assay the resistance of *L. brevis* biofilms to bile salt, 0.3% (w/v) bile salt was added to the suspensions and the mixtures were then incubated for 4 h at 37 °C. Finally, the

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percentage of bacterial cell survival was determined according to equation 1.

% survival = Nt/N0 × 100 …….. (1)

where N0: The number of viable bacteria biofilm at 0 h (CFU/mL); Nt: The number of viable bacteria biofilm at 4 h (CFU/mL)

Analysis of physicochemical characters of *L. brevis* biofilms

The physicochemical characters of LAB biofilms were investigated based on their adhesion, texture and chemical composition. The textures and chemical compositions were analyzed by Scanning Electron Microscope-Energy Dispersion X-ray Spectroscopy (SEM-EDS), whereas the adhesion of LAB biofilms was investigated by observing the adhesion strength of the biofilm on the zeolite surface.

The bacterial culture suspension was previously prepared by transferring the colonies from agar plates into 9 mL of MRS broth medium in 25 mL Erlenmeyer flasks and incubating them at 37 °C for 24 h. These initial cultures were subsequently diluted into fresh sterile MRS broth media at a bacterial number of 10^7-10^8 CFU/mL, which was equivalent to a bacterial turbidity of 0.25 ± 0.05 on a 600 nm wavelength spectrophotometer measurement. A bacterial suspension of 3 mL was then inoculated into 100 mL fresh sterile MRS broth medium containing 10 g of zeolite with a diameter of 0.4 to 0.6 cm (Retnaningrum and Wilopo, 2017). After incubation for 48 h at 37 °C, biofilm that attached on the zeolite surfaces was observed in an adhesion test by vortex treatment (Genie 2, Scientific Industries) at speeds of 600, 1200 and 1800 rpm for 20 sec (Siradje *et al*., 2017). Subsequently, optical density was measured using a spectrophotometer (Thermo Scientific) with a 600 nm wavelength. The percentage of viable cell bacteria released after vortex treatments was determined by the pour plate method in NA.

Scanning electron microscope-energy dispersion X-ray spectroscopy (SEM-EDS) analysis of LAB biofilms

The texture and chemical composition of *L. brevis* LAB biofilm that produced the highest inhibitory effect against pathogenic bacteria indicators, tolerance to acidic pH and bile salts, and strongest adhesion was further observed using Scanning Electron Microscope-Energy Dispersion X-ray Spectroscopy (SEM-EDS) (JEOL JSM-T300). A 3 mL aliquot of the selected bacterial suspension (10^7-10^8 CFU/mL) was inoculated into an Erlenmeyer flask containing 30 mL MRS broth medium and a glass cover slip. After 48 h of incubation at 37 °C, biofilm formed and attached itself to the surface of the glass cover slip. The broth was then removed, and the biofilms on the glass cover slips were rinsed twice in 100 mM phosphate buffer (pH 7.4) for 1 h at room temperature. The biofilms were then fixed for 24 h at room temperature in 0.5 mL 2.5% glutaraldehyde and 100 mM phosphate buffer (pH 7.4). The fixed biofilms were subsequently dehydrated using a graded ethanol series, which included 30 min at 50%, 70%, 80%, 90%, 95% and 100% ethanol, followed by 3 h in t-butyl alcohol. The dehydrated biofilms were then sputter-coated with gold/palladium after freeze drying. The biofilm was then attached to the cover slip and dried for 30 min. The coating process was carried out using platinum elements. The surface texture of the biofilm was observed by SEM to determine its morphological and topographic characteristics. The elements or chemical characteristics of the biofilm were analyzed by EDS.

Statistical analysis

All of the data is given as a three-replicate mean and standard deviation. Analysis of variance was used to establish treatment effects and Duncan's multiple range test was used to determine significant differences. Statistical significance was defined as a difference of $p<0.05$.

RESULTS AND DISCUSSION

Inhibitory effect of *L. brevis* biofilms against pathogenic bacteria

The inhibitory effect and superiority of LAB biofilms against pathogenic bacteria are an important characteristic for the development of biopreservation agents. The inhibitory effect of *L. brevis* biofilms against *S. aureus* can be shown from the growth profile of *S. aureus* in the exposure of their biofilms compared with a control (without addition of *L. brevis* biofilms) (Figure 1). The growth of *S. aureus* exposed to *L. brevis* biofilm was lower than that of the control, showing that *L. brevis* biofilms could inhibit the growth of *S. aureus*. All biofilms of *L. brevis* strains significantly reduced pathogenic bacterial growth ($p<0.05$). In addition, each biofilm had a substantially different pathogenic bacterial growth inhibition value, which was heavily affected by the strain type and length of exposure (Margalho *et al*., 2021). Growth of *S. aureus* was significantly reduced after 72 h of exposure to *L. brevis* biofilms. *Lactobacillus brevis* KA2 biofilm induced the greatest inhibition of growth of that pathogenic bacterium of all strains of biofilm, as much as 2.29 ± 0.056 log CFU/mL (78.3%). Growth of the pathogenic bacterium *E. coli* was also strongly inhibited after 72 h of exposure to the *L. brevis* biofilms, as shown in Figure 2. The pathogenic bacteria had the highest inhibitory value of 3.49 ± 0.049 log CFU/mL (66.51%) when exposed to *L. brevis* KB1 biofilm (Solichah and Retnaningrum, 2020).

The results of growth inhibition of the pathogenic bacteria *S. aureus* and *E. coli* after exposure to LAB biofilms were concordant with the results of previous investigations (Gómez *et al*., 2016; Kumar *et al*., 2017). Several other researchers also reported similar results that *L. brevis* biofilm can suppress the growth of *S. aureus* more strongly than that of *E. coli* (Sadeghi *et al*.,

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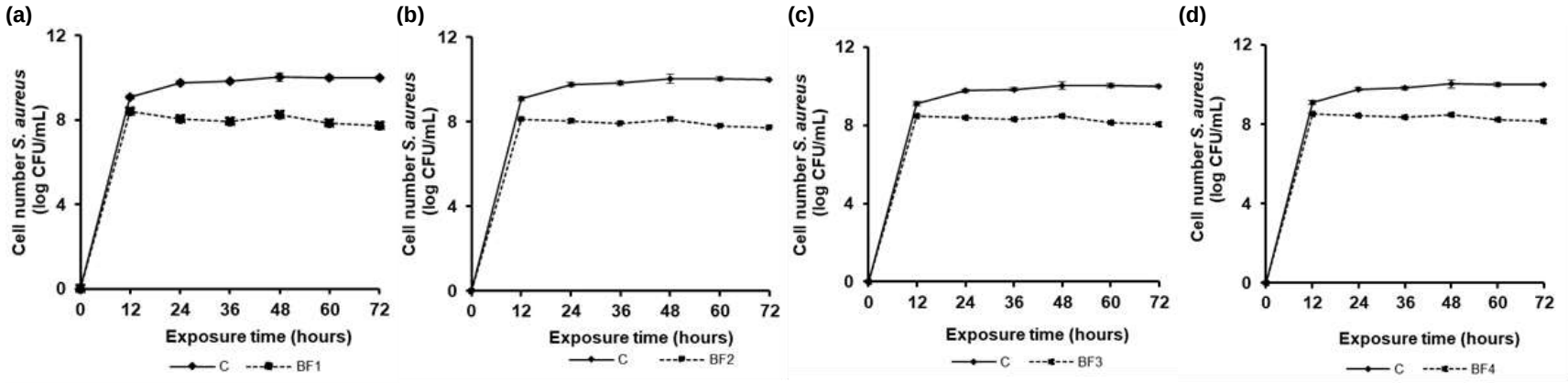

Figure 1: The growth of *S. aureus* during periods of biofilm exposure. (a) *L. brevis* KA2, (b) *L. brevis* KA5, (c) *L. brevis* KB1 and (d) *L. brevis* KC4. The treatments BF1, BF2, BF3 and BF4 represent biofilm exposure of *L. brevis* KA2, *L. brevis* KA5, *L. brevis* KB1 and *L. brevis* KC4, whereas the control (C) was without biofilm exposure.

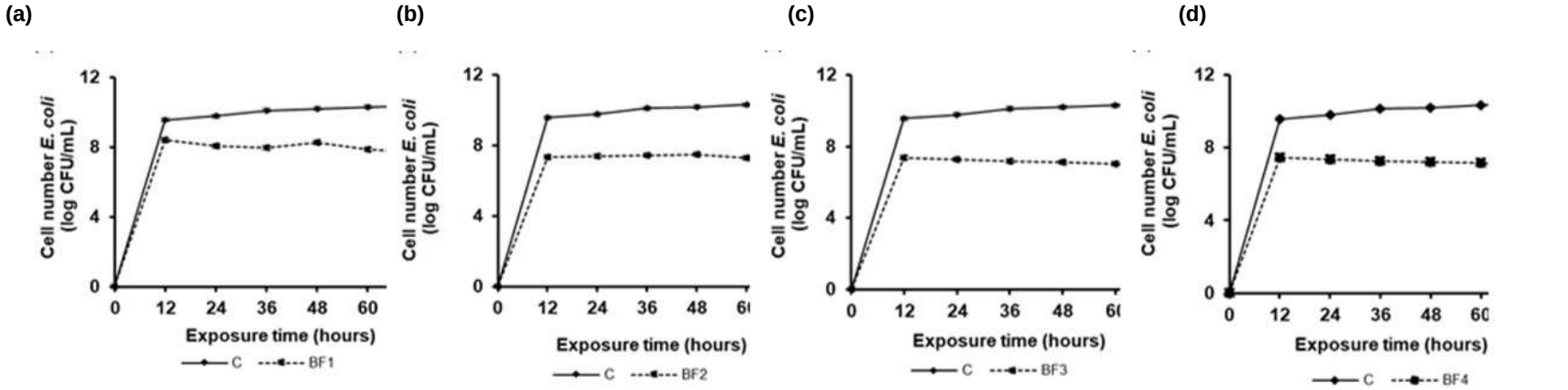

Figure 2: The growth of *E. coli* during periods of biofilm exposure. (a) *L. brevis* KA2, (b) *L. brevis* KA5, (c) *L. brevis* KB1 and (d) *L. brevis* KC4. The treatments BF1, BF2, BF3 and BF4 represent biofilm exposure of *L. brevis* KA2, *L. brevis* KA5, *L. brevis* KB1 and *L. brevis* KC4, whereas the control (C) was without biofilm exposure.

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Table 1: Percentage survival of *L. brevis* biofilms in pH 2.5 incubated at 37 °C for 4 h.

L. brevis biofilms strains	Viable cells (10^9 CFU/mL)		Survival (%) (4 h/0 h)
	0 h	4 h	
KA2	3.33 ± 0.006	3.15 ± 0.003	94.59[b]
KA5	2.70 ± 0.002	2.34 ± 0.005	86.67[c]
KB1	2.34 ± 0.001	2.26 ± 0.004	96.58[a]
KC4	2.90 ± 0.001	2.32 ± 0.001	80.00[d]

Values are expressed as means ± SD. The means in the same column with different superscript characters are substantially significant different (p<0.05).

Table 2: Percentage survival of *L. brevis* biofilms in 0.3% bile salt incubated at 37 °C for 4 h.

L. brevis biofilms strains	Viable cells (10^9 CFU/mL)		Survival (%) (4 h/0 h)
	0 h	4 h	
KA2	4.28 ± 0.002	3.92 ± 0.003	91.59[b]
KA5	3.26 ± 0.004	2.64 ± 0.001	80.98[c]
KB1	3.92 ± 0.001	3.71 ± 0.003	94.64[a]
KC4	3.65 ± 0.002	2.73 ± 0.002	74.79[d]

Values are expressed as means ± SD. The means in the same column with different superscript characters are substantially significant different (p<0.05).

2019; Hojjati *et al.*, 2020). Noohi *et al.* (2021) found contrasting results, *L. brevis* biofilm was able to inhibit *E. coli* growth more effectively than that of *S. aureus*. Besides being able to inhibit *S. aureus* and *E. coli*, *L. brevis* biofilm has been observed to inhibit pathogenic bacteria from various species, including *Listeria monocytogenes* and *Salmonella enterica* (Sadeghi *et al*., 2019). Jang *et al*. (2021) reported on the ability of *L. brevis* biofilm to inhibit *Streptococcus mutans*, which causes dental caries. *Salmonella enterica*, *Staphylococcus epidermidis*, multiple drug resistant *S. enterica* and *Shigella flexneri* have all been reported to be inhibited by *L. brevis* biofilm (Rahmeh *et al*., 2019).

The ability of LAB biofilms to inhibit pathogenic bacteria is linked to the quorum sensing system, in which each of these cells interacts with one another in order to thrive in both nutritional competition and pathogenic bacteria (Guerrieri *et al*., 2009). In addition, the LAB biofilms that also produce substances such as biosurfactants and bacteriocins and have probiotic properties were more effective at inhibiting the growth of pathogenic bacteria (Gómez *et al*., 2016). *Staphylococcus aureus* and *E. coli* pathogens can be further inhibited by all these substances (Singh and Cameotra, 2004).

Tolerance of *L. brevis* biofilms strains to acidic pH and bile salts

Lactobacillus brevis biofilms were subjected to a range of assays, including acidic pH and bile salt tolerance, to assess their probiotic qualities for biopreservative development. As indicated in Table 1, they had a high survival rate in pH 2.5 incubated for 4 h at 37 °C. At pH 2.5, all LAB biofilms demonstrated good growth, with numbers of viable LAB cells in the range of 2.26-3.15 × 10^9 CFU/mL after 4 h of incubation. Meanwhile, *L. brevis* biofilm KB1 showed the highest tolerance significantly with a survival value of 96.85% at pH 4, followed by *L. brevis* KA2, *L. brevis* KA5 and *L. brevis* KC4 with survival values of 94.59%, 86.67% and 80%, respectively (p<0.05).

Lactobacillus brevis biofilms also demonstrated good growth ability after being cultured for 4 h at 37 °C in the test of tolerance to 0.3% bile salt, as shown in Table 2. They can grow in these conditions of bile salt stress and maintain a significant number of viable cells, ranging from 2.64 to 3.92 × 10^9 CFU/mL. With a survival ability of 94.64%, *L. brevis* biofilm KB1 demonstrated the strongest resistance to 0.3% bile salt, followed by *L. brevis* biofilm KA2, *L. brevis* biofilm KA5 and *L. brevis* biofilm KC4 at 91.59%, 80.98% and 74.79% (p<0.05).

The ability of LAB biofilms to survive at acidic pH and in the presence of bile salts is a crucial trait for their application as food biopreservatives. A similar ability in LAB was also published by Aarti *et al*. (2017) who investigated that *L. brevis* LAP2 from a fermented fish product (hentak) of Manipur, India and *L. brevis* LB062 from cheese were able to survive in low acid and bile salt environments to 90% and 85%, respectively (Zhang *et al*., 2020). Acid and bile salt resistance have been discovered in *Lactobacillus* spp. isolated from human saliva (Azizian *et al*., 2021).

LAB strains were able to successfully gain stress resistance to acidic pH and bile salt by regulating their cell physiology, including regulating the intracellular microenvironment, maintaining cellular membrane functionality and bile salt hydrolase production. The regulation of the intracellular environment was carried out by accumulating amino acids and regulating intracellular pH. Valine, leucine and isoleucine were among the amino acids that LAB accumulates. Furthermore, under LAB stress exposure, the amino acids proline and arginine were appropriate solutes (Huang *et al*., 2016; Wang *et al*., 2018). Amino acid metabolism via the arginine deiminase (ADI) and glutamate decarboxylase (GAD) pathways also contributed to BAL's intracellular pH regulation. In the ADI

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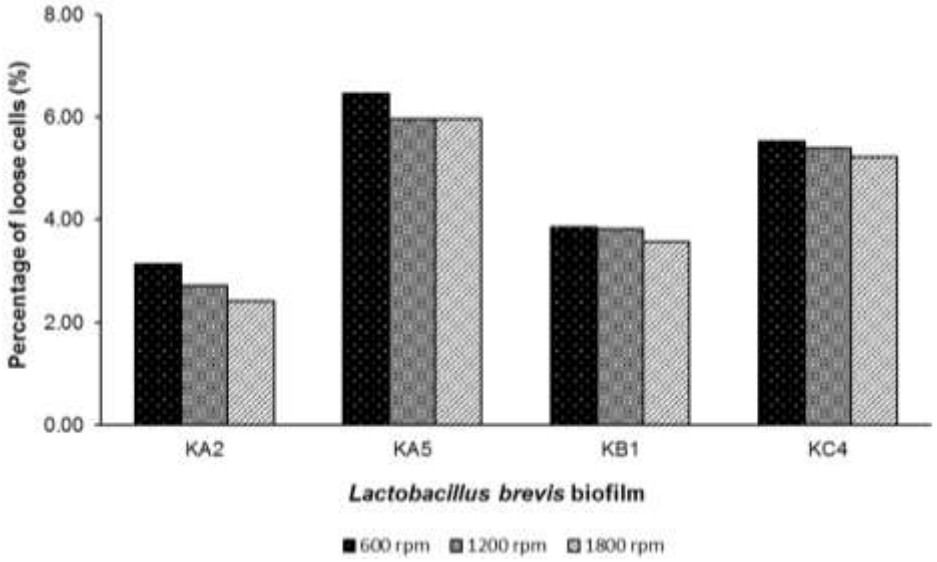

Figure 3: Percentage of cells released in *L. brevis* biofilm with different vortex speed (a) 600 rpm, (b) 1200 rpm and (c) 1800 rpm.

route, arginine is transformed to ornithine, which results in the generation of NH_3, CO_2 and ATP. Meanwhile, glutamate decarboxylase (GAD) converts glutamate to ɣ-aminobutyrate (GABA), allowing intracellular pH to be increased (Wang *et al*., 2018).

Fatty acid distribution, fluidity and membrane integrity all play a role in the regulation of LAB cellular membrane function. Membrane fatty acid synthesis was regulated to enhance the ratio of unsaturated to saturated fatty acid biosynthesis in the cell membrane (Bonomo *et al*., 2018; Guo *et al*., 2020). LAB's ability to produce bile salt hydrolase, which catalyzes the deconjugation of conjugated bile salts, also plays a role in bile salt resistance. According to reports, the *bsh1* and *bsh3* genes influence the activity of those enzymes (Wang *et al*., 2021a).

Adhesion characteristic of *L. brevis* biofilms

The effectiveness of *L. brevis* biofilm as a biopreservative can also be determined based on their aggregation strength, which was investigated by an adhesion test using vortex treatment. Although vortex treatment is a traditional method, it is inexpensive and simple to implement, and it is successful in releasing bacterial cells that have adhered to the substrate (Kobayashi *et al*., 2009). In addition, the biofilm adhesion test using vortex has been used successfully to analyze the aggregation of several biofilm-forming bacteria (Webber *et al*., 2015; Siradje *et al*., 2017).

At different vortex speeds, adhesion measurements of four *L. brevis* strains at 24 h, which provided the highest biofilm aggregation, revealed less than 8% loose cells (Figure 3). These findings suggest that in the formation of biofilms, those four *L. brevis* strains have a high level of cell aggregation. In *L. brevis* KA2, KA5, KB1 and KC4 biofilms, the average percentages of cell release were 2.76%, 6.13%, 3.75% and 5.38%, respectively. The yield of cells released from LAB biofilms was less than 6.46% in the vortex treatment at 600 rpm, while it was 5.96% in the vortex treatment at 1200 rpm and 1800 rpm which conducted once (Sapalina and Retnaningrum, 2019). These findings showed that increasing the vortex speed of the biofilm treatments did not result in an increase in cell release from the biofilms.

The difference in the percentage of cells released from the LAB biofilms were influenced by several factors, the properties of substrate, such as roughness and hydrophobic interactions, as well as the biofilm's adhesive factor, which includes protein, DNA and polysaccharides (Elbourne *et al*., 2019). In addition, the confounding factors such as metabolite repression, reduced nutrient availability, changes in internal bacterial biochemistry and endogenous enzyme degradation, on the other hand, can reduce the intensity of biofilm aggregation (Viljoen *et al*., 2020).

Surface texture and element composition of *L. brevis* biofilm

The surface texture and element composition of *L. brevis* biofilm KA2 and KB1 that produced the highest inhibitory effect of *L. brevis* biofilms against pathogenic bacteria indicators, tolerance to acidic pH and bile salts, and strongest adhesion were further analyzed through SEM-EDS. The SEM images showed both biofilm structures clearly, as shown in Figure 4. The bacterial cells that formed biofilm attached to each other and they formed a bacterial community enveloped by the EPS matrix. The morphology of LAB cells was clearly visible in the form of rods with a smooth surface biofilm textures. Similar results were reported by previous researchers who

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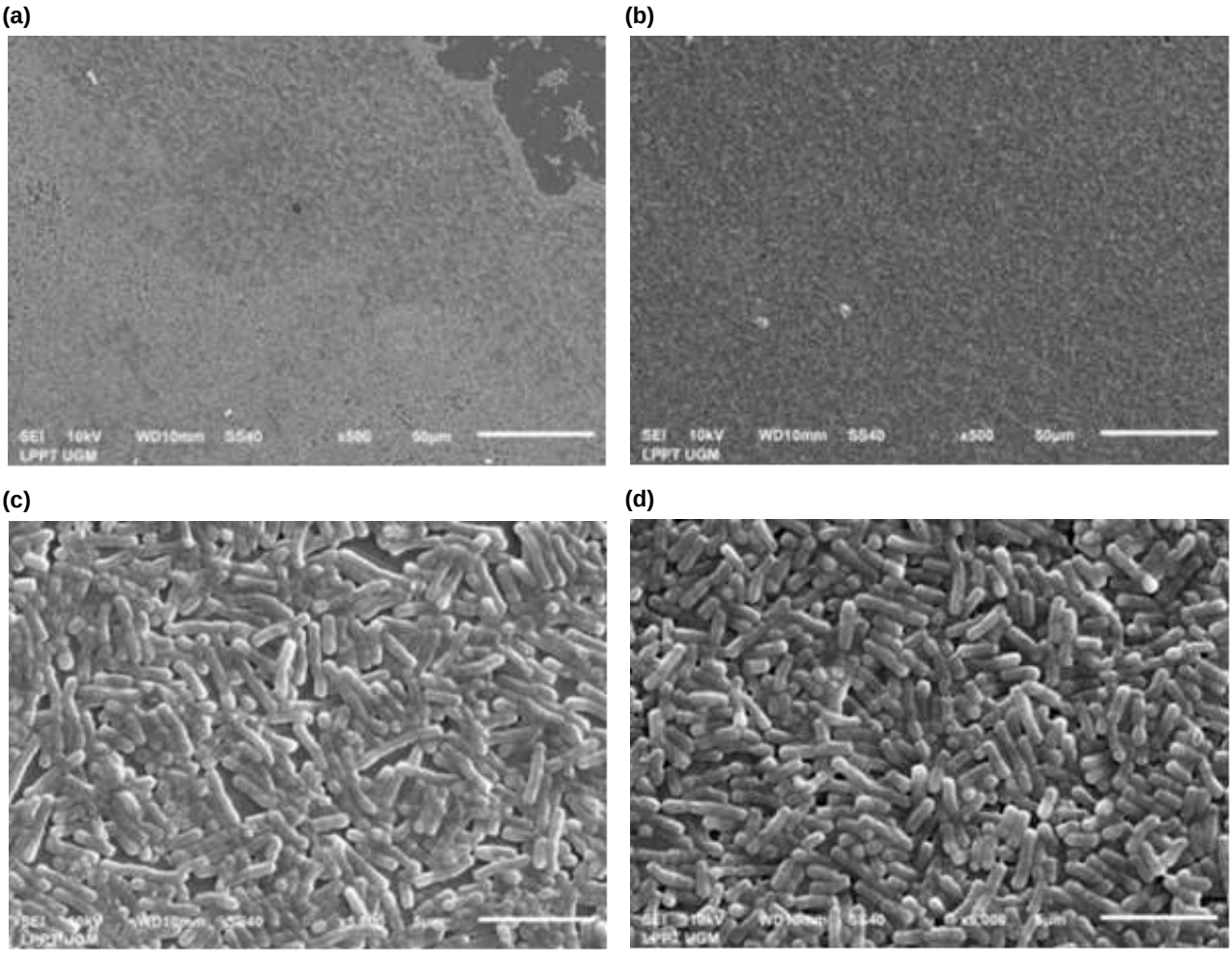

Figure 4: The SEM of *L. brevis* biofilm. (a) Biofilm surfaces of *L. brevis* KA2, (b) Biofilm surfaces of *L. brevis* KB1, (c) Biofilm cells of *L. brevis* KA2 and (d) Biofilm cells of *L. brevis* KB1. Magnification (a, b) 500× and (c, d) 5000×. Scale bars (a, b) 50 µm and (c, d) 5 µm**.**

investigated the rod LAB with smooth biofilm textures (Kubota *et al*., 2008; Pinaria *et al*., 2016; Kumar *et al*., 2017). In addition, both *L. brevis* strains showed strong aggregation. These SEM results were consistent with their aggregation strength results, which were investigated by an adhesion test using vortex treatment. Bacterial aggregation is crucial in a number of biological niches (Merino *et al*., 2019). The aggregation ability of *L. brevis* strains is related to their attachment to surface of biotic and abiotic materials, thereby causing an inhibitory effect on colonization and infection of pathogenic bacteria, as well as foodborne pathogens (Arena *et al*., 2017; Hossain *et al*., 2017). The formation of biofilms in food and on food-contact surfaces by *L. brevis* strains could limit the adhesion and proliferation of foodborne pathogenic bacteria, minimizing food contamination during processing.

When compared with those of bacteriocin, the qualities of LAB biofilms as a food biopreservative are superior. EPS, which are elements of LAB biofilms, are responsible for this outstanding property. EPS-producing whole cells and purified EPS are used in the food industry, among others, to avoid the growth of spoilage bacteria and foodborne pathogens. Furthermore, they serve a critical function in increasing the texture, rheology and mouth feel of food formulations (London *et al*., 2015; Zarour *et al*., 2017; Zhou *et al*., 2019). Furthermore, LAB biofilms are resistant to environmental stress and several process conditions, such as culture media, temperature and pH. While these harsh conditions have a significant impact on bacteriocin's antibacterial action. Muruzović *et al*. (2018) showed a reduction in bacteriocin production by the LAB genera of *Lactobacillus* and *Lactococcus*.

Figure 5 shows the findings of the elemental composition analysis of the LAB biofilm. Each of the elements discovered had a distinct peak height, with the element detected from the sample examined represented by the green peak. However, the elements Na, Al, Si, S,

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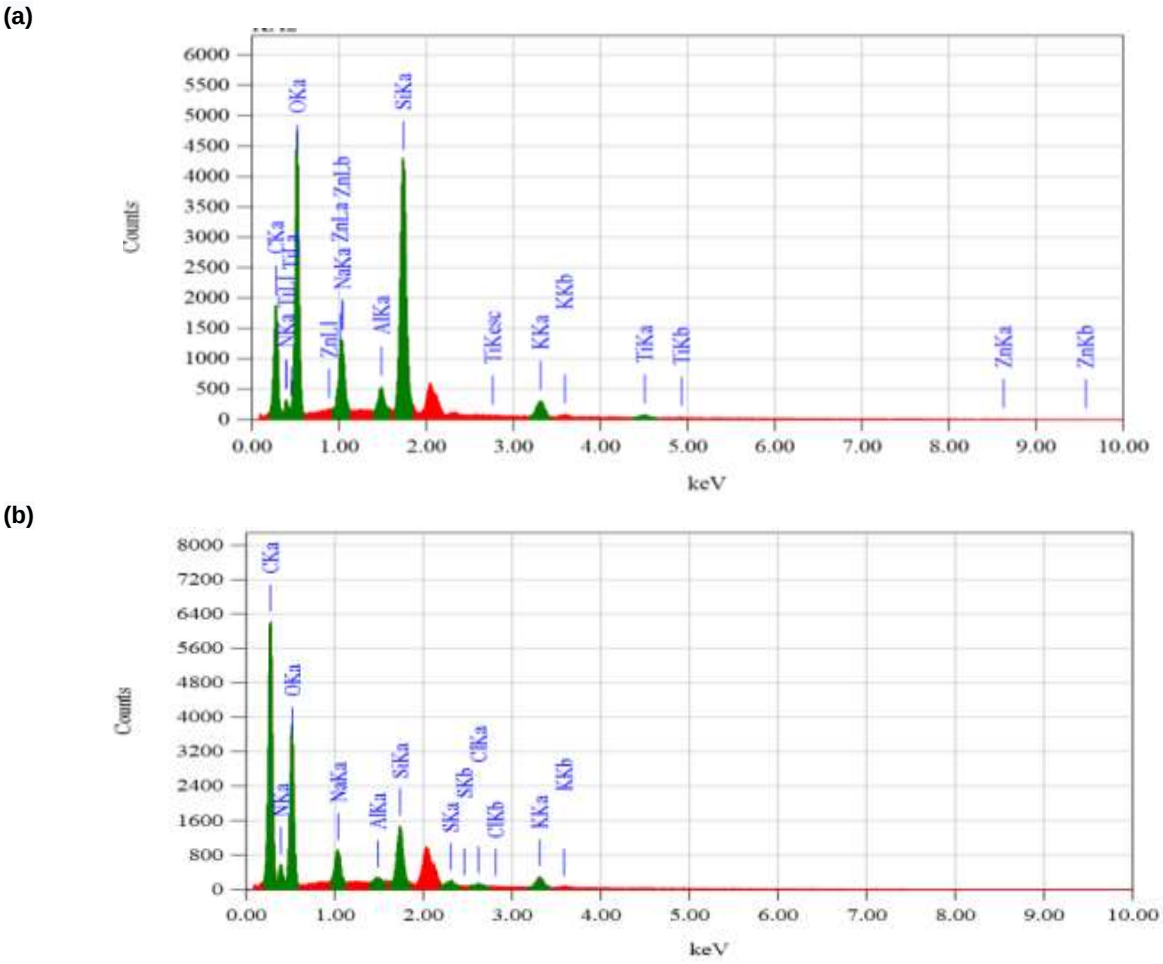

Figure 5: Element composition of *L. brevis* biofilm determined by SEM-EDS analysis. (a) Element composition of *L. brevis* KA2 biofilm and (b) Element composition of *L. brevis* KB1 biofilm.

Cl, K, Ti and Zn found in this study were impurities. Some elements including Si, Al, Ti and Zn were found on the surface of the cover slip. Meanwhile, the components Na, S, K and Cl came from the MRS medium broth. These impurity components were also discovered in the SEM-EDS study of EPS generated by *Lactobacillus casei* AL15 by a prior study (Pinaria *et al*., 2016).

The SEM-EDS analysis showed that the *L. brevis* KA2 biofilm consisted of 22.02% carbon, 38.08% oxygen and 11.02% nitrogen, whereas *L. brevis* KB1 biofilm consisted of 37.96% carbon, 31.12% oxygen and 20.75% nitrogen (Sapalina and Retnaningrum, 2019). Carbon and oxygen are known as carbohydrate constituents. Nitrogen is known as the main constituent of proteins, but some complex carbohydrates also contain nitrogen. Thus, the existence of the elements C, O and N were related to LAB biofilm composition, because LAB biofilm contains EPS, which consists of polysaccharides and proteins. According to Di Martino (2018), one of the important elements in the formation and maintenance of biofilm structures is EPS, which contain polysaccharides, proteins and DNA. The presence of EPS as a constituent of LAB biofilms influences their ability to survive in an acidic pH and bile salt environment (Vamanu, 2017). Those biofilms also function as a barrier for gases, vapors, solutes and lipids, as a protective structure to prevent biological, chemical and physical hazards (Moradi *et al*., 2021).

The availability of particular molecules in their surface as an EPS, which worked as ligands to enable them to attach to pathogens, could explain the variance in percentages of C, N and O values of two *L. brevis* strains (Tsai *et al*., 2018). Several researchers reported the components and compositions of EPS in the genus *Lactobacillus*, which were unique with bioactive abilities so that they have lot of promise in the food, biomedical and pharmaceutical industries (Ibarburu *et al*., 2015; Moscovici, 2015). Rajoka *et al*. (2019) published that the heteropolysaccharide composition of EPS from *L. kefiri* MSR101 isolated from kefir grains consisted of glucose and galactose, and detected carboxyl hydroxyl groups, respectively. That purified EPS was able to inhibit the

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growth of HT-29 cancerous cells by 44.1% through up-regulation of caspase3, caspase8, caspase9, Cyto-c, BAX and BAD gene expression.

Bomfim *et al*. (2020) informed that *L. plantarum* CNPC003 from dairy milk produced heteropolysaccharide EPS notably formed by mannose and glucose, with minor amounts of galactose. Its EPS at 8 mg/mL has antioxidant activity with 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical-scavenging activity of 90.88 ± 0.80%. The EPS homopolysaccharides in the form of glucans with a molecular weight of 104 kDa were generated by *L. pentosus* EPS47FE and EPS68FE. The anticoagulant and fibrinolytic activity of the EPS bioactivity was impressive. In addition, the EPS of 10 mg/mL showed rather high DPPH radicals in the range of 54.50-68.90; moreover, the emulsifying activity ranged from 53.33 to 83.33 after 48 h (Abo Saif and Sakr, 2020).

Recent research obtained purified EPS (10 mg/mL) of *L. paracasei* isolated from sauerkraut samples showing a total antioxidant capacity of 71.15%, while the antioxidant activity using hydrogen peroxide and 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) was 68.65% and 60.31%, respectively. The presence of components in the form of sulfate compounds, carboxyl groups and hydrogen gives EPS its antioxidant properties. The elements carbon, hydrogen, nitrogen and sulfur were detected by mass spectrometry at 54.36%, 21.74%, 9.63% and 18.03%, respectively (Shankar *et al*., 2021).

CONCLUSION

From the investigations, four strains of *L. brevis* biofilms isolated from kimchi could inhibit the growth of *S. aureus* FNCC 0047 and *E. coli* FNCC 0091. All the LAB biofilms also showed probiotic properties, which were tolerance to pH 2.5 and 0.3% bile salt, and strong adhesion. The *L. brevis* biofilm KA2 and *L. brevis* KB1 produced the highest inhibitory ability against the growth of those pathogenic bacteria. *Lactobacillus brevis* biofilm KA2 could inhibit the growth of *S. aureus* FNCC 0047 with a reduction value of 2.29 ± 0.56 log CFU/mL, whereas *L. brevis* KB1 could inhibit the growth of *E. coli* FNCC 0091, with a reduction value of 3.49 ± 0.49 log CFU/mL. *Lactobacillus brevis* biofilm KA2 and KB1 showed higher tolerance to pH 2.5 and 0.3% bile salt at 37 °C for 4 h compared with two other strains (*L. brevis* KA5 and *L. brevis* KC4). Both *L. brevis* biofilms also had higher aggregation. As a result, during vortex treatments at speed of 600 rpm, 1200 rpm and 1800 rpm, very few cells were released. The percentage values of cells released from the *L. brevis* biofilm KA2 and *L. brevis* biofilm KB1 were 2.76% and 3.75%, respectively. According to SEM-EDS analysis, both biofilm strains showed a smooth surface texture and rod-shaped cell morphology and contained many components such as carbon, oxygen and nitrogen that were constructed of extracellular polymeric compounds (EPS). All these excellent characteristics will be extremely beneficial in terms of biopreservation.

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