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LACTIC ACID BACTERIA ISOLATED FROM LOCALLY PRODUCED VINEGARS AND THEIR ANTIBACTERIAL ACTIVITY AGAINST FOODBORNE BACTERIA

AGNES LEE CHIU NEE[1], MOHD NIZAM LANI[1*], ROZILA ALIAS[2] AND ZAITON HASSAN[C3]

[1]*School of Food Science and Technology, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia.*
[2]*International Halal Institute Universiti Selangor, Universiti Selangor, Seksyen 7, 40000 Shah Alam, Selangor, Malaysia.*
[3]*Faculty of Science and Technology, Universiti Sains Islam Malaysia (USIM), 71800 Bandar Baru Nilai, Nilai, Negeri Sembilan, Malaysia.*

[*]*Corresponding author: nizamlani@umt.edu.my*

Abstract: Vinegars are most widely used as preservatives in food industry. Vinegars are known for their health benefits; however, the roles of vinegar-associated microflora in locally produced vinegars are not well established. The objectives of this study are to isolate and identify the lactic acid bacteria (LAB) from black rice vinegar and coconut vinegar, measure their pH and titratable acidity, and determine their antibacterial activity. LAB was isolated using cultural method. Phenotypic characterization of LAB was carried out using Gram-staining, oxidase test, catalase test and API 50 CHL Kit. Results from API 50 CHL Kit confirmed that BRV03M strain from black rice vinegar and CV03M strain from coconut vinegar were *Lactobacillus paracasei* ssp. *paracasei*. The identified bacteria in both samples were consistent as *L. paracasei* using 16S rDNA gene sequences with 93% and 99% similarity, respectively. The pH and titratable acidity percentage of both vinegars were also determined. The stability of Cell Free Supernatant-Lactic Acid Bacteria (CFS-LAB) strains within 14 days on their inhibition against selected pathogenic bacteria was determined using agar well diffusion method. The CFS-LAB strain isolated from black rice vinegar (BRV03M) was more stable within 14 days than coconut vinegar in inhibiting tested bacteria, suggesting this strain has great potential as natural antibacterial agents.

Keywords: antibacterial activity, black rice vinegar, coconut vinegar, pH, titratable acidity

Introduction

Vinegar is a product of two-stage successive microbial fermentation involving carbohydrate fermentation by yeasts to produce ethanol, followed by oxidative fermentation of ethanol by lactic acid bacteria (LAB) and acetic acid bacteria (AAB) to produce acetic acid (Yetiman & Kesmen, 2015). The varieties of vinegars can be defined based on the different types of raw materials used in their production. Black rice vinegar (kurosu) is produced by rice saccharification, alcohol fermentation, and oxidative fermentation of ethanol to acetic acid. Kurosu is produced using either submerged fermentation process or traditional static fermentation (Murooka *et al*., 2009).

Coconut vinegar is usually low in acidity compared to other vinegars and used widely in the cooking of Thai dishes. It has a musty flavour with a very unique aftertaste. The production of coconut vinegar involves sugar of the coconut sap or the mature coconut water fermented to ethanol within 8-12 hours by yeasts and lactic acid bacteria. Formation of ethanol creates a highly suitable medium for the growth of acetic acid bacteria (AAB). During fermentation, the acetic acid bacteria appear after 2-3 days. The microbial diversity in this vinegar reflects the variety of raw materials, sugar sources and processes, as well as the diversity of the physicochemical characteristics such as temperature, pH, and water activity. Nanda et al (2001) has reported detailed analysis of microorganisms during kurosu fermentation which belongs to *Acetobacter pasteurianus*.

Previous study has highlighted the roles of acetic acid bacteria (AAB) in vinegars (Murooka & Yamshita, 2008). AAB are usually the only bacteria that can grow and survive in fermenting vinegar broth that contains more than 2 to 3% acetic acid. Based on a report by Murooka & Yamshita (2008), *Acetobacter pasteurianus* are the dominant bacteria in black rice vinegar. Perumpuli et al. (2014) have isolated *Acetobacter pasteurianus* and *Gluconobacter frateurii* from coconut vinegar.

LAB are the brewery bacteria that are normally found in vinegars and fermented products (Liu & Han, 2014). LAB comprise of an ecologically diverse group of non-sporing Gram positive bacteria and produce lactic acid as primary metabolites of growth of either homofermentative or heterofermentative pathways (Fugelsang & Edwards, 2007). LAB are rod-shaped or cocci-shaped Gram-positive, acid-tolerant, non-spore forming, anaerobic bacteria that share common metabolic and physiological characteristics (Aween *et al.*, 2012). LAB can produce bioactive components such

as organic acid, free fatty acids, ethanol, benzoate, enzymes, hydrogen peroxide, antimicrobial peptides and antibiotics that have inhibitory spectrum against pathogenic bacteria (Olofsson*et al.,* 2016; Lani et al., 2015). These bacteria have strong antagonistic activity against bacteria and fungi (Aween *et al.,* 2012, Bulgasem *et al.,* 2016, Bulgasem *et al.,* 2017). As there are limited studies on LAB isolated from locally produced vinegars, this study was carried out to isolate, identify and characterise the microbiological and chemical properties and their antibacterial activity against foodborne bacteria.

Materials and Methods

Vinegar Samples

The samples used in this study were obtained from traditional homemade vinegars. In Northeast region of Malaysia, coconut vinegar is widely found and produced manually in villages in Penang, whereas the black rice vinegar is locally produced by a factory in Perak, Malaysia.

Isolation of LAB from Vinegar Samples

Firstly, 10 ml of vinegar samples with 90 ml of the de Man Rogosa Sharpe (MRS) broth were incubated anaerobically at 30°C for 24-48 h (Aween et al., 2012). Serial dilution was carried out by adding 1 ml of sample-broth solution into 9 ml of 0.1% buffered peptone water (BPW). Then, 0.1 ml of diluent was spread on MRS agar plates and incubated anaerobically at 30°C for 24-48 h. All isolates were checked for catalase negative and Gram-positive bacteria. Catalase test and oxidase test are orientation tests prior to the selection of API kit.

Identification of LAB using API 50 CHL

All presumptive LAB isolates were identified phenotypically by using API CHL 50 kit (API system, BioMerieux, France) following the methods described by the manufacturer. Strips were incubated at 37°C as recommended by the manufacturer. Changes in colors either to yellow or blue were recorded after 24 and 48 h and the results were analyzed based on numerical profiles and the these profiles were interpreted using API Web ™ (BioMerieux, France).

Polymerase Chain Reaction of LAB

An overnight culture cells grown in 20 mL MRS broth incubated at 30°C were used for total genomic DNA extraction using Qiagen Mini DNA Extraction Kit (Germany) following the methods described by the manufacturer. The preparation of PCR of samples was according to the method described by Yetiman & Kesmen, 2015. Each sample of purified DNA of LAB isolates was processed to the PCR using BiotekePowerTaq 2X MasterMix (Biolution® company). The primers used were WBAC1 (5'- GTC GTC AGC TCG TGT CGT GAG A – 3') and WBAC2 (5'- CCC GGG AAC GTA TTC ACC GCG – 3'). The setting of PCR was carried out as follows: initial at 95°C for 5 min, denaturation at 92°C for 30 s, annealing at 67°C for 1 min and extension at 72°C for 30 s, with 30 cycles for each step and a final extension at 72°C for 5 min. A 2 µL of each amplification mixture was subjected to electrophoresis in 0.8% (w/v) agarose gels in 1.0 × TBE buffer for 45 min and 75 V. Then, the gel was stained using Sybr safe DNA stain (Invitrogen, Germany) and was visualized to clear band and photographed with e-gel imager with blue light based (BioRAD, USA). The partial 16S rDNA sequences were determined by Apical Scientific Sdn. Bhd. Malaysia and sequences were compared with databases (Gen-Bank).

pH and Titratable Acidity

The pH values of the vinegars were measured using digital pH meter and the titratable acidity (TA) of both vinegars were determined as ml of 1N NaOH used to obtain a pink colour endpoint with phenolphthalein (AOAC, 2000). The black rice vinegar was prepared by diluting with distilled water at 1:29 ratio. The equation to calculate TA% as acetic acid is as follows:

$$\%TA = \frac{(\text{ml of NaOH}) \times (\text{N of NaOH}) \times (60.05)}{10 \times \text{Sample Weight}}$$

Antibacterial activity of LAB

Preparation of Cells Free Supernatant from LAB

LAB isolates were inoculated into de Man Rogosa and Sharpe (MRS) broth (Oxoid, CM0359, United Kingdom) and incubated for 24-48 hours at 30°C in anaerobic incubator. The LAB-CFS was prepared by centrifuging the broth at 10000 rpm for 1 minute at 27°C. The supernatant of CFS-LAB was kept at 4ºC until further used.

Quantitative Evaluation for Antibacterial Activity by Agar Well Diffusion Method

Antibacterial activity of the LAB isolates against foodborne bacteria was determined using the agar well diffusion method as described by Balouiri *et al.,*(2015). Initially, LAB strains were cultured in de Man Rogosa and Sharpe (MRS) broth and incubated anaerobically at 30°C for 24-48 h to obtain the supernatant. The foodborne bacteria were swabbed onto the Mueller Hilton Agar (MHA) plates and several wells with a diameter of 6 to 8 were punched using a sterile cork's borer. Then, 50 µL of CFS-LAB and streptomycin were inserted into the wells as tested samples and positive control respectively and incubated aerobically at 30°C for 24 h. Clear zone indicative of bacteria inhibition was measured (mm). The experiment was done in duplicate. The antibacterial activity was monitored for a period of 14 days to observe the stability of CFS-LAB against foodborne bacteria.

Statistical Analysis

Minitab 17 was used to analyze raw data of growth inhibition against foodborne bacteria by CFS-LAB isolated from vinegars. One-way ANOVA was chosen in order to determine the significant effect of a factor (types of sample). Then, the means were further analysed using Fisher's test to identify the significant difference between means at $p \leq 0.05$ for different samples. The statistical analyses were performed using Minitab 17 software.

Results and Discussion

Isolation and Identification of LAB

Two LAB isolates from coconut vinegar and black rice vinegar samples were further identified. These isolates showed colonies creamy white in colore elevated, entire and circular shaped on the MRS agar, catalase negative and oxidase negative. All organisms were observed under a light microscope using 1000x magnification and confirmed as Gram positive rod.

Phenotypic and Genotypic Identification LAB Isolates

Phenotypic identification of the LAB isolates using API 50 CHL kit showed that the isolates of Black Rice Vinegar (BRV03M) and Coconut Vinegar (CV03M) had 92.3% to 99.5% similarity to *L. paracasei* ssp. *paracasei* 2 as shown in Table 1.

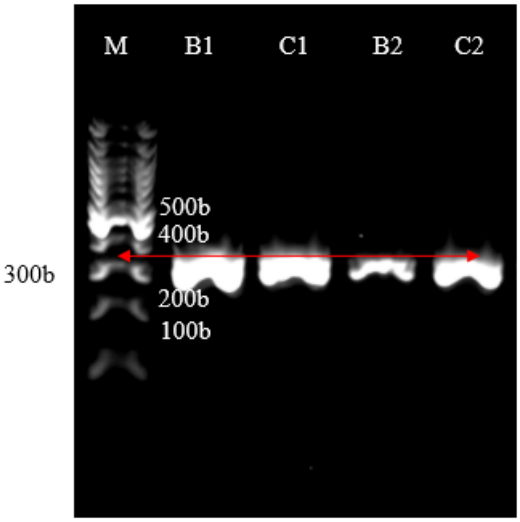
Table 1: Identification of LAB isolates using API 50 CHL kit

Sample	Similarity	Identification using API 50 CHL	Level of identification
BRV03M	92.3%	*L. paracasei* ssp. *paracasei* 2	Very Good Identification
CV03M	99.5%	*L. paracasei* ssp. *paracasei* 2	Very Good Identification

For genotypic identification using 16S rDNA, it was found that *L. paracasei* had the highest percentage of similarity, which was similar to API 50 CHL results (Table 2). For example, the isolates BRV03M had 93% similarity to *L. paracasei* compared to *L. rhamnosus* and *L. casei* (both had 92% similarity). For strains of coconut vinegar (CV03M), *L. paracasei* also showed the highest similarity. Therefore, both strains were confirmed as *L. paracasei*.

Table 2: Identification of LAB isolates using 16S rDNA

LAB isolates	16S rDNA		
	Similarity	Identification	Accession
Black rice vinegar	**93%**	***L.paracasei***	**MF611813.1**
	92%	*L.rhamnosus*	KP942850.1
	92%	*L.casei*	MF108773.1
Coconut vinegar	**99%**	***Lactobacillus paracasei***	**KX388386.1**
	99%	*L.rhamnosus*	MG685875.1
	99%	*L.casei*	MF108773.1



*Note:
Primers: WBAC1 and WBAC2
M: Marker Ladder
Sample B1 and B2 (Black Rice Vinegar, BR03M in duplicate)
Sample C1 and C2 (Coconut Vinegar, CV03M in duplicate)

Figure 1: Visual of LAB gel by e-gel image (blue-light based)

pH and Titratable Acidity

The pH values of all three locally produced vinegar were obtained by using digital pH meter. As shown in Table 3, black rice vinegar had lower pH than coconut vinegar. The higher the pH value, the lower the acidity of the vinegar. The titratable acidity (TA) percentages of the three locally produced vinegar were obtained, and black rice vinegar had the highest acidity. Black rice vinegar had higher percentage of TA (5.46%) than coconut vinegar (1.54%). The pH value and titratable acidity of both vinegars showed positive relationship with their antibacterial properties. This finding was in agreement with the research conducted by Ewadh, *et al*. (2013) who reported that antibacterial activity of vinegar was attributed to direct pH reduction of the substrate.

Table 3: pH value and titratable acidity (%) of locally produced vinegar sample.

Sample	pH value	Titratable acidity (%)
Coconut vinegar	3.56 ± 0.02	1.54
Black Rice vinegar	2.64 ±0.01	5.46

Quantitative Evaluation for Antibacterial Activity by Agar Well Diffusion Method

Agar well diffusion method is one the promising methods to determine the antibacterial activity. In this study, Streptomycin with concentration of 0.01g/ml was used as the control. The inhibitory effect shown by Streptomycin is due to its effects on ribosomes through inhibition or faulty synthesis to cellular proteins of the pathogenic bacteria (Kogut & Harris, 1969). Streptomycin also has high degree of toxicity and exhibits strong action against certain Gram-negative and Gram-positive bacteria *in vivo* (Robinson, 2014). The stability of CFS-LAB in inhibiting foodborne pathogenic bacteria was monitored for 14 days.

Table 4 shows the inhibition activity of CFS-LAB of *L. paracasei* against *E. coli* ATCC 11775. The CFS of *L. paracasei* isolated from black rice vinegar showed inhibition activity that ranged from 10 ± 0.0 mm to 15 ± 1.0 mm where no inhibition activity against *E. coli* was observed at day-3 and day-5. The CFS-LAB isolated from coconut vinegar exhibited inhibition activity that ranged from 12.0 ± 1.0 mm to 14.7 ± 0.6 mm against the growth of *E. coli*, but no inhibition was observed at day-1, day-3 and day-11. The range of inhibition against *E. coli* was in agreement with study by Rahman et al., (2017) who reported inhibitory effect against the growth *E. coli* with inhibition zone that ranged from 5-17 mm.

Table 4: Antibacterial activity of LAB against *E. coli* (ATCC 11775)

Pathogens	*E. coli* (ATCC 11775)						
Inhibition Zone (mm)	Day 1	Day 3	Day 5	Day 7	Day 9	Day 11	Day 14
Positive control	31.7 ±1.2	45±1.0	34.7±1.5	26.7±1.2	36.3±2.1	35.7±2.1	35.3±2.5
Sample BRV03M	10	-	-	15±1.0	13±1.0	13±0.0	12.7±0.6
Sample CV03M	-	-	13.7±0.6	14.7±0.6	12.7±0.6	-	12.0±1.0

Positive control = Streptomycin (0.1mg/ml)
Control of each plate= 0mm
Mean diameter of inhibition zone (mm) after 24hour incubation at 30°C
* (-) indicates no inhibition zones; ≤15mm (low effect); 16-20mm (moderate effect); ≥21mm (strong effect)

(Vlková *et al*., 2006)

Isolates of LAB from both coconut vinegar and black rice vinegar possessed weak inhibitory effect against *B. cereus* ATCC 11778 compared to the positive control, streptomycin (Table 5). The CFS of *L. paracasei* isolated from black rice vinegar exhibited inhibition diameter that ranged from 9.0 ± 1.0 mm to 15.0 ± 1.0 mm with no inhibition activity at day-3. The CFS-LAB of *L. paracasei* isolated from coconut vinegar showed inhibition activity that ranged from 13.7 ± 0.6 mm to 16.0 ± 1.0mm with no inhibition activity at day-1, day-3, day-5, and day-11. The range of inhibition activity was similar to a report by Rahman *et al.* (2017) who found inhibitory effect against *B. cereus* with 8-18 mm.

Table 5: Antibacterial activity of LAB against *B. cereus* (ATCC 11778)

Pathogens	*B. cereus* (ATCC 11778)						
Inhibition Zone (mm)	Day 1	Day 3	Day 5	Day 7	Day 9	Day 11	Day 14
Positive control	36.7 ±1.2	45.3±2.1	34.7±1.2	33.7±0.6	42.0±1.0	35.7±2.1	30.0±2.0
Sample BRV03M	9.0±1.0	-	13.0±0	14.7±1.2	10.3±0.6	15.0±1.0	13.0±1.0
Sample CV03M	-	-	-	14.7±1.2	13.7±0.6	-	16.0±1.0

Positive control = Streptomycin (0.1mg/ml)
Control of each plate= 0mm
Mean diameter of inhibition zone (mm) after 24hour incubation at 30°C
* (-) indicates no inhibition zones; ≤15mm (low effect); 16-20mm (moderate effect); ≥21mm (strong effect)

(Vlková *et al*., 2006)

Table 6 exhibits the antibacterial activity of CFS-LAB in black rice vinegar and coconut vinegar against *S. aureus* ATCC 33862. The inhibition activity of CFS-LAB from black rice vinegar against *S. aureus* ranged from 10.3 ± 0.6 mm to 14.3 ± 1.5 mm, with no inhibition activity at day-5. The inhibition activity of CFS-LAB isolated from coconut vinegar ranged from 9.0 ± 0.0 mm to 15.0 ± 1.0 mm with no inhibition activity at day-1, day-5 and day-11. The result of this research was in agreement with previous study which stated that isolates of LAB exhibited antibacterial effect against the growth of *S. aureus* with 6-14 mm inhibition zone.

Table 6: Antibacterial activity of LAB against *S. aureus* (ATCC 33862)

Pathogens	*S. aureus* (ATCC 33862)						
Inhibition Zone (mm)	Day 1	Day 3	Day 5	Day 7	Day 9	Day 11	Day 14
Positive control	34.3 ±1.5	48.3 ±1.5	37.7 ±1.5	34.7 ±0.6	36.7 ±1.5	37.7 ±1.2	38.3 ±2.1
Sample BRV03M	13.7 ±0.6	10.3 ±0.6	-	11.3 ±1.2	13.3 ±0.6	14.3 ±1.5	12.3 ±0.6
Sample CV03M	-	9.0 ±0	-	13.0 ±0.6	12.0 ±0	-	15.0 ±1.0

Positive control = Streptomycin (0.1mg/ml)
Control of each plate= 0mm
Mean diameter of inhibition zone (mm) after 24hour incubation at 30°C
* (-) indicates no inhibition zones; ≤15mm (low effect); 16-20mm (moderate effect); ≥21mm (strong effect)

(Vlková *et al*., 2006)

Antibacterial activity of LAB strains against *P. aeruginosa* ATCC 10145 is shown in Table 7. The inhibition of CFS-LAB from black rice vinegar against *P. aeruginosa* ranged from 9.0 ± 1.0 to 16.3 ± 0.6 mm, whereas the inhibition of CFS-LAB from coconut vinegar against *P. aeruginosa* ranged from 10.0 ± 1.0 to 15.3 ± 0.6 mm with no inhibition activity at day-1. The results were supported by previous finding where the growth of *P. aeruginosa*, *B. cereus*, and *S. aureus* were inhibited by the CFS-LAB of *L. paracasei*. The inhibition against *P. aeruginosa* strain was attributed to the low pH by the undissociated lactic acid and production of other primary and secondary antimicrobial metabolites by LAB (Okorhi, 2014).

Table 7: Antibacterial activity of LAB against *P. aeruginosa* (ATCC 10145)

Pathogens	*P. aeruginosa* (ATCC 10145)						
Inhibition Zone (mm)	Day 1	Day 3	Day 5	Day 7	Day 9	Day 11	Day 14
Positive control	29.3 ±2.1	32.3 ±0.6	29.7 ±1.2	23.0 ±1.0	26.7 ±1.2	30.0 ±1.7	25.3 ±0.6
Sample BRV03M	9.0 ±1.0	11.3 ±0.6	13.0 ±0	15.3 ±1.2	12.0 ±1.7	16.3 ±0.6	12.0 ±1.0
Sample CV03M	-	10.0 ±1.0	13.7 ±0.6	15.3 ±0.6	12.0 ±0	13.0 ±1.0	13.3 ±1.2

Positive control = Streptomycin (0.1mg/ml)
Control of each plate= 0mm
Mean diameter of inhibition zone (mm) after 24hour incubation at 30°C
* (-) indicates no inhibition zones; ≤15mm (low effect); 16-20mm (moderate effect); ≥21mm (strong effect)

(Vlková *et al*., 2006)

Overall, the CFS-LAB of *L. paracasei* of black rice vinegar sample (BRV03M) was more stable than coconut vinegar (CV03M). Both LAB strains showed low to moderate antibacterial effect by possessed inhibition zone ranging from 8-16 mm. The greater antibacterial activity of *L. paracasei* by BRV03M corresponded to its lower pH (2.64) and greater percentage of lactic acid as measured by titratable acidity (5.46%). Lactic acid, a major metabolite produced by *L. paracasei*, is the major compound responsible for inhibiting pathogenic bacteria (Lozo et al., 2004). Mechanism of action by lactic acid against bacteria is similar to acetic acid. It involves disruption of cytoplasmic membrane and increase permeability of outer membrane of Gram-negative bacteria (Okorhi, 2014).

Conclusion

In conclusion, the isolation and identification of LAB using API 50 CHL and 16S rDNA isolated from black rice vinegar (BRV03M) and coconut vinegar (CV03M) confirmed it as *Lactobacillus paracasei.* The antibacterial activity of CFS-LAB of *L. paracasei* by BRV03M and CV03M strains against *E. coli*, *B. cereus*, *S. aureus* and *P. aeruginosa* showed moderate inhibitory effect against all the tested pathogens. The stability of CFS-LAB strain isolated from black rice vinegar in inhibiting pathogenic bacteria was higher within 14 days than coconut vinegar, suggesting this strain has great potential as natural antibacterial agents.

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