Sains Malaysiana 51(10)(2022): 3401-3414
http://doi.org/10.17576/jsm-2022-5110-23

Isolation of Lactic Acid Bacteria from Cocoa Bean Fermentation as Potential Antibacterial Agent against ESKAPE Pathogens

(Pemencilan Asid Laktik Bakteria daripada Penapaian Biji Koko sebagai Agen Antibakteria yang Berpotensi terhadap Patogen ESKAPE)

NORAZIAH MOHAMAD ZIN[1,*], AUNI NADZIRAH ABD RASHID[1], NUR ASYIKIN ZULKHAIRI[1], NUR AQILAH RIDZMAN[1], KHAIRUL BARIAH SULAIMAN[2], NUR FAIZAH ABU BAKAR[1] & ASIF SUKRI[1]

[1]Programme of Biomedical Science, Centre of Diagnostic, Therapeutic & Investigative Studies (CODTIS), Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Federal Territory, Malaysia
[2]Cocoa Research & Development Centre Hilir Perak, Malaysian Cocoa Board, P. O. Box 30, Sg. Dulang Road, 36307 Sg Sumun, Perak Darul Ridzuan, Malaysia

Received: 26 April 2022/Accepted: 2 August 2022

ABSTRACT

This study aimed to evaluate the antibacterial activity of microorganisms isolated before and during cocoa fermentation against ESKAPE pathogens. Microorganisms from cocoa fermentation process were isolated on the selective media and were tested against ESKAPE pathogens. Total titratable acidity, minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of cell-free supernatant from lactic acid bacteria (LAB) were determined. The presence of bacteriocin and antibacterial activity were determined. LAB was identified through molecular and biochemical tests. Ninety-five and 134 isolates were obtained from the fermentation of Sungai Balung 25 (BR25) clone and mixed clones, respectively. Screening of antibacterial activity showed that 26 isolates of LAB from the cocoa fermentation process had antibacterial activity against ESKAPE pathogens (zone of inhibition ≥11 mm). Cell-free supernatant from some LAB demonstrated potent antibacterial activity against some ESKAPE pathogens through MIC and MBC and evaluation. Furthermore, one isolate (mix48M01) exhibited antibacterial activity against *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterobacter* spp., methicillin-resistant *Staphylococcus aureus*, and *Enterococcus raffinousus*. An increase in the diameter of inhibition zone was observed as the lactic acid production increased. Gram-positive bacteria isolates were more resistant to LAB than Gram-negative bacteria. Bacteriocin with good antibacterial activity was also detected from the LAB examined. Molecular analysis showed that 13 LAB shared >99% sequence similarity to *Lactobacillus fermentum* CECT 562 while one isolate shared sequence similarity <98%, indicating that it might be a novel *Lactobacillus* species. Isolated LAB from this study exhibited high antibacterial activity against ESKAPE pathogens and could be investigated further as potential probiotics and antibacterial agents in the future.

Keywords: Antibacterial; cocoa fermentation; ESKAPE pathogens; lactic acid bacteria; *Lactobacillus*

ABSTRAK

Kajian ini bertujuan menilai aktiviti antibakteria mikroorganisma dipencilkan daripada sebelum dan selepas penapaian koko. Mikoorganisma daripada penapaian koko dipencilkan di atas media selektif dan diuji ke atas patogen ESKAPE. Keasidan boleh titrat, kepekatan perencat minimum (MIC) dan kepekatan bakterisid minimum (MBC) daripada supernatan tanpa sel bakteria laktik asid (LAB) ditentukan. Kehadiran bakteriosin dan aktiviti antibakteria ditentukan. LAB dikenal pasti menggunakan kaedah biokimia dan molekul. Sembilan puluh lima dan 134 pencilan masing-masing dipencilkan daripada klon Sungai Balung (BR25) dan klon campuran. Saringan aktiviti antibakteria menunjukkan 26 pencilan mempunyai aktiviti terhadap patogen ESKAPE (zon perencatan ≥11 mm). Supernatan tanpa sel menunjukkan aktiviti antibakteria melalui penilaian MIC dan MBC. Satu pencilan (mix48M01) menunjukkan aktiviti terhadap *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterobacter* spp., rintangan

metisilin *Staphylococcus aureus* dan *Enterococcus raffinousus.* Peningkatan diameter zon perencatan dapat diperhatikan apabila kepekatan asid laktik bertambah. Bakteria Gram positif adalah lebih rintang terhadap LAB berbanding dengan Gram negatif. Bakteriosin dengan aktiviti antibakteria yang baik turut dikesan. Analisis molekul menunjukkan 13 pencilan mempunyai penjujukan DNA sama >99% dengan *Lactobacillus fermentum* CECT 562, manakala satu pencilan mempunyai penjujukan <98%, mencadangkan spesis *Lactobacillus* yang baharu. Pencilan LAB yang dipencilkan dalam kajian ini menunjukkan aktiviti antibakteria yang baik terhadap patogen ESKAPE dan boleh dikaji dengan lebih mendalam lagi pada masa hadapan.

Kata kunci: Antibakteria; asid laktik bakteria; *Lactobacillus*; patogen ESKAPE; penapaian koko

Introduction

ESKAPE is an acronym given to *Enterococcus faecium*, *Staphyloccocus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa,* and *Enterobacter* sp. that are usually responsible for nosocomial infections and resistant to multiple antibiotics (Mulani et al. 2019). The rise in antibiotic resistance among ESKAPE pathogens contribute to worldwide economic burden and high mortality and morbidity (Zhen et al. 2019). World Health Organization (WHO) has prioritized the research and discovery of new antibiotics against carbapenem-resistant *A. baumannii*, carbapenem-resistant *P. aeruginosa*, carbapenem-resistant *Enterobacteriaceae*, methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (Tacconelli et al. 2018). Given the importance of research and discovery of novel antibiotics against ESKAPE pathogens, multiple approaches such as multiple antibiotics combination for treatment, bacteriophage therapy, antibacterial peptides and silver nanoparticles have been employed (Mulani et al. 2019). Lactic acid bacteria (LAB) from food fermentation can serve as a new antibacterial agent because they are generally regarded as safe (GRAS) by Food and Drug Administration (FDA). Studies have since demonstrated antibacterial properties of *Lactobacillus* sp. isolated from food fermentation against pathogenic bacteria, i.e., *Listeria monocytogenes*, *Escherichia coli*, *S. aureus*, *Salmonella typhimurium,* and carbapenem-resistant *Enterobacteriaceae* (Ahmad et al. 2018; Chen et al. 2019; Tamang et al. 2016). Furthermore, a study conducted on goat milk fermentation also showed that metabolites released by probiotics during fermentation process also possessed antioxidant activity that can be beneficial to the host, apart from their antibacterial activity (Moreno-Montoro et al. 2017). Clinical trial on probiotics in post-surgery colorectal cancer also showed that the supplementation of probiotics reduced the pro-inflammatory cytokines secretion in the patients for promising application in cancer immunotherapy (Zaharuddin et al. 2019). Taken together, these results demonstrate the promising application of probiotics and traditional fermented foods for the host's health benefits. In Malaysia, cocoa is considered a legacy that has been commercialized since 1950, and this country was once one of the biggest cocoa producers in the world (Azhar & Lee 2004). Cocoa bean fermentation is an essential step in the production of aromatic flavor for chocolate production. Multiple microorganisms are involved during cocoa fermentation that usually lasts for six days. Initially, yeast dominates cocoa fermentation, resulting in ethanol production and an increase in temperature and pH. This condition is suitable for LAB to eliminate the yeast population. Production of acetic acid by LAB favors the growth of acetic acid bacteria (AAB). Microbial diversity during multiple steps of cocoa bean fermentation presents a golden opportunity for isolation of bacteria and fungi groups, namely LAB, acetic acid bacteria and yeasts, to discover new antibacterial agents for research (Vuyst & Leroy 2020). Cocoa crude husks extract, products from aerobic fermentation, has exhibited antibacterial properties against *P. aeruginosa* and *Salmonella choleraesuis* (Santos et al. 2014), and LAB and yeasts isolated from cocoa fermentation inhibited the growth of filamentous fungi (Romanens et al. 2019). However, research and discovery of new antibacterial agent from microorganisms isolated from cocoa fermentation is scarce. Given the promising application of live bacteria from cocoa bean fermentation as a source of new antibacterial agents, this research was undertaken 1) to identify microbial groups during cocoa fermentation, 2) to screen antibacterial activity of isolated microorganisms from cocoa fermentation against ESKAPE pathogens, and 3) to identify microbial species with antibacterial activity against ESKAPE pathogens.

Materials and Methods

Bacterial strain and growth conditions of ESKAPE pathogens

ESKAPE pathogens, namely *E. raffinosus* (ATCC 49464), methicillin-resistant *S. aureus* (MRSA) (ATCC 43300), *K. pneumoniae* (ATCC 13883), and *E. aerogenes* (ATCC 51697), were purchased from American Type Culture Collection (ATCC, Virginia, USA) while *A. baumannii* and *P. aeruginosa* were frozen clinical isolates obtained from the Bacteriology Unit, Department of Diagnostic Laboratory Service, Hospital Canselor Tuanku Muhriz, Kuala Lumpur. All bacteria were cultured on Mueller-Hinton agar (Oxoid, Basingstoke, UK). Antibacterial susceptibility test on *A. baumannii* and *P. aeruginosa* was conducted according to standard protocol (CLSI 2017).

Sample collection

All samples were collected from Malaysian Cocoa Board plantations located at Bagan Datoh, Perak, Malaysia. BR25 and KKM22 clones were used for the fermentation process. Both BR25 and KKM22 are clones that originated from Malaysia. Cocoa pods were collected from *Theobroma cacao* trees. Pods were cut open using a machete; beans were scooped out from pods and placed into a bucket. Fermentation of cocoa beans was conducted in a wooden heap box with 18 kg of beans for the BR25 clone and 9 kg each for mixed clones of BR25 and KKM22. The fermentation of the KKM22 clone alone was not conducted because the fermentation of this clone is already well understood. Fermentation was conducted for 120 h at room temperature (28-29 °C), and samples were taken at 0, 12, 24, 36, 48, 60, 72, 84, 96, 108, and 120 h of fermentation. Five cocoa beans were taken at each depth of three different depths of layers (5, 15 and 30 cm) at each sampling interval, were weighed and placed into a sterile Falcon© tube with 45 mL of 0.1% peptone water. Thermometers were placed at a depth of 5 cm (top layer), 15 cm (middle layer) and 30 cm (bottom layer) in the fermentation box and temperatures were recorded at days 0, 1, 2, 3, 4, and 5. Swabs from the environment were taken from five different points: the outer layer of cocoa pods, basket for collecting cocoa pods, machetes, farmer's gloves, and fermentation box using sterile cotton swabs. The swab samples were then placed into a sterile centrifuge tube containing 0.1% of peptone water (weight/volume).

Enumeration of microorganisms from cocoa before and during fermentation

Isolation and enumeration of microorganisms from cocoa fermentation were conducted using protocol as described previously with modifications (Meersman et al. 2013). Cocoa beans were homogenized aseptically for three minutes using sterile mortar and pestle. Then 1.0 mL homogenate was serially diluted in 0.1% peptone water at 10-fold serial dilution (from 10^{-1} to 10^{-8} dilution factors). A total of 0.1 mL aliquots were aseptically spread on different selective agar media and incubated at 37 °C for 1-4 days. All plates were incubated aerobically. The monitoring, enumeration, and isolation of a specific group of microorganisms were conducted using the plate count agar (PCA) (Merck, Darmstadt, Germany) for the total aerobic bacterial count, yeast extract agar (YEA) (Merck, Darmstadt, Germany) for yeast, YEA agar containing 22% (weight/volume) calcium carbonate for acetic acid bacteria (AAB) and de Man-Rogosa-Sharpe (MRS) (Merck, Darmstadt, Germany) agar for LAB. The experiment was performed in duplicates. Enumeration of bacteria on plates using plate count method was calculated using colony-forming unit (CFU)/mL.

For isolation of bacteria, each colony formed on the plates was picked and recultured onto the respective agar media to isolate a single colony and incubated at the conditions described above.

Preparation of cell-free supernatant from LAB strains

Preparation of bacteria in suspension and cell-free supernatant from LAB strains were conducted as previously described with modification (Ahmad et al. 2018). The LAB isolated from cocoa fermentation (10^8 CFU/mL) was cultured in 10 mL MRS broth suspension (Merck, Darmstadt, Germany) aerobically at 37 °C for 24 h using orbital shaker incubator at 100 rpm shaking (Stuart, Staffordshire, UK). *K. pneumoniae*, *P. aeruginosa*, and *Enterobacter* spp. were cultured in 10 mL nutrient broth (Merck, Darmstadt, Germany) at a similar condition to the LAB isolates. To determine cell concentration of each bacteria, we generated the respective standard growth curve in our laboratory. Bacterial absorbance was measured at 600 nm (A_{600nm}) using spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). To collect cell-free supernatant, the LAB culture was centrifuged at 800 x g for 3 min and then filtered.

ANTIBACTERIAL ASSAY AGAINST ESKAPE PATHOGEN

A perpendicular streak test was carried out for all bacteria obtained from PCA and AAB from GYC agar. Each ESKAPE bacteria was streaked horizontally while the isolates from cocoa fermentation were streaked vertically on Mueller-Hinton agar (MHA) (Oxoid, Basingstoke, UK). For LAB, the agar well diffusion method was performed. A total of 100 µL (10^8 CFU/mL) of the pathogenic bacterial suspension was spread on the entire MHA surface using a plate spreader or 'hockey stick'. A 7-mm diameter well was punched aseptically onto the MH agar by using a blue micropipette tip. Next, 100 µL of MRS broth containing an overnight LAB culture (10^8 CFU/mL) was seeded into the well, and the plate was incubated at 37 °C for 24 h. LAB isolated from commercialized yoghurt products (Yakult, Japan), identified as *Lactobacillus casei* was used as a positive control in this experiment. LAB isolated from commercialized yoghurt products from Yakult was used because numerous studies have shown the efficacy of this strain as an antibacterial agent (Liévin-Le Moal & Servin 2014). A similar agar well diffusion method was performed to determine the antibacterial activity of cell-free supernatant obtained from LAB against ESKAPE pathogens. However, cell-free supernatant was pipetted into well instead of broth culture containing LAB isolates.

pH AND TITRATABLE ACIDITY OF CELL-FREE SUPERNATANTS

A pH of cell-free supernatants obtained from four LAB strains (BR48M01, BR72M01, BR84M01, and mix48M01) was measured using a pH meter (Sartorius, Göttingen, Germany). To determine titratable acidity of cell-free supernatants, 1 mL cell-free supernatant was titrated with 0.1 mol/L sodium hydroxide with phenolphthalein as an indicator. Titratable acidity was observed and calculated at four different time points: 10, 14, 18, and 22 h of growth.

MINIMAL INHIBITORY CONCENTRATION (MIC) AND MINIMAL BACTERICIDAL CONCENTRATION (MBC) OF CELL-FREE SUPERNATANT

MIC and MBC of cell-free supernatant produced by LAB (mix48M01) in our study were determined using the procedure as previously described with modifications (Basri et al. 2012). *K. pneumoniae*, *P. aeruginosa*, and *Enterobacter* spp. at a concentration of 1.5×10^8 CFU/mL in 100 µL nutrient broth was pipetted into 96-well plates. The concentration of cell-free supernatant was diluted using MRS broth to result in 10% to 100% concentrations. LAB from commercialized yoghurt and sterile nutrient broth served as the positive and negative control, respectively. Then, 100 µL cell-free supernatants at different concentrations were pipetted into each well containing each ESKAPE pathogen. The plates were incubated at 37 °C for 24 h. The growth of the pathogens was observed after the incubation period. MIC is defined as the lowest concentration of cell-free supernatant that inhibits the visible growth of the pathogen. To determine MBC, the cultured broth without visible growth of the bacteria was cultured onto nutrient agar using the spread plate method and incubated for 24 h. MBC is defined as the lowest concentration of cell-free supernatant at which no growth of the bacterial colony was observed. All experiments were performed in duplicates.

DETERMINATION OF PRESENCE OF BACTERIOCIN

LAB namely BRM01, BR72M01, BR84M01, mix48M01, and SY1 (LAB from commercial yogurt) were cultured in broth suspension as described above. Then, 1 mL of culture was taken at 8^{th}, 10^{th} and 12^{th} hour of exponential phase and centrifuged at 10000 × g for 15 min at 4 °C, followed by filtration using 0.22 µm filter to obtain crude extract. SDS PAGE was prepared using 15% separating gel and 6% stacking gel. Fifteen µL sample was mixed with loading buffer and loaded into gel well. The gel was stained silver stain and visualized using gel documentation.

ANTIBACTERIAL ACTIVITY OF CRUDE BACTERIOCIN EXTRACT

To determine antibacterial activity of crude bacteriocin extract, well diffusion assay was performed as described above. The ESKAPE pathogens were spread onto the agar plates and 100 µL of crude bacteriocin extract was placed into the well. The plates were incubated at 37 °C overnight.

IDENTIFICATION OF LAB USING API50 CH

Identification of LAB using API50 CH was performed as described by the manufacturer (Biomerieux, Marcy-l'Étoile, France).

IDENTIFICATION OF LAB AT THE SPECIES LEVEL

Identification of LAB was performed by 16S rRNA sequencing. DNA of LAB was extracted using a Wizard® Genomic DNA Purification Kit according to manufacturer (Promega, Wisconsin, USA). Amplification of the gene and sequencing of 16S rRNA were performed

as described previously (Sukri et al. 2021). The sequences have been deposited in National Center for Biotechnology Information (NCBI) database under accession number: MW325958 to MW325971.

Results

Temperature and time for sampling of BR25 clone and mixed clone

A slight difference in the average starting temperature between the BR25 clone (31 °C) and the mixed clone (31.3 °C) was observed and recorded as in Table Supplementary 1 (Table S1). The highest temperature in the cocoa fermentation process was recorded at 96 h, with an average temperature of 46.3 °C for both clones. At the last hour of 120 h, the BR25 clone had an average temperature of 44.7 °C, while the mixed clone had an average temperature of 44.3 °C. BR25 clone and mixed clone were comparably different in initial temperature rise (1 to 2 °C). This result suggests that cocoa fermentation begins as early as 24 h for a mixed clone than BR25 clone (single clone).

Enumeration of microorganisms before cocoa fermentation

The highest number of bacteria (7.5×10^6 CFU/mL) was isolated from the basket, followed by machetes (5.6×10^6 CFU/mL) and outer layer of cocoa fruit (1.2×10^6 CFU/mL). The number of LAB was similar in both baskets and machetes (3×10^6 CFU/mL) (Figure S1). For the fermentation box samples, only LAB were observed (1.3×10^6 CFU/mL). For glove samples, the concentrations for all types of bacteria, LAB, AAB and yeast were 2×10^6 CFU/mL, 0.8×10^6 CFU/mL, 0.1×10^6 CFU/mL and 0.2×10^6 CFU/mL, respectively. Further, for the machete samples, the concentrations for all types of bacteria: LAB and yeast were 5.6×10^6 CFU/mL, 3×10^6 CFU/mL and 0.1×10^6 CFU/mL, respectively (Figure S1).

Enumeration of microorganisms during BR25 clone fermentation

The growth of all types of bacteria and LAB was observed to be the highest at 60 h of fermentation, while a low number throughout fermentation was observed in AAB. The growth of yeast was the highest at 60 h and 108 h (Figure S2). At 48 h, the growth of all types of bacteria, LAB and yeast were 1.95×10^6 CFU/mL, 4×10^6 CFU/mL and 2.85×10^6 CFU/mL, respectively. At 60 h, the growth of LAB became the most prominent with 2.13×10^7 CFU/mL, followed by all types of bacteria with 6.8×10^6 CFU/mL and yeast with 2.75×10^6 CFU/mL. Additionally, at 108 h, the most noticeable growth was yeast with 4.55×10^6 CFU/mL and LAB with 1.15×10^6 CFU/mL. At 120 h, only the growth of LAB was observed at 2.55×10^6 CFU/mL.

Enumeration of microorganisms during mixed clone fermentation

Based on Figure S3, the growth of all types of bacteria and LAB was the highest at 108 h with 2.9×10^7 CFU/mL and 1.98×10^7 CFU/mL, respectively. For yeast, the growth was low throughout the mixed clone fermentation process. In addition, the growth of AAB peaked at 0 h, followed by 60 h and 96 h (4.65×10^6 CFU/mL, 2.5×10^6 CFU/mL and 2.75×10^6 CFU/mL). At 48 h, the growth of LAB (5.35×10^6 CFU/mL), growth of AAB (2×10^6 CFU/mL) and growth of all types of bacteria (2×10^6 CFU/mL) were observed during cocoa fermentation. At 60 h, the growth of LAB was 3.25×10^6 CFU/mL. In addition, at 72 h, the growth for all types of bacteria was 2.25×10^6 CFU/mL, while for AAB, the growth was 2.8×10^6 CFU/mL. The optimal conditions for LAB growth from mixed clones were at 48 h and 108 h.

Antibacterial assay against ESKAPE pathogens

The result showed that *A. baumannii* used in our study was resistant to carbapenem while *P. aeruginosa* was susceptible. ESKAPE pathogens were cultured onto standard selective agar media, respectively. Fifteen LAB isolates obtained from the fermentation of mixed clones (BR25 and KKM22) demonstrated zone of inhibition against at least one type of six ESKAPE pathogens examined, while only 11 LAB isolates from the fermentation of single clone (BR25) were observed to have zone of inhibition against at least one type of six ESKAPE pathogens examined. For Gram-negative bacteria, 14 LAB isolates had inhibition zones against *P. aeruginosa*, 11 isolates had inhibition zones against *Enterobacter* sp., 13 isolates had inhibition zones against *K. pneumonia,* and only 4 isolates had inhibition zone against *A. baumannii*. For Gram-positive bacteria, only two LAB isolates demonstrated an inhibition zone against *E. raffinosus,* and one isolate demonstrated an inhibition zone against MRSA. Isolate C48M02 had antibacterial activity to almost all ESKAPE pathogens except *Acinetobacter baumannii*. Of 26 LAB isolates that exhibited antibacterial activity to at least one ESKAPE pathogens, seven had antibacterial activity against two ESKAPE pathogens, and two had activity against three ESKAPE pathogens. LAB isolates mix48M01, and

mix48M02 from the fermentation of mixed clones had antibacterial activity against four ESKAPE pathogens (*K. pneumoniae*, *P. aeruginosa*, *Enterobacter* sp., and *E. raffinosus*) and five ESKAPE pathogens (*K. pneumoniae*, *P. aeruginosa*, *Enterobacter* sp., *E. raffinosus* and MRSA), respectively. Interestingly, strain mix48M02 was the only LAB isolate that conferred antibacterial activity against MRSA from this study, with a zone of inhibition diameter at 15 mm.

No inhibition zones against the ESKAPE pathogen were observed in 78 isolates of all bacteria from PCA agar and 60 isolates of AAB from GYC. The test could not be conducted for yeasts because they were not cultivable during the subculturing process. Twenty-six LAB isolates out of 118 isolates from MRS agar demonstrated inhibition zones to at least one of ESKAPE pathogens (Table 1).

TABLE 1. Zone of inhibition diameter against ESKAPE pathogens and identification of LAB

Zone of Inhibition Diameter (mm)							
Isolation no. (Lactic acid bacteria)	*K. pneumoniae*	*A. Baumannii*	*P. aeruginosa*	*Enterobacter* spp.	*E. raffinosus*	MRSA ATCC 33591	Identification of LAB using 16S rRNA
BR36M01	-	-	-	18	-	-	*Lactobacillus fermentum* CECT 562 (>99%)
BR36M02	15	-	-	-	-	-	*Lactobacillus fermentum* CECT 562 (>99%)
BR48M01	14	-	12	-	-	-	*Lactobacillus fermentum* CECT 562 (97.77%)
BR48M02	16	-	13	-	-	-	*Lactobacillus fermentum* CECT 562 (>99%)
BR48M05/1	-	-	-	19	-	-	*Lactobacillus fermentum* CECT 562 (>99%)
BR48M05/2	-	-	-	20	-	-	-
BR72M01	19	-	13	17	-	-	*Lactobacillus fermentum* CECT 562 (>99%)
BR84M01	12	-	-	18	-	-	*Lactobacillus fermentum* CECT 562 (>99%)
BR96M01	11	-	12	-	-	-	-
BR108M01	10	11	-	-	-	-	-
BR108M02	-	-	13	-	-	-	-
mix48M01	18	-	15	20	11	-	*Lactobacillus fermentum* CECT 562 (>99%)
mix48M02	13	-	11	20	11	15	-
mix48M03	-	-	-	19	-	-	*Lactobacillus fermentum* CECT 562 (>99%)
mix60M01/1	-	-	13	-	-	-	*Lactobacillus fermentum* CECT 562 (>99%)
mix60M01/2	-	-	15	-	-	-	*Lactobacillus fermentum* CECT 562 (>99%)
mix84M03	-	-	15	17	-	-	*Lactobacillus fermentum* CECT 562 (>99%)
mix96M02	19	-	-	-	-	-	*Lactobacillus fermentum* CECT 562 (>99%)
mix96M03	18	11	11	-	-	-	-
mix108M01	15	-	-	-	-	-	-
mix108M07/1	-	14	-	-	-	-	-
mix108M07/2	-	12	-	-	-	-	-
mix108M07/3	19	-	-	-	-	-	-
mix108M09	-	-	13	-	-	-	-
mix108M10	-	-	13	-	-	-	*Lactobacillus fermentum* CECT 562 (>99%)
mix120M05	-	-	15	15	-	-	-

TABLE 2. pH value (mean±SD) and total titratable acidity of cell-free supernatant obtained from four LAB strains

Isolates	pH				Titratable acidity (mg/mL)			
	10 h	14 h	18 h	22 h	10 h	14 h	18 h	22 h
BR48M01	4.25 ± 0.005	4.29 ± 0.008	4.25 ± 0.008	4.22 ± 0.012	10.81	11.53	8.29	11.17
BR72M01	4.25 ± 0.008	4.22 ± 0.005	3.97 ± 0.005	3.98 ± 0.012	10.81	11.53	10.09	15.49
BR84M01	4.26 ± 0.008	4.27 ± 0.016	4.21 ± 0.008	4.24 ± 0.012	10.45	10.81	7.57	10.09
mix48M01	4.21 ± 0.008	4.21 ± 0.009	3.94 ± 0.005	3.90 ± 0.008	10.09	13.69	12.97	16.57

All experiments were performed in duplicates

Antibacterial activity of cell-free supernatants

Our data showed that cell-free supernatants obtained from BR48M01, BR72M01, BR84M01, and mix48M01 had a large zone of inhibition against *K. pneumoniae*, *P. aeruginosa,* and *Enterobacter* spp. as the time of incubation increased from 10 h to 22 h (supplementary Table S2, S3 and S4). Of note, cell-free supernatant obtained from BR72M01 and mix48M01 had a zone of inhibition diameters 20 mm and 22 mm, respectively. Furthermore, cell-free supernatant obtained from mix48M01 also demonstrated a large zone of inhibition diameter against *K. pneumoniae* at 18 mm.

pH measurement and titratable acidity of cell-free supernatants

The pH of cell-free supernatants obtained from LAB strains BR48M01 and BR84M01 were stable from 10 to 22 h (pH=4.24 to 4.29). However, the pH of cell-free supernatants obtained from strains BR72M01 and mix48M01 slightly decreased as time progressed from 10 to 22 h (pH decreased from 4.29 to 3.98 for BR72M01 and 4.21 to 3.90 for mix48M01). Table 2 shows the total titratable acidity of lactic acid of cell-free supernatants from those LAB strains examined. Increased total titratable acidity was observed in all cell-free supernatants as time progressed, in which a notable increase was observed for BR72M01 (from 10.81 mg/mL at 10 h to 15.49 mg/mL at 22 h) and mix48M01 (from 10.09 mg/mL at 10 h to 16.57 mg/mL at 22 h). No notable increase was observed for strains BR48M01 and BR84M01.

MIC AND MBC OF CELL-FREE SUPERNATANT

MIC and MBC of cell-free supernatant obtained from LAB strain mix48M01 were determined because the strain demonstrated a large zone of inhibition against *K. pneumoniae*, *P. aureginosa*, *Enterobacter* spp., and *E. raffinosus*. Table S5 shows the result for MIC and MBC of cell-free supernatant obtained from 48-hour culture of LAB. MIC of cell-free supernatant against *K. pneumoniae* was at 40% concentration while MBC was 50%. For *P. aeruginosa*, MIC was at concentration 20%, while MBC was at concentration 30%. Meanwhile, MIC and MBC of cell-free supernatant were respectively at a concentration of 30% and 40% for *Enterobacter* spp.

DETERMINATION OF BACTERIOCIN IN LAB

We detected the presence of proteins at molecular weight of 75 kDa and 45 kDa from crude bacteriocin extracts of BR48M01 LAB isolate at 8th, 10th and 12th h of exponential phase. In addition, we also detected the presence of proteins at molecular weight of 34 kDa and 18 kDa from BR48M01 12th h of exponential phase. The presence of proteins for other LAB isolates examined were not detected.

ANTIBACTERIAL ASSAY OF CRUDE BACTERIOCIN EXTRACT

We determined the antibacterial assay of crude bacteriocin extract isolated from four LAB isolates against *K. pneumoniae*, *P. aeruginosa,* and *Enterobacter* sp. All crude bacteriocin extracts showed antibacterial activity

against the pathogens with zone of inhibition >9 mm. Notably, BR72M01 and mix48M01 exhibited good zone of inhibition against all the pathogens tested.

IDENTIFICATION OF LAB

Thirteen out of 14 LAB were identified as *Lactobacillus fermentum* CECT 562 with more than 99% sequence similarity, while one clone (BR48M01) was also identified *Lactobacillus fermentum* CECT 562 with less than 98% sequence similarity (97.86%) (Table 1). As BR48M01 shared less than 98% sequence similarity to that of *Lactobacillus fermentum* CECT 562, we furthered identified this strain using a standard kit of API50 CH. From biochemical test result, BR48M01 was identified as *Lactobacillus brevis* 2 with a percentage of identification of 94.3%.

DISCUSSION

ESKAPE pathogens are resistant to multiple antibiotics and contribute to high mortality and economic burden in healthcare settings (Mulani et al. 2019; Taconelli et al. 2018). We aimed to isolate live bacteria from fermented cocoa beans and evaluate their potential as producers of antibacterial agents for ESKAPE pathogens. The highest count was seen in the basket for all types of bacteria, followed by machetes and gloves. A high abundance of LAB was detected in all environmental samples, suggesting environmental factors as a source of LAB during cocoa beans fermentation. In the box samples during the fermentation, only LAB growth was recorded because the box was washed every time after use to favour a clean state that can reduce the contamination with bacteria from the surroundings (Papalexandratou et al. 2013). LAB highly resilient in a different environment and can survive under dry conditions (George et al. 2018). Growth profiles of microorganisms isolated from cocoa fermentation at different time points were different for LAB, yeast, and AAB. The temperature increases for the mixed clone occurred first because the mixed clone contained the pods of the KKM22 clone, which has a sweeter layer of pulp and used as pulp juice (Chin 2006). The juice from the pulps attracts more yeast to ferment sugar into ethanol, leading to the temperature rising faster than the fermentation of the BR25 clone alone. The high growth of LAB in the mid-fermentation phase was observed because the yeast converts sugar into ethanol and carbon dioxide (Thompson et al. 2007). The growth of LAB at the end of the fermentation process in mixed clone was due to the high presence of lactic acid at the end of fermentation while the growth of LAB declined after 60 h in BR25 clone was due to the depletion of lactic acid after that time point (Pereira et al. 2012). We did not observe the growth of AAB and yeast in the mixed clone because the samples were stored at unsuitable temperature (-18 °C). Thus, the microorganisms could not survive (Bullerman 2004; Gomes et al. 2018).

A larger zone of inhibition against ESKAPE pathogens was observed in LAB isolated from mixed clones than single clones because mixed fermentation may cause microorganisms to synthesize more effective secondary metabolites, including pestalone, levorin, istamycins and rhodostreptomycins, to increase bactericidal or bacteriostatic activity than just a single fermentation (Pettit 2009). LAB isolates at 48 h of cocoa fermentation process have an inhibition zone for ESKAPE pathogens because the time and condition were optimum for the growth of LAB (Camu et al. 2007).

Studies have shown that LAB has antibacterial activity against pathogens as they inhibit the binding of bacteria to the mucosa, improve the host's immune response and manufacture antibacterial metabolites and factors (Mathur et al. 2017). Bacteriocin is one of the factors produced by LAB that can interfere with the regulation of biofilm formation (Sharma et al. 2018). Some strains of LAB demonstrated antibacterial activity against *P. aeruginosa*, suggesting the efficacy of these bacteria in combating the biofilm formation of bacteria (Rasamiravaka et al. 2015). Interestingly, some strains of LAB isolated from cocoa fermentation had antibacterial activity against *A. baumannii* but not against other ESKAPE pathogens or vice versa. Antibacterial activity of LAB can be narrow or broad depending on the types of metabolites or factors they produce (Mathur et al. 2017). A striking difference between antibacterial activity of LAB against Gram-positive and Gram-negative was also observed. Gram-negative bacteria were more susceptible to LAB isolated from this study as compared to Gram-positive bacteria. Mechanisms of antibiotic resistance of bacteria include efflux pump at the cell membrane and gene mutation (Mutani et al. 2016). This result suggests a different mechanism of antibacterial activity of LAB against ESKAPE pathogens based on their cell membrane or efflux pump mechanism. We hypothesize that antibacterial compounds such as organic acids produced by LAB against ESKAPE pathogens might have better activity against the cell wall of Gram-negative bacteria, which has a thin peptidoglycan layer than Gram-positive bacteria. Further study on this phenomenon should be conducted in

the future. Of note, strain mix48M02 was the only strain that demonstrated antibacterial activity against five out of six ESKAPE pathogens which included MRSA. This strain also was the only isolate that exhibited inhibition zones against MRSA. Recent studies suggest that the pore-forming action leading to ATP efflux on biofilm cell activity is critical, and bacteriocins that can make stable pores on biofilm cells are highly effective as a treatment of MRSA (Okuda et al. 2013). Further studies have to be conducted to elucidate the antibacterial mechanisms of LAB against ESKAPE pathogens.

Our data showed that cell-free supernatants had high potency to inhibit the growth and kill some ESKAPE pathogens, namely *K. pneumoniae*, *P. aeruginosa,* and *Enterobacter* spp. through MIC and MBC evaluation. We attempted to elucidate the mechanism of antibacterial activity through the measurement of total titratable acidity. Zone of inhibition diameter of *K. pneumoniae*, *P. aeruginosa,* and *Enterobacter* spp. were observed to be larger as the concentration of lactic acid increased. Total titratable acidity increased during the 22 h in the LAB cultures of some isolates with good antibacterial activity against ESKAPE pathogens, suggesting that one of the antibacterial activity mechanisms of LAB cultures with good antibacterial activity could be attributed to increased acidity and pH lowering. A recent study also produced a similar result, in which the antibacterial activity mechanism of LAB could be attributed to the production of lactic acid that inhibited and killed the carbapenem-resistant *Enterobacteriaceae* (Chen et al. 2019). Furthermore, we also detected the presence of bacteriocin in the LAB examined with good antibacterial activity against *K. pneumoniae*, *P. aeruginosa*, and *Enterobacter* spp. However, we did not profile the metabolites produced by LAB that may have antibacterial activity against the pathogens. Future studies should be conducted to profile antibacterial molecules produced by LAB.

The cut-off value to identify the novel species is less than 98% similar to known species (Yoon et al. 2000). This study found that the clone BR48M01 was identified as *Lactobacillus fermentum* CECT 562 (97.86%) and might be novel species of *Lactobacillus*. Identification of BR48M01 using API50 CH identified BR48M01 as *Lactobacillus brevis* 2 with an identification percentage of 94.3%. This discrepancy can be explained by techniques employed to identify bacterial species, in which molecular test identification is more specific and robust compared to the traditional method of biochemical test. However, the identification of species could not be finalized because both tests resulted in low similarity identification. Further study to identify this species using more robust techniques such as whole genome sequencing should be conducted in the future.

Conclusion

In conclusion, LAB from cocoa fermentation demonstrated promising application as new antibacterial agent against ESKAPE pathogens and can be investigated further.

ACKNOWLEDGEMENTS

This study was supported by Universiti Kebangsaan Malaysia under grant no. GUP-2018-119. All authors declare no conflict of interest.

REFERENCES

Ahmad, A., Yap, W.B., Kofli, N.T. & Ghazali, A.R. 2018. Probiotic potentials of *Lactobacillus plantarum* isolated from fermented durian (Tempoyak), a Malaysian traditional condiment. *Food Sci. Nutr*. 6(6): 1370-1377.

Azhar, I. & Lee, M.T. 2004. Perspective for cocoa cultivation in Malaysia: Re-look at the economic indicators. *Malaysian Cocoa Journal* 1: 1-18.

Basri, D.F., Tan, L.S., Shafiei, Z. & Zin, N.M. 2012. *In vitro* antibacterial activity of galls of *Quercus infectoria* Olivier against oral pathogens. *Evid. Based Complement Alternat. Med*. 2012: 632796.

Bullerman, L.B. 2004. SPOILAGE | Fungi in food - An overview. In *Encyclopedia of Food Sciences and Nutrition,* 2nd ed., edited by Caballero, B. Massachusetts: Academic Press. pp. 5511-5522. https://doi.org/10.1016/B0-12-227055-X/01129-9

Camu, N., De Winter, T., Verbrugghe, K., Cleenwerck, I., Vandamme, P., Takrama, J.S., Vancanneyt, M. & De Vuyst, L. 2007. Dynamics and biodiversity of populations of lactic acid bacteria and acetic acid bacteria involved in spontaneous heap fermentation of cocoa beans in Ghana. *Appl. Environ. Microbiol*. 73(6): 1809-1824.

Chen, C.C., Lai, C.C., Huang, H.L., Huang, W.Y., Toh, H.S., Weng, T.C., Chuang, Y.C., Lu, Y.C. & Tang, H.J. 2019. Antimicrobial activity of *Lactobacillus* species against carbapenem-resistant *Enterobacteriaceae*. *Front Microbiol*. 10: 789.

Chin, H.H. 2006. Cocoa pulp juice. *Malaysia Cocoa* 3(1): 8-9.

CLSI. 2017. *Performance Standards for Antimicrobial Susceptibility Testing.* 27th ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute.

George, F., Daniel, C., Thomas, M., Singer, E., Guilbaud, A., Tessier, F.J., Revol-Junelles, A-M., Borges, F. & Foligné, B. 2018. Occurrence and dynamism of lactic acid bacteria in distinct ecological niches: A multifaceted functional health perspective. *Front Microbiol*. 9: 2899.

Gomes, R.J., Borges, M.F., Rosa, M.F., Castro-Gómez, R. & Spinosa, W.A. 2018. Acetic acid bacteria in the food industry: Systematics, characteristics and applications. *Food Technol. Biotechnol*. 56(2): 139-151.

Liévin-Le Moal, V. & Servin, A.L. 2014. Anti-infective activities of lactobacillus strains in the human intestinal microbiota: From probiotics to gastrointestinal anti-infectious biotherapeutic agents. *Clin. Microbiol. Rev*. 27(2): 167-199.

Mathur, H., Field, D., Rea, M.C., Cotter, P.D., Hill, C. & Ross, R.P. 2017. Bacteriocin-antimicrobial synergy: A medical and food perspective. *Front Microbiol*. 8: 1205.

Meersman, E., Steensels, J., Mathawan, M., Wittocx, P.J., Saels, V., Struyf, N., Bernaert, H., Vrancken, G. & Verstrepen, K.J. 2013. Detailed analysis of the microbial population in Malaysian spontaneous cocoa pulp fermentations reveals a core and variable microbiota. *PLoS ONE* 8(12): e81559.

Moreno-Montoro, M., Olalla-Herrera, M., Rufián-Henares, J.Á., Martínez, R.G., Miralles, B., Bergillos, T., Navarro-Alarcón, M. & Jauregi, P. 2017. Antioxidant, ACE-inhibitory and antimicrobial activity of fermented goat milk: Activity and physicochemical property relationship of the peptide components. *Food Funct*. 8(8): 2783-2791.

Mulani, M.S., Kamble, E.E., Kumkar, S.N., Tawre, M.S. & Pardesi, K.R. 2019. Emerging strategies to combat ESKAPE pathogens in the era of antimicrobial resistance: A review. *Front Microbiol*. 10: 539.

Mutani, J.M. & Arias, C.A. 2016. Mechanisms of antibiotic resistance. *Microbiol. Spectr*. 4(2): VMBF-0016-2015.

Okuda, K-I., Zendo, T., Sugimoto, S., Iwase, T., Tajima, A., Yamada, S., Sonomoto, K. & Mizunoe, Y. 2013. Effects of bacteriocins on methicillin-resistant *Staphylococcus aureus* biofilm. *Antimicrob. Agents Chemother.* 57(11): 5572-5579.

Papalexandratou, Z., Lefeber, T., Bakhtiar, B., Ong, S.L., Daniel, H.M. & De Vuyst, L. 2013. *Hanseniaspora opuntiae*, *Saccharomyces cerevisiae*, *Lactobacillus fermentum*, and *Acetobacter pasteurianus* predominate during well-performed Malaysian cocoa bean box fermentations, underlining the importance of these microbial species for a successful cocoa bean fermentation process. *Food Microbiol*. 35(2): 73-85.

Pereira, G.V., Miguel, M.G., Ramos, C.L. & Schwan, R.F. 2012. Microbiological and physicochemical characterization of small-scale cocoa fermentations and screening of yeast and bacterial strains to develop a defined starter culture. *Appl. Environ. Microbiol*. 78(15): 5395-5405.

Pettit, R.K. 2009. Mixed fermentation for natural product drug discovery. *Appl. Microbiol. Biotechnol.* 83(1): 19-25.

Rasamiravaka, T., Labtani, Q., Duez, P. & El Jaziri, M. 2015. The formation of biofilms by *Pseudomonas aeruginosa*: A review of the natural and synthetic compounds interfering with control mechanisms. *Biomed. Res. Int*. 2015: 759348.

Romanens, E., Freimüller Leischtfeld, S., Volland, A., Stevens, M.J.A., Krähenmann, U., Isele, D., Fischer, B., Meile, L. & Miescher Schwenninger, S. 2019. Screening of lactic acid bacteria and yeast strains to select adapted anti-fungal co-cultures for cocoa bean fermentation. *Int. J. Food Microbiol*. 290: 262-272.

Sharma, V., Harjai, K. & Shukla, G. 2018. Effect of bacteriocin and exopolysaccharides isolated from probiotic on *P. aeruginosa* PAO1 biofilm. *Folia Microbiol. (Praha)*. 63(2): 181-190.

Santos, R.X., Oliveira, D.A., Sodré, G.A., Gosmann, G., Brendel, M. & Pungartnik, C. 2014. Antimicrobial activity of fermented *Theobroma cacao* pod husk extract. *Genet. Mol. Res*. 13(3): 7725-7735.

Sukri, A., Saat, M.N.F., Mohd Yusof, N.A., Zin, N.M. & Abdul Rachman, A.R. 2021. Differences in antibiotic resistance profiles of methicillin-susceptible and–resistant *Staphylococcus aureus* isolated from the teaching hospital in Kuala Lumpur, Malaysia. *J. Appl. Biol. Biotechnol*. 9(4): 98-103.

Tacconelli, E., Carrara, E., Savoldi, A., Harbarth, S., Mendelson, M., Monnet, D.L., Pulcini, C., Kahlmeter, G., Kluytmans, J., Carmeli, Y., Ouellette, M., Outterson, K., Patel, J., Cavaleri, M., Cox, E.M., Houchens, C.R., Grayson, M.L., Hansen, P., Singh, N., Theuretzbacher, U., Magrini, N. & WHO Pathogens Priority List Working Group. 2018. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect Dis*. 18(3): 318-327.

Tamang, J.P., Watanabe, K. & Holzapfel, W.H. 2016. Review: Diversity of microorganisms in global fermented foods and beverages. *Front Microbiol*. 7: 377.

Thompson, S.S., Miller, K.B. & Lopez, A.S. 2007. Cocoa and coffee. In *Food Microbiology: Fundamental and Frontiers*, edited by Doyle, M. & Beuchat, L. ASM Press. pp. 837-850.

Vuyst, L.D. & Leroy, F. 2020. Functional role of yeasts, lactic acid bacteria and acetic acid bacteria in cocoa fermentation processes. *FEMS Microbiol Rev*. 44(4): 432-453.

Yoon, J.H., Kang, S.S., Mheen, T.I., Ahn, J.S., Lee, H.J., Kim, T.K., Park, C.S., Kho, Y.H., Kang, K.H. & Park, Y.H. 2000. *Lactobacillus kimchii* sp. nov., a new species from kimchi. *Int. J. Syst. Evol. Microbiol*. 50(5): 1789-1795.

Zaharuddin, L., Mokhtar, N.M., Muhammad Nawawi, K.N. & Raja Ali, R.A. 2019. A randomized double-blind placebo-controlled trial of probiotics in post-surgical colorectal cancer. *BMC Gastroenterol*. 19(1): 131.

Zhen, X., Lundborg, C.S., Sun, X., Hu, X. & Dong, H. 2019. Economic burden of antibiotic resistance in ESKAPE organisms: A systematic review. *Antimicrob. Resist. Infect. Control*. 8: 137.

*Corresponding author; email: noraziah.zin@ukm.edu.my

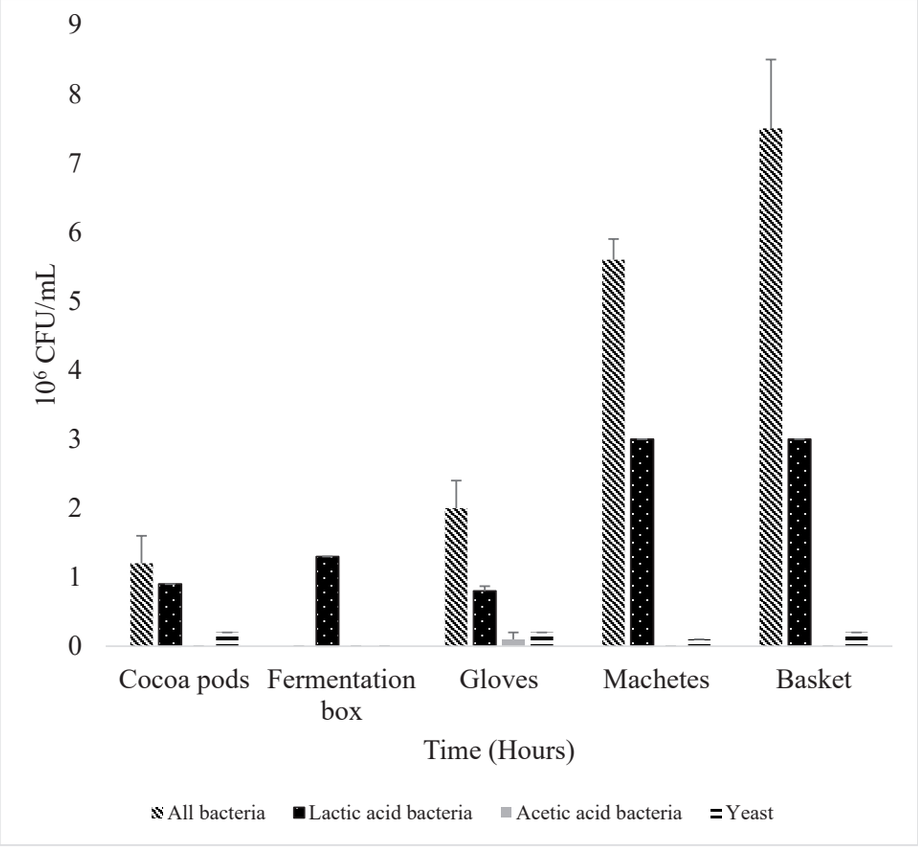

FIGURE S1. Average number of microbes from the environment before cocoa fermentation process. Error bars represent standard deviation

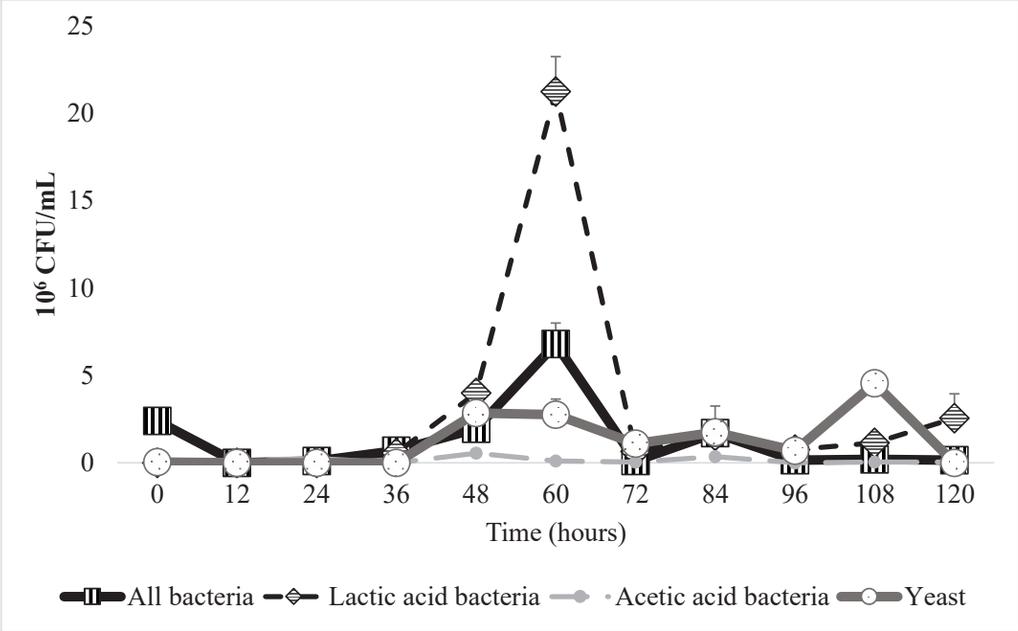

FIGURE S2. Average number of microbes from BR25 clone cocoa fermentation at different time points. The concentration of the bacteria was averaged from fermentation of cocoa beans at three different layers of fermentation. Error bars represent standard deviation from three different layers of cocoa fermentation performed in duplicates

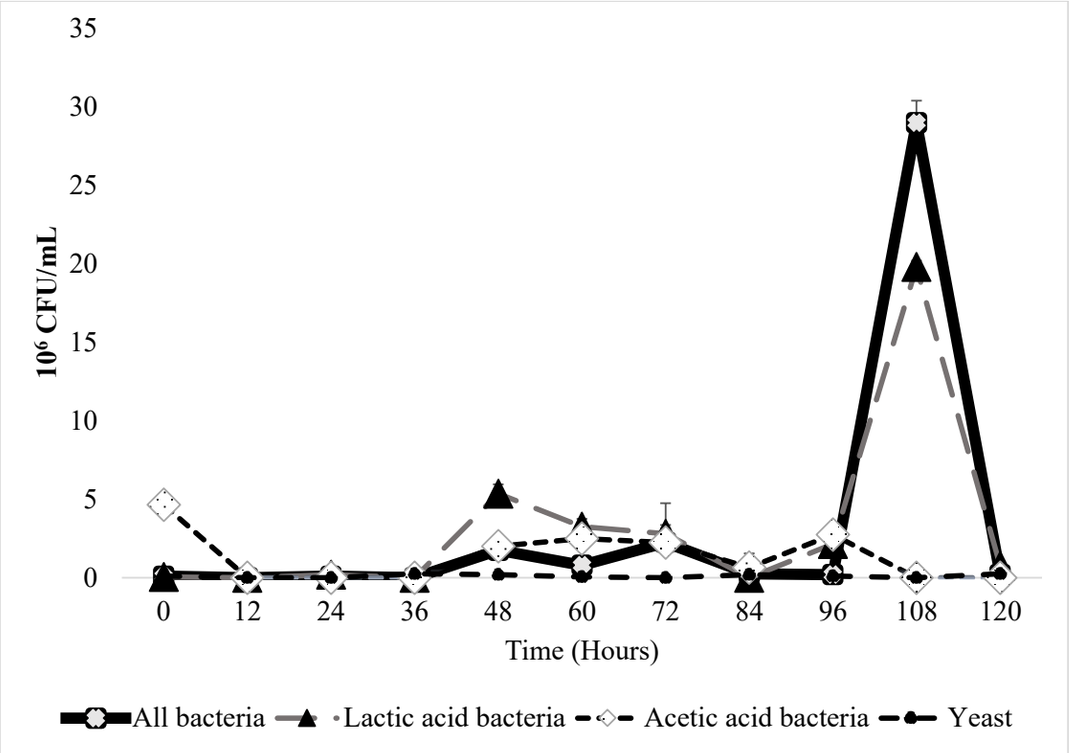

FIGURE S3. Average number of microbes from mixed clone cocoa fermentation at different time points. The concentration of the bacteria was averaged from fermentation of cocoa beans at three different layers of fermentation. Error bars represent standard deviation from three different layers of cocoa fermentation performed in duplicates

TABLE S1. Time and temperature of cocoa fermentation. Above denotes 5 cm of depth, middle denotes 15 cm of depth and bottom denotes 30 cm of depth

Time (Hour)	Temperature BR25 clone (°C)	Average (°C)	Temperature mixed clone (°C)	Average (°C)
0	Above: 31 Middle: 31 Bottom: 31	31	Above: 32 Middle: 31 Bottom: 31	31.3
12	Above: 31 Middle: 32 Bottom: 31	31.3	Above: 32 Middle: 32 Bottom: 31	31.7
24	Above: 33 Middle: 31 Bottom: 31	31.7	Above: 35 Middle: 37 Bottom: 34	35.3
36	Above: 40 Middle: 38 Bottom: 38	38.7	Above: 40 Middle: 38 Bottom: 38	38.7

48	Above: 43 Middle: 45 Bottom: 45	44.3	Above: 40 Middle: 42 Bottom: 45	42.3
60	Above: 42 Middle: 45 Bottom: 45	44	Above: 46 Middle: 45 Bottom: 45	45.3
72	Above: 45 Middle: 46 Bottom: 47	46	Above: 46 Middle: 45 Bottom: 44	45
84	Above: 46 Middle: 45 Bottom: 45	45.3	Above: 46 Middle: 47 Bottom: 45	46
96	Above: 47 Middle: 47 Bottom: 45	46.3	Above: 47 Middle: 47 Bottom: 45	46.3
108	Above: 47 Middle: 46 Bottom: 45	46	Above: 48 Middle: 45 Bottom: 45	46
120	Above: 46 Middle: 46 Bottom: 42	44.7	Above: 44 Middle: 46 Bottom: 43	44.3

TABLE S2. Zone of inhibition diameter cell-free supernatant against *Klebsiella pneumoniae*

Isolates	Diameter of inhibition zone (mm)			
	Klebsiella pneumoniae			
	10 h	14 h	18 h	22 h
(+) Control	13	13	14	18
(-) Control	0	0	0	0
BRM01	12	14	13	15
BR72M01	13	14	17	17
BR84M01	9	14	15	17
mix48M01	14	16	16	18

TABLE S3. Zone of inhibition diameter of cell-free supernatant against *Pseudomonas aeruginosa*

Isolates	Diameter of inhibition zone (mm)			
	Pseudomonas aeruginosa			
	10 h	14 h	18 h	22 h
(+) Control	19	18	18	19
(-) Control	0	0	0	0
BRM01	15	16	15	15
BR72M01	19	16	17	20
BR84M01	17	15	15	17
mix48M01	18	17	18	22

TABLE S4. Zone of inhibition diameter of cell-free supernatant against *Enterobacter* spp.

Isolates	Diameter of inhibition zone (mm)			
	Enterobacter spp.			
	10 h	14 h	18 h	22 h
(+) Control	18	17	18	19
(-) Control	0	0	0	0
BRM01	17	15	16	18
BR72M01	18	16	17	19
BR84M01	18	17	16	17
mix48M01	18	17	17	18

TABLE S5. MIC and MBC of cell-free supernatant obtained from LAB strain mix48M01

Pathogen	Concentration of cell-free supernatant (%)									
	100	90	80	70	60	50	40	30	20	10
K. pneumoniae	-	-	-	-	-	MBC	MIC	+	+	+
P. aeruginosa	-	-	-	-	-	-	-	MBC	MIC	+
Enterobacter spp.	-	-	-	-	-	-	MBC	MIC	+	+

-denotes no growth; + denotes normal growth; MBC denotes minimal bactericidal concentration and MIC denotes minimal inhibitory concentration