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Ale beer containing free and immobilized *Lactobacillus brevis*, a potential delivery system for probiotics

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Abstract

Probiotics in ale beer may be attractive to health-conscious consumers. However, beer conditions may decrease probiotic viability. Powder produced from durian (*Durio zibethinus*) rind, a by-product that is currently unutilized, can be used for the immobilization of probiotics. MRS medium was incubated with *Lactobacillus brevis* and periodically sampled to obtain the growth curve. Ale beer with free *L. brevis* and cells immobilized in durian rind powder was produced and separately assessed during storage at 21 °C for 24 days. The physico-chemical parameters of both beers did not differ significantly. Durian rind powder conferred protection up to 12 days of storage with the immobilized cells in the beer having a significantly higher count than the free cells, which can be due to the acid detergent fiber content (19.67%). Free and immobilized cells remained viable with counts of 4.89 and 5.00 log CFU/mL of beer, respectively, at the end of the storage period. Both treatments had approximate counts of 5 log CFU/mL after 120 min in simulated gastric and intestinal fluids. The predominant bacterial species present at the end of storage were *L. brevis* and *L. farciminis*. This study suggests that ale beer could be a potential delivery system for free and immobilized probiotic bacteria. This is one of the few studies demonstrating the use of probiotic lactic acid bacteria in beer brewing.

Keywords: Probiotics, Beer, Durian, Viability

Introduction

The increasing awareness about the importance of a healthy diet has driven the market for health-oriented food and beverages (Augustin and Sanguansri 2015). Probiotic products have gained interest throughout the years because of their reported role in immunological, digestive, and respiratory health and in easing infectious diseases (Vasudha and Mishra 2013). Probiotics are defined by the Food and Agriculture Organization and World Health Organization (2002) as "live microorganisms which when administered in adequate amounts confer a health benefit on the host." Probiotics are thought to confer health benefits primarily through

modulating the immune system of the intestine and displacing pathogens such as *Salmonella typhimurium*, *Helicobacter pylori*, and *Escherichia coli* (Govender et al. 2014; Hove et al. 2014).

While most probiotic products available are dairy, alternative raw materials are being investigated (Vasudha and Mishra 2013). Allergy and lactose intolerance are considered as the major disadvantages of dairy probiotic products, whereas probiotic survival poses a huge challenge when dealing with non-dairy foods (Vijaya Kumar et al. 2015). One possible probiotic drink is beer which is the most consumed alcoholic drink globally and the third most popular drink after water and tea. To date, only one study has investigated the survival of probiotic bacteria in a novel beer product (Alcine Chan et al. 2019). The worldwide production of beer is estimated to be 1.7 billion liters per year (Colen and Swinnen 2016;

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Martínez et al. 2017). China and the USA are the top two beer-producing countries in the world followed by Germany, Brazil, and Russia, all accounting for more than half of the total output (da Silva et al. 2008). Moreover, the consumption of alcoholic drinks has been linked to the disruption of intestinal microbiome homeostasis in both rodent models and humans (Engen et al. 2012). Alcoholic drinks influence gut microbiota and gut inflammations (Bishehsari et al. 2017).

Microorganisms including probiotic bacteria are generally susceptible to stress conditions (G-Alegría et al. 2004). Alcohol is reported to cause cell death by increasing membrane fluidity, thereby inhibiting microbial cell growth (Jia et al. 2010). However, gram-positive bacteria, especially lactic acid bacteria, are known to survive and grow in the presence of high alcohol concentrations (Jia et al. 2010). The genus *Lactobacillus* generally manifests significant ethanol tolerance when grown in the presence of up to 16% ethanol by volume (Gold et al. 1992). In addition, the *Lactobacillus brevis* species used in the study carried out by Knoshaug and Zhang (2009) manifested growth in environments with up to 3% butanol. According to the Beer Judge Certification Program (2015), the alcohol percent by volume of blonde ale beer that is the type of beer produced in this study is between 3.8 and 5.5% which is lower than the alcohol concentration (16%, v/v) the *L. brevis* was exposed to in the study of Gold et al. (1992). Therefore, *Lactobacillus brevis* was selected in the current study.

Incorporating probiotics in beer will potentially confer health benefits that typical beer in the market cannot provide. However, the presence of hop iso-α-acids in beer is also detrimental to the survival of probiotics (Alcine Chan et al. 2019), signifying the need for some support. The study of Alcine Chan et al. (2019) aimed to overcome the antimicrobial effect of hops iso-α-acids by carrying out first a co-fermentation step through co-culturing the starter culture *L. paracasei* L26 with *S. cerevisiae* S-04 in unhopped wort for 10 days. Isomerized hop extract was then added to the co-culture and the viability was evaluated at 5 and 25 °C during 28 days of storage. According to the authors, during this co-fermentation step, yeasts may help protect the probiotic bacteria due to the formation of mixed-species biofilms that have been demonstrated in other acidic food products. Moreover, immobilization, which refers to the trapping of material in a matrix, has been shown to improve the survival and growth of probiotics in various products (Mitropoulou et al. 2013). Several immobilization supports have been considered with a particular preference for biopolymers and natural supports that are food-grade (Mitropoulou et al. 2013). In a recent study, hi-maize starch, which behaves similarly to fiber, was used to immobilize probiotics (Bradford et al. 2019).

Durian (*Durio zibethinus*) is one of the most popular tropical fruits in Southeast Asia (Espino and Espino 2014). Only one-third of the fruit is consumed, with the fiber-rich rind comprising more than half of the total fruit weight (Hameed and Hakimi 2008; Penjumras et al. 2014). Since direct disposal of durian wastes causes social and environmental issues (Nuithitikul et al. 2010), utilizing them could provide economic value. Teh et al. (2009) evaluated durian rind, along with that of mangosteen and jackfruit, as immobilizers for probiotics in soy milk. In the current study, powder produced from durian rind was used as immobilization support for probiotic bacteria in beer. This study aimed to produce ale beer with either free *Lactobacillus brevis* or immobilized cells on durian rind powder to serve as a delivery system for probiotics.

Materials and methods
Microorganism
A lyophilized strain kindly provided by the USDA Agricultural Research Service Culture Collection (Washington, DC, USA) was identified as *Lactobacillus brevis* using 16 s rRNA sequencing (MR DNA, Shallowater, TX, USA). The culture was activated in De Man, Rogosa, and Sharpe (MRS) broth (Neogen Corporation, Lansing, MI, USA). For the free cells, seventy-five mL of the culture was inoculated in 1.5 L MRS broth and incubated at 37 °C for 16 h to reach stationary phase and to achieve a minimum cell count of 8.0 log CFU/mL. The cultures were harvested by centrifugation at 7500 x g for 10 min at 4 °C (Beckman J2-HC, Beckman Coulter, Inc., Brea, CA, USA). The pellets were washed twice with sterile 0.85% NaCl solution, centrifuged, and suspended in sterile distilled water.

Growth curve and growth parameters
One mL of *L. brevis* was inoculated in 100 mL of MRS broth and incubated at 37 °C for 24 h. A growth curve was constructed by measuring the optical density at 600 nm (OD_{600}) of the cell culture using a spectrophotometer (Genesis 20, Thermo Scientific Instruments, LLC, Madison, WI, USA). The absorbance was correlated with the concentration acquired (X, expressed in g/L) using the equation obtained from the standard curve. The maximum cell density (OD_{max}), maximum concentration (X_{max}), maximum specific growth rate (μ_{max}), and doubling time (T_d) were obtained as described by Mis et al. (2019).

Preparation of durian rind powder
Frozen durian fruit (*Durio zibethinus*), Mornthong variety (Thailand), was purchased from a local Asian store in 2017 (Baton Rouge, LA, USA). The whole fruit was thawed, cut using a knife to obtain the thorn-covered

rind, after which the thorny part was removed. The rind portions were sliced into smaller pieces before oven-drying at 70 °C for 20 h. The dried rind was ground for 10–12 min using a commercial blender to obtain the durian rind powder (DRP) which was sieved through a Tyler Standard Screen No. 48 (W.S. Tyler, Mentor, OH, USA). The resultant powder with an average particle size of 295 μm was stored at 4 °C until further use.

Proximate analysis of DRP

DRP was analyzed for moisture content, fat, protein, ash, and crude fiber. Moisture content was determined using a microwave-type moisture analyzer (Model 907,875, CEM Corporation, Inc., Matthews, NC, USA) (Reyes et al. 2018). Fat content was quantified using AOAC official method 2003.06 (AOAC 2006). For the protein analysis, the sample was digested following the EPA method 351.2 (EPA, 1993), then it was subjected to AOAC (1995) method 976.06. Ash content was measured using AOAC (1990) method 942.05. Crude fiber was analyzed by the filter bag technique using AOCS (2006) approved procedure Ba 6a-05.

Preparation of immobilized Lactobacillus brevis

DRP in MRS broth was sterilized in an autoclave before immobilization. After activating the culture in MRS broth, 75 mL was inoculated in 1.5 L sterile MRS broth with 15 g DRP to achieve a cell count of at least 8.0 log
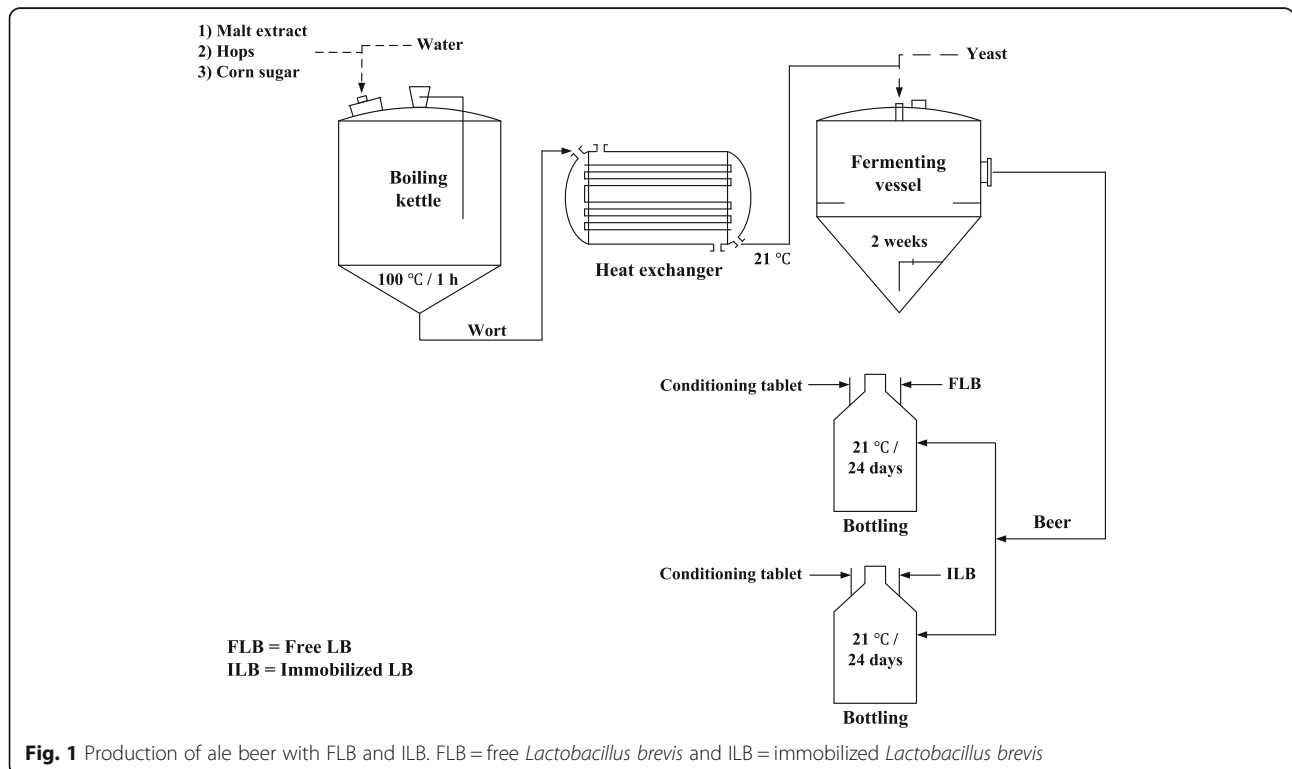
CFU/mL. Immobilization was carried out through shaking at 150 rpm using a shaker (Lab-line incubator shaker model 3525, Fisher Scientific Inc., Pittsburgh, PA, USA) at 37 °C for 16 h. The immobilized LB on DRP was washed twice with sterile 0.85% NaCl solution, centrifuged at 7500 x *g* for 10 min at 4 °C to obtain the pellets, and suspended in sterile distilled water.

Scanning electron microscopy (SEM)

SEM was carried out following the method of Reyes et al. (2018). The morphology of FLB and ILB was observed under a scanning electron microscope (JSM-6610LV, JEOL Ltd., Tokyo, Japan).

Production of ale beer

The ale beer was produced following the standard procedure described by Lordan et al. (2019) and Parker (2012) with some modifications. The production process for ale beer is outlined in Fig. 1. Pale malt extract (3.6 kg) (Alexander's Sun Country, CA, USA) was dissolved in 38 L of water in a brew kettle (Model MKEL100T, Cleveland Range Ltd., Toronto, ON, Canada) and brought to boiling. After 1 min of boiling, 12 g of pelleted hops (Northern Brewer, Roseville, MN, USA) was added for bitterness and flavor. After 15 min of boiling, 24 g hops was further added, and the remaining 12 g was added after another 15 min of boiling. The specific gravity (SG) was measured using a triple scale hydrometer

Fig. 1 Production of ale beer with FLB and ILB. FLB = free *Lactobacillus brevis* and ILB = immobilized *Lactobacillus brevis*

(Alla France, Chemillé-en-Anjou, France). Corn sugar was added until an SG value of 1.040–1.060 was reached. The wort was transferred to a conical fermenter with a capacity of 26.5 L (SS Brewtech, CA, USA). It was then cooled to 21 °C using a VWR refrigerated recirculator (Model 1176, VWR Scientific, Niles, IL, USA). Once cool, dry brewing yeast (*Saccharomyces cerevisiae*) (SafAle S-3, Fermentis, France) was added to the wort. Fermentation was carried out for 2 weeks at 20 °C.

Inoculation of FLB and ILB into beer
Fifteen mL of the free cells (FLB) and immobilized cells (ILB) was separately inoculated in 285 mL of ale beer corresponding to counts of 8.92 ± 0.04 log CFU/mL and 9.94 ± 0.04 log CFU/mL of beer, respectively. A conditioning tablet made of dextrose was added during bottling to give carbonation. The beer was stored for 24 days at 21 °C to simulate ambient storage. Ale beer with free cells was used as the control.

Physico-chemical properties of beer
Pure beer (without FLB and ILB) and beer with FLB and ILB were analyzed for specific gravity, total soluble solids, pH, and titratable acidity. Specific gravity was measured using a triple scale wine and beer hydrometer (Alla France, Chemillé-en-Anjou, France). The SG of the wort (OG), as well as the final SG (FG) after yeast fermentation before the addition of probiotic bacteria, were used to calculate the alcohol percentage by volume (ABV) of the beer.

Equation 1 was used with a value of approximately 0.79 as the specific gravity of alcohol (Brick 2006; Stange 2015). This formula considers 1.05 g of ethanol produced per gram of carbon dioxide lost during fermentation according to the chemical reaction. Dividing this value by 0.79 and multiplying by 100 yields 132.91 which is the factor used to calculate ABV.

$$ABV = 132.91(OG - FG) \qquad (1)$$

The total soluble solids as expressed in °Brix were determined using a digital handheld refractometer (AR200, Reichert, Inc., Depew, NY, USA). pH was measured using an EcoSense pH pen (YSI Inc., Yellow Springs, OH, USA). Titratable acidity (TA) was determined using the method described by Mis et al. (2019). TA was expressed in terms of mg/mL lactic acid.

Carbonation was also measured using a beer carbonation tester (Model 2701-BCT, Taprite, San Antonio, TX, USA) at 24 days of storage at 21 °C for both beers inoculated with FLB and ILB.

Viability of FLB and ILB in ale beer during storage at 21 °C
Viability was measured on the free and immobilized cells every 3 days for 12 days, and then at 18 and 24 days. Beer samples were serially diluted in test tubes containing 0.85% NaCl solution. An aliquot of 0.1 mL was inoculated into MRS agar using the pour plating technique and measurements were obtained in triplicate. After incubation at 37 °C for 48 h, Petri plates with 25–250 colonies were counted and the results were expressed as log CFU/mL of beer.

Viability in simulated gastric and intestinal fluids of FLB and ILB in ale beer stored for 24 days at 21 °C
The viability under simulated gastric and intestinal conditions was measured at 18 and 24 days of storage at 21 °C. The simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) solutions were prepared according to the method used by Roberts et al. (2018) with slight modifications. SGF was prepared by dissolving 0.5 g of NaCl and 1.5 g of pepsin in 1.75 mL of 12 M HCl. The solution was diluted to 250 mL with sterile distilled water, adjusted to pH 3.0, and passed through a sterile 0.22-μm filter. The SIF solution was prepared by dissolving 1.7 g KH_2PO_4 in 62.5 mL of water. Then, 19.25 mL of 0.2 N NaOH was added and the solution was diluted to 250 mL with sterile distilled water. Pancreatin was added (1% w/v) and the pH was adjusted to 6.5. The solution was filtered using a sterile 0.22-μm filter. Beer samples of 1 mL each were separately introduced to 9 mL of pre-warmed SGF and 9 mL of pre-warmed SIF solutions and incubated at 37 °C under constant agitation. Samples were collected at 30, 60, and 120 min intervals for viability measurements. Results were expressed as log CFU/mL of beer.

Microbial diversity of beer with FLB and ILB
Beer samples with FLB and ILB were analyzed for bacterial and fungal diversity using bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) as described previously by Dowd et al. (2008) (MR DNA, Shallowater, TX, USA). Samples were sequenced at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on an Ion S5 Next-Generation Sequencing System (Thermo Fisher Scientific, Inc., USA) following the manufacturer's guidelines. Sequence data were processed using a proprietary analysis pipeline (MR DNA, Shallowater, TX, USA).

Operational taxonomic units (OTUs) were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTn in comparison with a curated high-quality 16 s rRNA gene database derived from RDP-II (http://rdp.cme.msu.edu) and the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov). Data were compiled and

relative percentages of bacteria and fungi were determined for each beer sample.

Statistical analysis

Data from triplicate experiments were statistically analyzed using the Statistical Analysis System (SAS Version 9.2, SAS Institute Inc., Cary, NC, USA). An independent t-test was used to determine the significant differences between the two samples. Analysis of variance and Tukey's test as post-hoc at an alpha level of 0.05 were used to determine significant differences within each treatment through time.

Results and discussion

Growth curve and growth parameters of Lactobacillus brevis

The growth curve of *L. brevis* is shown in Fig. 2. The microorganism reached a maximum cell density (OD_{max}) of 1.09 ± 0.03 and a maximum concentration (X_{max}) of 0.39 ± 0.01 g/L at 16 h of fermentation. The OD_{max} was comparable to that of *L. brevis* S354 subjected to different carbon sources (Guo et al. 2010). In this study, stationary phase was reached after 16 h so the pellets were harvested at this time before adding to the beer. Moreover, *L. brevis* displayed a maximum specific growth rate (μ_{max}) of $0.12\,h^{-1}$ and a doubling time of 5.82 h based on concentration.

Proximate composition of DRP

As presented in Table 1, the major dry components of the DRP were acid detergent fiber (ADF) (19.67%), crude fiber (17.10%), ash (6.06%), protein (4.77%), and fat (0.46%). Previous studies reported comparable values for the composition of durian peel (Foo and Hameed 2011; Unhasirikul et al. 2012). The main components of ADF

Table 1 Proximate composition of DRP

Composition	% (wet basis)
Protein	4.77 ± 0.58
Fat	0.46 ± 0.10
Crude fiber	17.10 ± 0.40
Acid detergent fiber	19.67 ± 0.26
Carbohydrate[a]	85.83 ± 1.72
Ash	6.06 ± 0.98
Moisture	2.89 ± 0.27

Values are means ± standard deviations of triplicate measurements
[a]Estimated by difference

are homogeneous polysaccharides, which can easily undergo cleavage to produce monosaccharides. These, in turn, could serve as a fermentation substrate for the bacterial community in the gut and promote gut health (Zhao et al. 2019). Moreover, according to Madhu et al. (2017), crude fiber is predominantly 60–80% cellulose and 4–6% lignin plus some mineral matter. It was reported that dried durian peel had a high hemicellulose content at 15.5% (Masrol et al. 2015). The forms of cellulose present in DRP could help improve probiotic stability by providing some protection as mentioned by Perricone et al. (2015). Nonetheless, the actual sugar composition of DRP should be evaluated in future research. It was also noted that the DRP had a minimal moisture content of 3.04% corresponding to a low water activity comparable to the results of Kha et al. (2010). This dictates good storage quality of the powder (Shyamala and Jamuna 2010).

Scanning electron microscopy

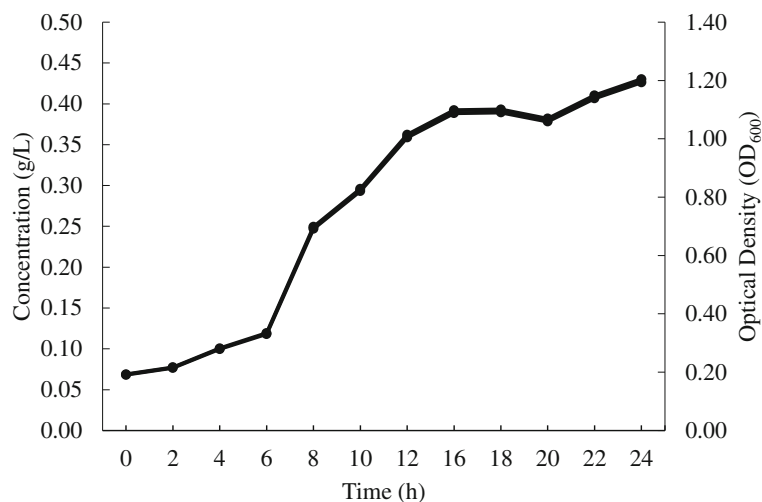Scanning electron micrographs of FLB and ILB are shown in Figs. 3 and 4, respectively. Free cells appear to

Fig. 2 Growth of *Lactobacillus brevis* measured as concentration (g/L) and optical density (OD_{600}) using MRS media
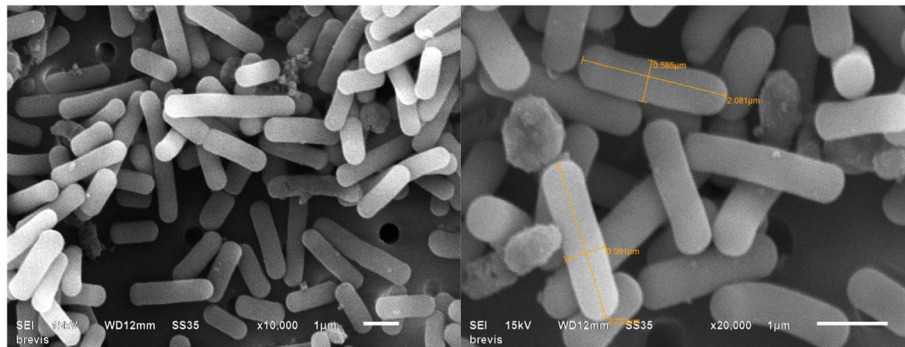
Fig. 3 Scanning electron micrographs of free *L. brevis*

have a rod shape (Fig. 3). This morphology agreed with the results of Kimoto-nira et al. (2015) and Elez-Martinez et al. (2005). As illustrated in Fig. 4a, it was evident that the bacterial cells were randomly distributed and attached to the DRP. This indicated that DRP could provide an adherence matrix for the probiotic bacteria. The DRP had rough and uneven surfaces to which the probiotic bacteria attached (Fig. 4b). The spaces introduced by the bumpy surface of the powder matrix provided room for the attachment of the probiotic as seen in a zoomed out micrograph in Fig. 4c with the encircled portion magnified as shown in Fig. 4d. Results were similar to those from a study by Jagannath et al. (2010) wherein the disorganized arrangement of cellulose strands were illustrated to hold many bacteria in the spaces and on the surface. Another study described the penetration of bacterial cells into the open pores of different immobilization matrices particularly inorganic porous supports (El Enshasy and Moawad 2011). In our study, the high number of cells attaching to the surface and going into open spaces signified immobilization of *L. brevis* on DRP. The adherence of probiotic cells on the powder despite several washings upon fixing the sample for SEM indicated sufficient immobilization, similar to the results of Xiudong et al. (2016).

Microorganisms such as probiotic bacteria adsorb spontaneously on various organic and inorganic supports with intermolecular forces of attraction responsible for
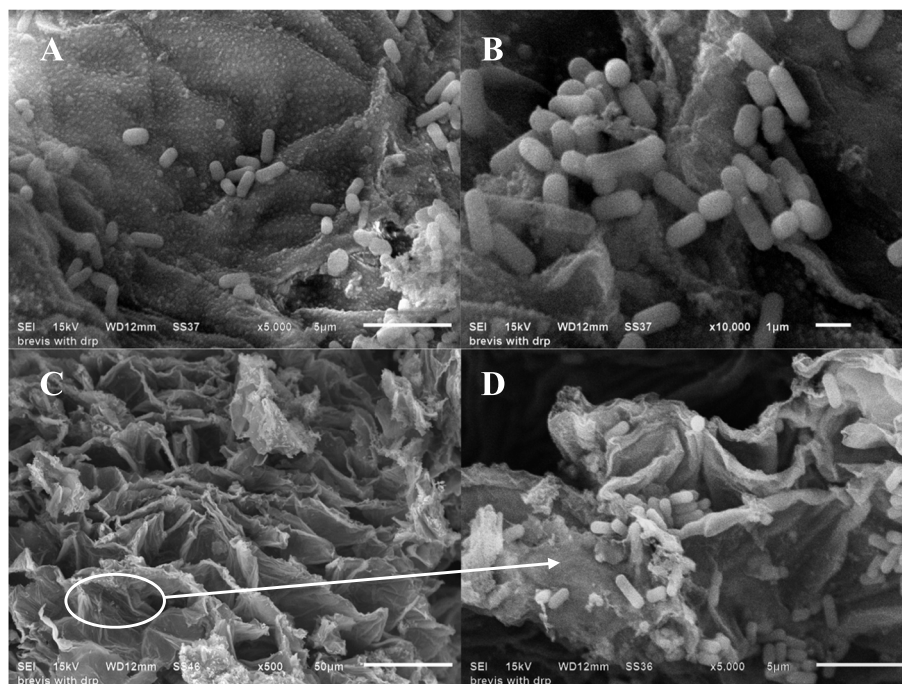

Fig. 4 Scanning electron micrographs of cross-sections of DRP loaded with *L. brevis*

cell binding. Cell physiology greatly affects the strength of adhesion (Nedovic and Willaert 2005). As mentioned by Xiudong et al. (2016), covalent bonding or physical adsorption by electrostatic forces could be responsible for the immobilization of the probiotic. Also, Raghavendra et al. (2006) reported that grinding provides increased surface area for absorption, and the uneven surfaces shown in the SEM could have been produced by grinding the powder, thereby increasing available areas for attachment of *L. brevis* cells.

Physico-chemical properties of ale beer

In this study, the final carbonation of beer at 24 days of storage was 2.8 volumes CO_2, which is characteristic for ales according to a chart set for the carbonation tester used (Model 2701-BCT, Taprite, San Antonio, TX, USA). A carbonation level of "2 volumes" implies 2 cubic inches of CO_2 dissolved in every cubic inch of volume (Walsh et al. 2014). The level of CO_2 dissolved in beer largely affects its flavor and visual appeal. Around 2.6 volumes have been recommended for carbonation in beer packages, while breweries employ 0.2 volumes higher to account for a possible loss during packaging (Rohner and Tompkins 1970). The carbonation level measured for the ale beer produced in this study corresponded with the aforementioned literature. Carbon dioxide, a product of yeast fermentation during beer production, has been reported to have inhibitory and stimulatory effects on microorganisms depending on the product and strain (Walsh et al. 2014). In this study, both free and immobilized *L. brevis* in beer were shown to be resistant to CO_2 levels up to 2.8 volumes. CO_2 creates an anaerobic environment and *L. brevis* was able to tolerate such conditions as it is a facultatively anaerobic microorganism (Vriesekoop et al. 2013).

The specific gravity, total soluble solids, pH, and titratable acidity of beer with FLB and ILB are presented in Fig. 5. The specific gravity and total soluble solids on day 0 are similar to the values obtained for pure beer, while the pH and titratable acidity of pure beer were 4.66 and 1.80 mg/mL lactic acid, respectively. The significant changes in values during the initial days of storage may be due to the introduction of *L. brevis* cells which were adjusting to the conditions of the beer as well as the addition of a conditioning tablet. Specific gravity is a useful measurement in calculating the alcohol by volume of beer as it compares the density of a liquid to that of water. The final specific gravity after yeast fermentation is expected to be lower than the original specific gravity of the unfermented wort as the yeast has consumed most of the fermentable sugars, converting them to alcohol and leaving behind a proportion of starch that was not fermented (Bamforth 2004; Stange 2015). In this study, the wort had an original

specific gravity of 1.04 and the resulting beer had a final specific gravity of 1.007. Using Eq. 1, the alcohol percent by volume was calculated to be 4.39%. This agreed with the report of Bamforth (2004) stating that beer usually contains lower levels of ethanol compared to other alcoholic drinks with the UK mean alcohol content of 4.1% and the US average of 4.6%. Moreover, during the storage period of the beer inoculated with probiotic bacteria, some yeast, and probiotic bacteria could have consumed the residual sugar, explaining the slightly decreasing trend in specific gravity until day 24. No significant difference was observed in the SG of both types of beer.

The trend for the total soluble solids follows that of specific gravity with a final value of 6.2 °Brix for both beers. Brix scale measures the percentage of sugar and other dissolved solids in the solution. This value is also related to the alcohol content of beer (Ball 2006). Comparable to the trend for specific gravity, the increase in °Brix from day 0 to day 3 could be attributed to the changing environment. Similarly, the slight decrease in total soluble solids during the 24-day storage period could be due to the consumption of the remaining sugar in the beer. Beer with FLB had a decrease of 0.45 °Brix from day 3 to day 24, while the decrease was only 0.3 for beer with ILB. As explained by Zandi et al. (2016), free cells have easier access to sugar. In this study, however, no significant difference was observed between FLB and ILB at any storage time. These results indicated that immobilizing in DRP did not significantly affect the specific gravity, total soluble solids, and alcohol content of the beer.

The pH of pure beer was 4.66 and upon inoculation of the bacteria, this dropped to 4.40 for both beer with FLB and beer with ILB (Fig. 5). The decrease in pH coincided with the acid production by the lactic acid bacteria, which is similarly reported by Alcine Chan et al. (2019). The pH of both beers stayed at around 4.40 throughout the period of 24 days. This agreed with the values mentioned by Sakamoto and Konings (2003) stating that beer has pH from 3.8–4.7, making it an unfavorable medium for microbial growth. Controlling the pH of beer is necessary as pH influences beer flavor as well as physical and microbiological stability (Nimubona et al. 2013).

The general trend for titratable acidity was contrary to that for pH, owing to the production of lactic acid. The lactic acid concentration was determined and expressed in terms of the predominant organic acid produced by *L. brevis* in the beer. The titratable acidity of pure beer on day 0 was 1.80 mg/mL, this value increased to 4.27 and 4.64 mg/mL at day 3 for FLB and ILB, respectively. Titratable acidity of both beers after day 3 was relatively constant throughout time (Fig. 5). Beer with ILB had 4.59 mg/mL lactic acid after 24 days of storage compared to 4.26 mg/mL for beer with FLB. The titratable acidity
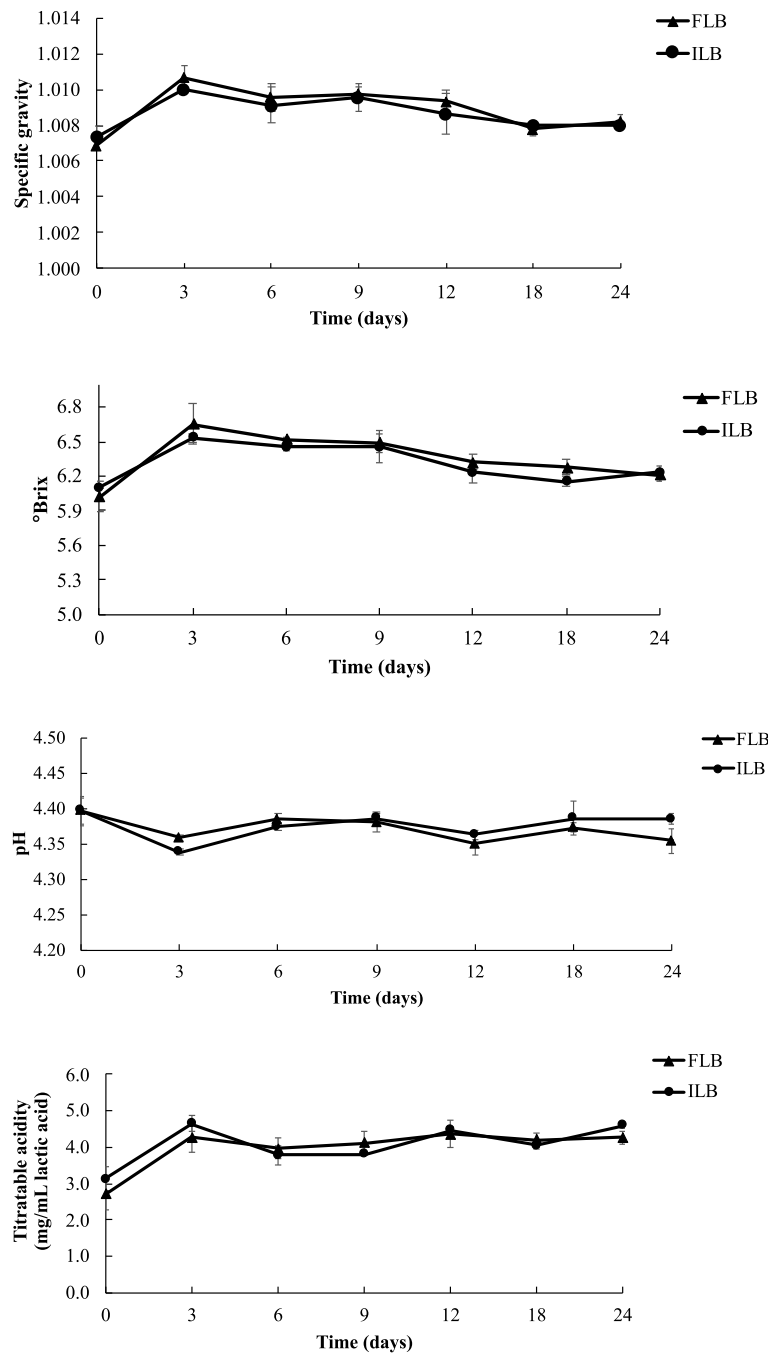
Fig. 5 Physico-chemical characteristics of beer with FLB and ILB. FLB (▲) = free *L. brevis* and ILB (●) = immobilized *L. brevis*

of both beers in terms of lactic acid did not differ significantly for all sampling times, signifying that immobilization in DRP did not have a significant influence on lactic acid production. Lactic acid production can be attributed to the growth of probiotic bacteria, consequently decreasing the pH (Fig. 5). As demonstrated in Fig. 5, the residual sugar must have been consumed by the yeast as well as the lactic

acid bacteria, similar to the results presented by Pakbin et al. (2014), thereby producing more lactic acid. Moreover, lactic acid production in the beer can be attributed to the growth of probiotic bacteria, consequently decreasing the pH of the substrate. Results showed that *L. brevis* both in free and immobilized states could survive and produce lactic acid in beer.

Viability of FLB and ILB in ale beer during storage at 21 °C

Viability counts of *L. brevis* followed a propagation in culture which is a standard culture-based technique that has been widely used and is regarded as the only validated operational test to determine bacterial viability (Bogosian and Bourneuf 2001). Other techniques such as PCR or real-time PCR are unable to determine whether cells are viable or non-viable since these techniques cannot differentiate between DNA arising from live or dead cells (Cangelosi and Meschke 2014). Initial cell counts of FLB and ILB before inoculating into the beer were 8.92 ± 0.04 log CFU/mL and 9.94 ± 0.04 log CFU/mL of beer, respectively. Results showed that free and immobilized *L. brevis* manifested survival over the storage period of 24 days with the FLB count falling to 4.89 log CFU/mL and ILB having a count of 5.00 log CFU/mL of beer at the end of 24 days (Fig. 6). The counts at day 0 for both FLB and ILB were significantly different from those for the other storage times. Immobilized cells had significantly higher counts than free cells at day 0, day 6, and day 12 of storage. On day 12, ILB had a count of 5.98 log CFU/mL of beer which was significantly higher than the count for FLB. These results indicate that compared to the control, immobilization was able to effectively protect the *L. brevis* up to 12 days of storage at 21 °C after which the counts decreased by 1 log CFU/mL. Given that at least 1 million CFU of *L. brevis* were viable in the beer at ambient temperature during the storage period, the product has the potential to render probiotic benefits since it meets the minimun concentration (10^6 CFU/mL or gram) required for a probiotic product to exert its beneficial effects (Kechagia et al. 2013).

While probiotic bacteria are generally reported to be susceptible to ethanol (G-Alegría et al. 2004), DRP in this study was shown to confer protection in 4.39% ABV

beer for up to 12 days. Immobilization has been described to be successful in protecting and improving the viability of probiotic bacteria (Mitropoulou et al. 2013). In the current study, *L. brevis* cells immobilized in DRP generally showed better survival compared to the control (free cells). Teh et al. (2009) suggested that the minerals and fibers present in durian rind supported the survival of probiotic bacteria. However, the difference between the viability of FLB and ILB was not significant at 24 days of storage. These results indicated that beer with free *L. brevis* or with cells immobilized on DRP could provide approximately 5 log CFU/mL of beer. In a recent study utilizing probiotics as a starter culture in beer brewing, refrigeration was shown to better support the viability of the microorganisms compared to ambient storage (Alcine Chan et al. 2019). Since the present study simulated ambient storage, it is expected that the probiotics would be more viable at lower temperatures.

Data from this investigation suggested that both FLB and ILB were able to tolerate ale beer conditions including the presence of ethanol and hop acids. In general, *Lactobacilli* are shown to be tolerant to 4% ethanol (Gold et al. 1992). In such a study, strains of *L. brevis* were reported to be tolerant to the said percentage but the tolerance decreased at 8% ethanol levels. Ethanol tolerance has been attributed to the stimulation of the release of stress proteins and changes in the fatty acid profile of the cell membrane (Gold et al. 1992; Yomano et al. 1998). This suggested a possible correlation between abnormality in the cellular lipids and characteristic ethanol tolerance of the microorganism as described by Uchida and Mogi (1973). Nonetheless, employing a more alcohol-tolerant *L. brevis* strain, a higher initial probiotic count, and refrigerated conditions can be explored in the future. Furthermore, despite the known antimicrobial activity of hop compounds, *L. brevis* strains are considered to be resistant due to immunity
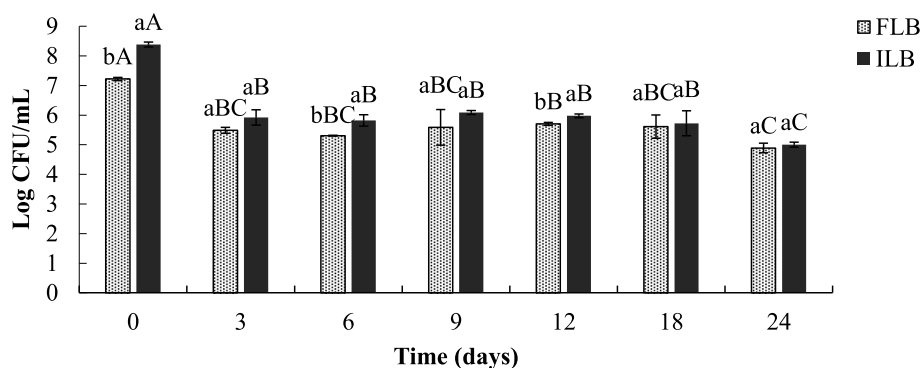

Fig. 6 Viability of FLB and ILB in ale beer during 24 days of storage at 21 °C. [a-b]Means with no letter in common between treatments within the same incubation time are significantly different (*P* < 0.05). [A-C]Means with no letter in common within the same treatment across incubation times are significantly different (*P* < 0.05). FLB (▦) = free *Lactobacillus brevis* and ILB (■) = immobilized *Lactobacillus brevis*

developed from prolonged contact with hop compounds during brewing. This hop resistance was associated with changes in membrane lipid components (Sakamoto and Konings 2003). Behr et al. (2007) further explained hop resistance mechanisms, including proton motive force depletion as well as complex changes in metabolism and structural improvements of cell wall components. Probiotic strains were not viable in hopped wort as shown recently (Alcine Chan et al. 2019), but the current study revealed the survival of *L. brevis* in hopped wort, signifying hop tolerance of the microorganism used. To the best of the authors' knowledge, this is the first study demonstrating the survival of probiotic bacteria in hopped fermented wort in the production of ale beer. The recent study by Alcine Chan et al. (2019) employed co-fermentation of unhopped wort with yeast and probiotic bacteria. Our study was able to manifest the survival of the *L. brevis* even in hopped wort.

Another possible scenario could be the enhancement of probiotic survival due to the presence of yeast, possibly forming biofilms that protected the lactic acid bacteria from external stress conditions. A combination of live *S. cerevisiae* S-04 and probiotic *L. paracasei* L26 in beer during refrigerated storage was shown to maximize probiotic viability (Alcine Chan et al. 2019).

Viability in simulated gastric and intestinal fluids of FLB and ILB in ale beer stored for 24 days at 21 °C

Figure 7 presents the probiotic viability in SGF and SIF. The pH conditions used in this study were in agreement with the report of Vandamme et al. (2002), which suggested that dosage forms must start passing through the stomach (pH 1.5–3.5) to the colon with pH ranging from 6.4 in the ascending colon to 7.0 in the descending colon. *Lactobacillus* strains must survive at least at pH 3.0 in the stomach (Fernandez et al. 2003). Survival at pH 3.0 for 2 h was reported to be the standard for optimal acid tolerance of probiotic strains (Liong and Shah 2005). Using the same pH condition, FLB and ILB in beer stored for 24 days had counts of 4.63 and 4.70 log CFU/mL of beer, respectively, after 120 min of exposure to SGF. These results were in accordance with findings from Sahadeva et al. (2011) which demonstrated that the survival of some *Lactobacillus* strains was due to the pH not causing the complete destruction of all the cells. Since disruption of metabolic and cytoplasmic activities normally restrict survival of probiotics under acidic conditions for 2 h (Kim et al. 2016), current results indicated that FLB and ILB met the criterion for gastric acid resistance.

Teh et al. (2009) postulated that immobilization on agrowastes, including durian rind, allows for higher stability in acidic conditions. The presence of cellulose in DRP could account for partial protection. Xiudong et al.

(2016) found that adsorbing *Lactobacillus* cells on or entrapping them in bacterial cellulose was effective in protecting the probiotics in simulated gastric juices. Additionally, FLB and ILB in beer stored for 24 days had counts of 4.64 and 5.14 log CFU/mL of beer, respectively, after 120 min of exposure to SIF. The polysaccharide nature of DRP could account for its dissolution by enzymatic hydrolysis upon reaching the small intestine to deliver the probiotic bacteria into the colon as described by Govender et al. (2014). Results showed that ILB had higher viable counts than FLB in simulated gastric and intestinal conditions at the end of storage but the differences were not significant. The slightly higher counts obtained for ILB after exposure to SIF compared to the initial concentration suggest the possible presence of new microbial species as explained in the succeeding section.

Microbial diversity of beer with FLB and ILB

bTEFAP was used to evaluate the microbial diversity of beer with FLB and ILB. This method has been used for the determination of bacteria and fungi present in different materials as described in different studies (Guass et al. 2016; Schmidt et al. 2018; Tekin et al. 2017). For bacteria, a total of 1,068,631 and 1,084,617 sequences were analyzed for beer with FLB and beer with ILB, respectively (Fig. 8). For fungi, 255,326 and 259,741 sequences were analyzed for beer with FLB and beer with ILB, respectively (Table 2). At the genus level, *Lactobacilli* were the most dominant bacteria and *Saccharomyces* were the most dominant fungi in both beers. Results showed that the predominant species present in both types of beer was *L. brevis*, which was the probiotic bacteria inoculated. At 24 days of storage at 21 °C, *L. brevis* was present at 61.88 and 55.68% in beer with FLB and beer with ILB, respectively. An interesting finding was the presence of *L. farciminis* at 35.31 and 41.63% in beer with FLB and beer with ILB, respectively, following *L. brevis* among the bacterial species identified. *L. farciminis* is a halophilic obligately homofermentative lactic acid bacterium that is closely related to *L. alimentarius* (Lee et al. 2010; Rachman et al. 2003; Yoon et al. 2018). It is considered as a probiotic microorganism with reported health benefits in suppressing stress-induced hypersensitivity. One study hypothesized that this mechanism is linked to the bacteria's protective effect on the intestinal barrier. This species was also the first probiotic found to suppress stress-induced visceral hypersensitivity in rats, suggesting the use of the strain in managing irritable bowel syndrome (Eutamene et al. 2009). Since this species is typically part of kimchi fermentation, it can act as a probiotic (Lee et al. 2010). Data from our study indicated that the ale beer conditions including the
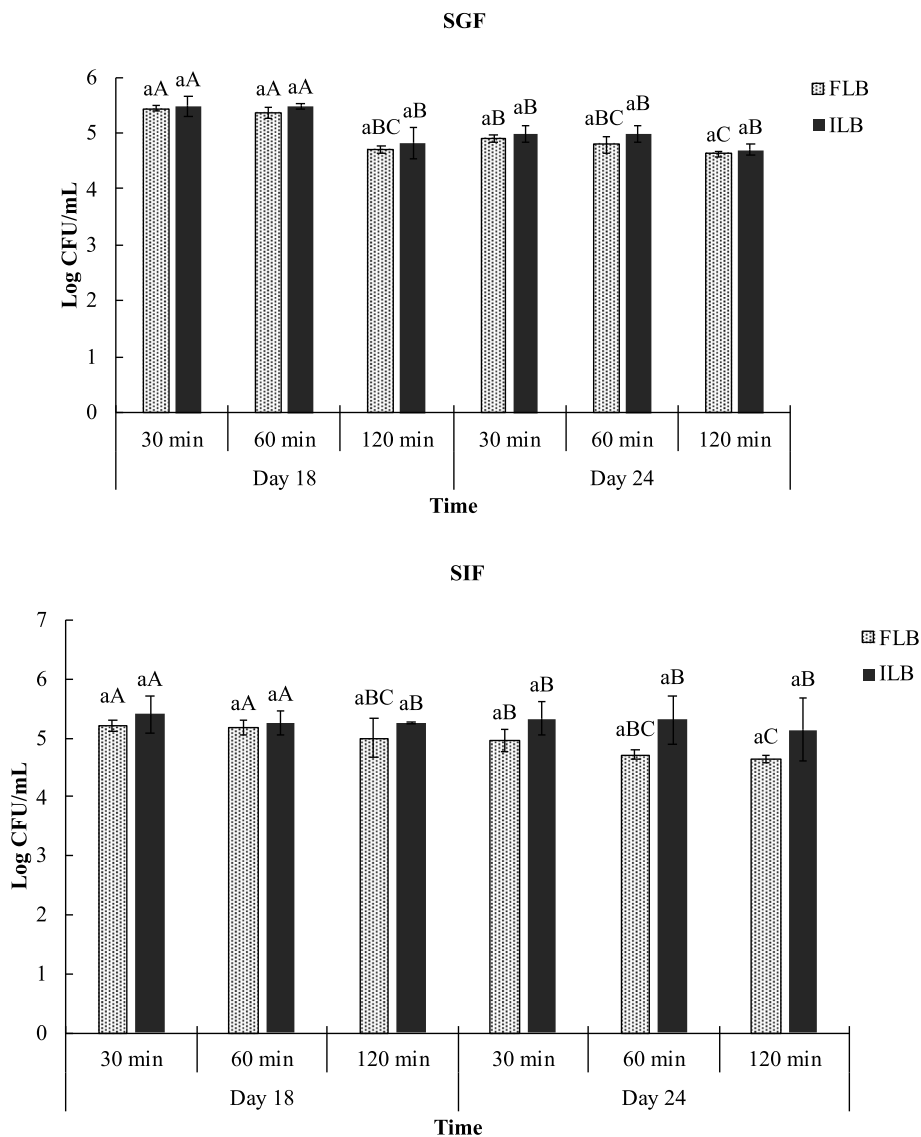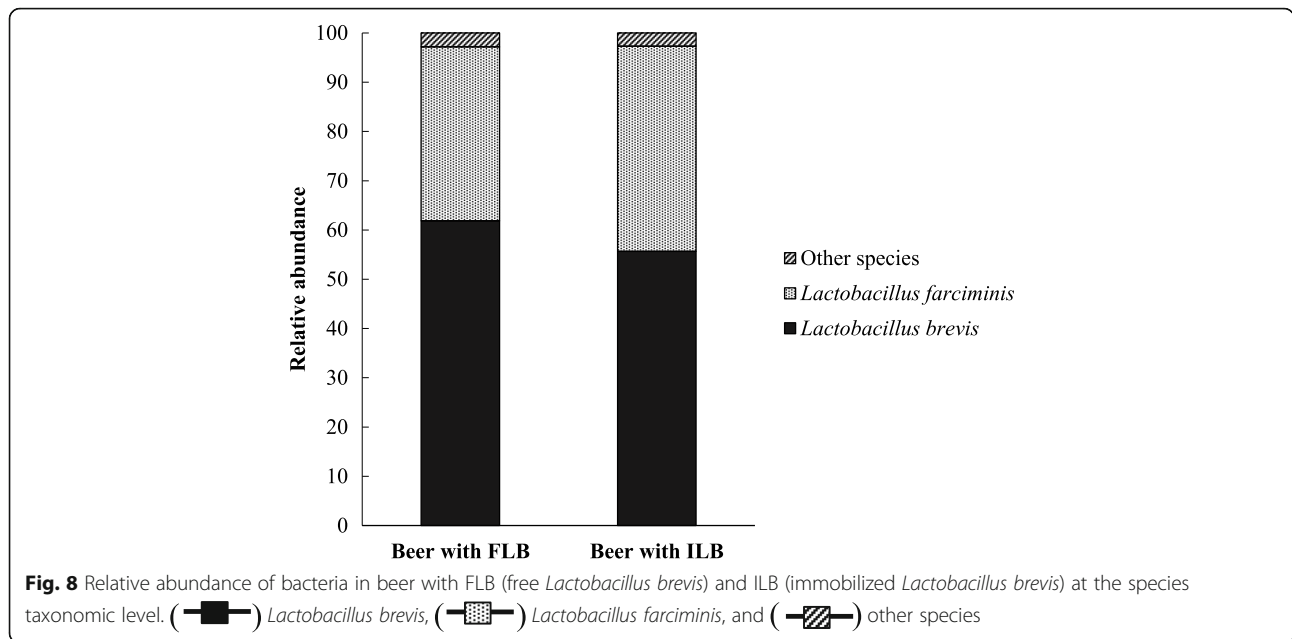
Fig. 7 Viability of FLB and ILB in ale beer during 24 days of storage at 21 °C followed by exposure to SGF (pH = 3.0) and SIF (pH = 6.5). [a]Means with no letter in common between treatments within the same incubation time are significantly different ($P < 0.05$). [A-C]Means with no letter in common within the same treatment across incubation times are significantly different ($P < 0.05$). FLB (⬛) = free *Lactobacillus brevis* and ILB (⬛) = immobilized *Lactobacillus brevis*

presence of *L. brevis* might have stimulated the growth of *L. farciminis* until the end of the storage period, with the latter having a slightly higher relative proportion when immobilized in DRP. While the microbial counts obtained from plating may have taken into account *L. farciminis*, the microbial diversity at different points of fermentation needs to be explored further. Nonetheless, the most dominant *L. brevis* and *L. farciminis* are probiotic strains, which show the potential for providing health benefits to beer consumers. Other bacterial species were present in minute amounts as shown in Fig. 8.

In this study, the fungal microbiome was characterized as presented in Table 2. Sequences associated with *S. cerevisiae* represented at least 99.9% in both beers. In a study by Spitaels et al. (2014), *S. cerevisiae* was the most prevalent species until 2 weeks of fermentation of lambic beer. Other fungal species were detected at less than 0.1% of the sequences using bTEFAP.

From this approach, it is proposed to study the synergistic relationships among the microbial communities present as well as the correlations between the abundance of particular microbes and the production of certain metabolites. Specific microorganisms may play a

Fig. 8 Relative abundance of bacteria in beer with FLB (free *Lactobacillus brevis*) and ILB (immobilized *Lactobacillus brevis*) at the species taxonomic level. (▬■▬) *Lactobacillus brevis*, (▬▦▬) *Lactobacillus farciminis*, and (▬▨▬) other species

role in metabolite production at different stages of beer production. The causality between the microbial diversity and organoleptic properties, such as beer flavor, can eventually be determined. Omics can therefore also be used to emphasize genes that are responsible for the health-associated benefits of the probiotic bacteria present (Walsh et al. 2016).

Conclusions

Ale beer containing free *L. brevis* and cells immobilized in DRP was developed. SEM micrographs illustrated random distribution and attachment of bacterial cells to DRP. The specific gravity, total soluble solids, pH, and titratable acidity of beer with FLB and beer with ILB did not differ significantly between the beers at 24 days of storage at 21 °C. ILB had significantly higher counts at day 0, day 6, and day 12, demonstrating that DRP conferred protection up to 12 days of ambient storage. However, the counts were not significantly different at the end of the storage period. The differences were not significant in simulated gastric and intestinal conditions, implying that beer with both forms of *L. brevis* could supply approximately 5 log CFU/mL. Results showed that the predominant species was *L. brevis* at 61.88 and

55.68% in beer with FLB and beer with ILB, respectively, followed by *L. farciminis* with respective relative percentages of 35.31 and 41.63%. Sequences with *S. cerevisiae* represented at least 99.99% in both beers. In conclusion, ale beer containing free *L. brevis* and immobilized cells in durian rind powder has potential as a delivery system for probiotics. Future research is needed to investigate the organoleptic properties and consumer acceptability of the beer as well as the cell viability in refrigerated conditions. It is important to highlight that in this study, no health benefits from the potential delivery system were investigated. However, it would be important in the future to investigate the effectiveness of the probiotic delivery system and its influence on gut microbiota and gut inflammation using in vivo models.

Table 2 Relative abundance of fungi in beer with FLB and ILB at the species taxonomic level

Genus	Species	Beer with FLB	Beer with ILB
Saccharomyces	*cerevisiae*	99.9	99.8
Brettanomyces	*bruxellensis*	0.05	0.04
	Other species	0.02	0.05

Availability of data and materials
All data generated during this study are included in this published article. Further details are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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