Kertas Asli/*Original Articles*

Identification and Characterization of Lactic Acid Bacteria (LAB) Isolated from Probiotic Drinks in Malaysia
(Pengenalpastian dan Pencirian Bakteria Laktik Asid (LAB) yang Dipencilkan daripada Minuman Probiotik di Malaysia)

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ABSTRACT

Many studies have shown that probiotic strains added to a number of probiotic products are not compatible to that of claimed. It is thus of note to validate probiotic strains added to probiotic products. In this study, three probiotic drinks, A, B and C, were cultured on MRS agar and the number of bacterial colonies was enumerated. The bacterial counts recovered from A (9.3 ± 6.9 log CFU/ml) and C (9.0 ± 6.9 log CFU/ml) were significantly higher than B (5.2 ± 3.5 log CFU/ml) and achieved the minimal amount recommended for probiotic bacteria. All of the isolates appeared as gram positive rods microscopically and were proven to be catalase negative. However, there were only A1, A2, B4 and C1 that were highly tolerant to the gastrointestinal pH 3 to 6. The four isolates produced and secreted antimicrobial substances which inhibited the growth of Escherichia coli (E. coli) and Staphylococcus aureus (S. aureus). C1 showed the greatest growth inhibition by forming 17.50-mm and 17.85-mm inhibition zones against E. coli and S. aureus, respectively. The 16s rDNA sequencing and phylogenetic analysis were performed to further identify the twelve isolates. The twelve isolates were found to be Lactobacillus (L.), particularly L. casei and L. paracasei. However, the bacteria isolated from drink B were incompatible to the labelled ones. In conclusion, probiotic drinks are possible to contain different bacterial counts and probiotic strains from the labelled ones. These differences might affect health benefits rendered by probiotic strains to consumers.

Keywords: Probiotics; lactic acid bacteria; antimicrobial; acid tolerances

ABSTRAK

Banyak kajian telah menunjukkan bahawa strain probiotik yang ditambah ke dalam kebanyakan produk probiotik adalah tidak sepadan dengan yang dinyatakan. Oleh itu, amatlah penting bagi mengesahkan strain probiotik yang ditambah ke dalam produk probiotik. Dalam kajian ini, tiga minuman probiotik iaitu A, B dan C dikulturkan di atas agar MRS dan jumlah koloni bakteria dikira. Jumlah bakteria yang dipulihkan daripada minuman probiotik A (9.3 ± 6.9 log CFU/ml) dan C (9.0 ± 6.9 log CFU/ml) adalah lebih tinggi secara signifikan daripada B (5.2 ± 3.5 log CFU/ml) serta mencapai bilangan minimum yang disarankan untuk bakteria probiotik. Kesemua pencilan muncul dalam bentuk rod gram positif secara mikroskopik dan juga dibuktikan negatif dalam ujian katalase. Namun, hanya pencilan A1, A2, B4 dan C1 yang bertahap toleransi yang tinggi terhadap pH gastrousus 3 hingga 6. Kempat-empat pencilan tersebut menghasilkan dan merembeskan bahan antimikrob yang dapat merencatkan pertumbuhan Escherichia coli (E. coli) serta Staphylococcus aureus (S. aureus). C1 menunjukkan tahap perencatan tertinggi dengan membentuk zon perencatan seluas 17.50-mm dan 17.85-mm masing-masing terhadap E. coli serta S. aureus. Penjujukan rDNA 16s dan analisis filogenetik dilakukan bagi mengenal pasti dua belas pencilan tersebut. Kesemua pencilan tersebut merupakan Lactobacillus (L.), terutamanya L. casei and L. paracasei. Namun bakteria yang dipencilkan daripada minuman B adalah tidak sepadan dengan yang dilabelkan. Kesimpulannya, minuman probiotik berkemungkinan untuk mengandungi bilangan bakteria dan strain probiotik yang berbeza daripada yang dilabelkan. Perbezaan demikian mungkin mempengaruhi manfaat kesihatan strain probiotik kepada pengguna.

Kata kunci: Probiotik; bakteria asid laktik; antimikrob; toleransi pH

INTRODUCTION

Increasing consumer awareness towards healthy diet has created a huge market demand for a variety of functional foods with beneficial health effects. Probiotic food is defined as "food containing live microorganisms which are believed to actively enhance health by improving the balance of microflora in the gut" (Mattila-Sandholm et al. 2002). Probiotic bacteria mainly consist of lactic acid bacteria (LAB) such as *Streptococcus thermophilus*, *Lactobacillus*

bulgaricus, *Lactobacillus lactis*, *Lactobacillus acidophilus* and *Lactobacillus casei* (Galal et al. 2012).

Numerous studies have reported that consumption of probiotic LAB confers many forms of health benefits to hosts. For instance, probiotic LAB have been shown to produce antimicrobial compounds, modulate host defense and exhibit antimutagenic properties (Adolfsson et al. 2004). The antimicrobial compounds produced by probiotic strains encompass lactic acid, acetic acid, hydrogen peroxide and bacteriocins (Saarela et al. 2000; Cotter et al. 2005). These antimicrobial compounds have been shown to prevent the colonization of pathogenic microorganisms such as *Salmonella enteritidis* (Thirabunyanon and Thongwittaya 2012) and *Candida albicans* (Wagner and Johnson 2012). Probiotic bacteria are also potent innate immune stimulators. They activate innate immune responses via interactions with pattern recognition receptors (PRRs) which then initiate the production of pro-inflammatory cytokines such as TNF-α and IL-1β. Pro-inflammatory cytokines are crucial for phagocyte recruitment in pathogen elimination (Kamada et al. 2013). Members of *Lactobacillus* and *Bifidobacterium* have been proven to possess significant antimutagenicity against food mutagens (Settanni and Moschetti 2010). Matsumoto and Benno (2004) reported that the gut mutagenicity in healthy adults was substantially reduced following the consumption of *Bifidobacterium lactis* LKM512 yogurt. These evidences prove that probiotic bacteria indeed bring goodness to human health. A sufficient amount of viable probiotic bacteria must reach the intestines in order to for the bacteria to establish optimal health benefits in hosts. Lourens et al. (2000) suggested that the minimum therapeutic dose of probiotic bacteria is 10^5 to 10^9 CFU/day. The bacteria must also be able to survive the acidic microenvironment in the stomach and resist bile acid at the beginning of the small intestine (Holzapfel et al. 1998).

Owing to the extensive use of probiotic strains in functional food, it has led to poor quality control by manufacturers. Several studies have reported that contents of probiotic products are often not accurately represented on their labels; for instance some products do not contain the labeled microorganisms but the other species whereas the others do not contain the stated LAB counts (Coeuret et al. 2004). In this light, this study was done to characterize and identify LAB in commercial probiotic drinks available in Malaysia. The viability of LAB was determined by enumerating the number of LAB recovered from the selected probiotic drinks. Probiotic drink B was found to contain approximately 4 log CFU/ml less bacterial count than A and C. The 16s rDNA sequencing and phylogeny results confirmed that the isolated bacteria were mainly *Lactobacillus*. However, the bacterial strain isolated from drink B was totally different from the labelled one. In spite of being one of the most prominent probiotic genera, there were only four isolates (A1, A2, B4 and C1) which could survive gastric pH 3. These four isolates produced and secreted antimicrobial substances that caused the formation of inhibition zones against *Escherichia coli* and *Staphylococcus aureus* on Mueller-Hinton (MH) agar. This study has shown that it is possible for probiotic products to contain different probiotic strains and counts from that of claimed. Furthermore, characteristics and benefits of probiotic bacteria such as pH tolerance and antimicrobial activities have also been substantiated to be strain- or isolate-dependent in this study. These evidences hence stress the importance of authenticating and characterizing probiotic strains added to probiotic products for the sake of consumers' health.

MATERIALS AND METHODS

CULTIVATION OF LAB FROM PROBIOTIC DRINKS

An amount of 0.5 ml of probiotic drinks A, B and C were diluted in 4.5 ml of phosphate buffer saline (PBS; 4.3 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, 1.4 mM KH_2PO_4; pH 7.6) and serial dilutions were made from 10^{-1} to 10^{-4}. Then, 0.1 ml of 10^{-3} and 10^{-4} dilutions were cultured on de Man, Rogosa and Sharpe (MRS) (Sigma-Aldrich, St. Louis, United States) agar plates and incubated for 48 h at 37°C. The bacterial colonies were enumerated and recorded as colony forming units (CFU). Twelve colonies were selected randomly as recommended in Bailey et al. (1979) from probiotic drinks A, B and C for gram-staining and catalase test.

CATALASE TEST

An amount of 3.0 ml (3%, v/v) hydrogen peroxide was aliquoted into test tubes. The twelve isolates were inoculated separately into the test tubes. For positive control, a single colony of *Staphylococcus aureus* (*S. aureus*) ATCC 25923 was inoculated into one of the test tubes. PBS buffer (pH7.6) was used as negative control. Formation of bubbles denoted the presence of catalase and the test was positive, otherwise, the results were recorded as negative.

pH TOLERANCE

Twelve isolates were inoculated separately into 5 ml of MRS broth and incubated at 37°C until OD_{600} reached 1.0 (equivalent to 8 log CFU/ml). The bacteria were spun down at 4000 x g (Beckman Coulter, California, United States) for 5 min and subsequently washed with PBS buffer. The centrifugation and washing steps were repeated twice. The pellet was dissolved in 5 ml of PBS at pH3-6 and incubated at 37°C for 2 h. The bacterial suspension was diluted 300 times and 10 μl of bacterial suspension were cultured on MRS agar plates. The plates were incubated at 37°C for 48 h. The viability of bacteria was determined by quantifying the CFU on the plates.

LAB must survive gastric pH in order to be functional in the gastrointestinal tract (Mattila-Sandholm et al. 2002). There were four isolates (A1, A2, B4 and C1) surviving pH3-6 in the pH tolerance test, they were therefore further analyzed for their antimicrobial ability. The isolates were inoculated separately into 5 ml of MRS broth. The cultures were incubated at 37°C until OD_{600} reached 1.0. The bacterial cells were spun down at 4000 x g for 5 min. The separated broth was sterilized with a membrane filter (0.2 μm; Eppendorf, Hamburg, Germany). The indicator bacteria, *Escherichia coli* (*E. coli*) strain Top 10 (Invitrogen, New York, United States), and *S. aureus* ATCC 25923, were initially cultured on MHA plates and allowed to dry for 30 min. Sterile 6-mm discs were dipped into 50 μl of sterilized broth and placed on MHA plates cultured with the indicator bacteria. The ampicillin discs (10 mg/ml; Oxoid Thermo Scientific, Leicestershire, United Kingdom) served as positive control. The plates were incubated at 37°C for 24 h. According to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Matuschek et al. 2013), *E. coli* and *S. aureus* are susceptible to ampicillin if the inhibition zone diameters are $\geq 14\pm1$ mm and $\geq 18\pm1$ mm, respectively. This standard was employed to validate the test reliability.

DNA EXTRACTION

Twelve isolates were cultured separately in MRS broth. A total of 1.5 ml bacterial culture was transferred into a sterile microcentrifuge tube and spun at 4000 xg for 5 min. The broth was discarded and the pellet was dissolved in 500 μl resuspending buffer [440 μl Tris-NaCl (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH8.0; 100 mM NaCl), 5 μl lysozyme (20 mg/ml), 50 μl 1% (w/v) sodium dodecyl sulfate (SDS) and 6 μl proteinase K (10 mg/ml)]. The suspension was incubated for 1 h at 37°C. After incubation, 470 μl of phenol:chloroform (1:1) was added into cell suspension and mixed evenly. The cell suspension was centrifuged at 4000 xg for 5 min. The aqueous phase was transferred into a new microcentrifuge tube. About 20 μl of sodium acetate (3 M, pH5.2) and 500 μl of isopropanol (Applied Biosystems, Foster City, United States) were added to the aqueous phase and mixed gently until the deposition of the white threads of DNA was observed. A glass Pasteur pipette was inserted and rotated slowly until DNA strands attached to it. The DNA threads were dipped into 70% (v/v) cold ethanol (Applied Biosystems, Foster City, United States), air-dried and dissolved in 50 μl cold distilled water containing RNase A (20 μg/ml). The DNA solution was stored at -20°C.

AMPLIFICATION AND SEQUENCING OF 16S RDNA

The bacterial 16S rDNA gene was amplified from the bacterial genome using the universal 16s rDNA primers, Forward (5´- GAG TTT GAT CCT GGC TCA G -3´) and Reverse (5´-CGG CTA CCT TGT TAC GAC TT-3´). The PCR reaction mixture (50 μl) consisting of 10 μl amplification buffer (5x), 1 μl dNTP (20 mM), 1.5 μl of each primer (20 μM), 0.5 μl Go *Taq* DNA Polymerase (Thermo Scientific, Leicestershire, UK), 5 μl $MgCl_2$ (25 mM), 29.5 μl nuclease free water and 1 μl template DNA was prepared. The thermo-cycling program was set as follows: 94°C for 5 min; 30 cycles of 94°C for 30 s, 55°C for 90 s, and 72°C for 30 s; and a final extension step at 72°C for 10 min. The PCR products were analyzed on 1% (w/v) agarose gel. The target fragments were excised and purified using the HiYield Gel/PCR DNA Fragments Extraction Kit (Yeastern Biotech, Taipei, Taiwan) according to the manufacturer's instructions. The purified 16S rDNA was sequenced (Nanolifequest, Kuala Lumpur, Malaysia) and the sequencing results were compared to that of the Genbank database using the Basic Local Alignment Search Tool (BLAST) (www.nbci.nlm.nih.gov/genbank).

PHYLOGENETIC TREE RECONSTRUCTION

Phylogeny.fr offers a user-friendly and automated pipeline to reconstruct phylogenetic trees for microorganisms of interest (Dereeper et al. 2008). The DNA sequences with 96-99% homology were selected from the BLAST results and used as inputs for the phylogenetic tree reconstruction. The DNA sequences of two commonly known probiotic bacteria were included as outgroups: *Bifidobacterium longum* subsp. *infantis* ATCC 15697 and *Lactobacillus acidophilus*. These sequences were then aligned and curated to A1-C4 DNA sequences. A phylogenetic tree was reconstructed subsequently with the PhyML (Phylogeny Maximum Likelihood) and presented using the TreeDyn. The PhyML was run with the aLRT (Likelihood Ratio Test) statistical test of branch support.

STATISTICAL ANALYSIS

The significance differences in bacterial counts recovered from drinks A, B and C were compared via Kuskal-Wallis and Mann-Whitney tests using the SPSS version 20.0 software. The differences in the inhibition zone diameters produced by isolate C1 and ampicillin were compared with One-Way ANOVA and Tukey's HSD tests using the SPSS version 20.0 software.

RESULTS AND DISCUSSION

BACTERIAL COUNTS IN PROBIOTIC DRINKS

The probiotic strains, minimum viable bacterial count and recovered bacterial count of each drink were listed in Table 1. According to the US Food and Drug Administration (FDA), it is important to label probiotic strains and minimum viable bacterial amount added to a probiotic product since probiotic health benefits are

strain specific and also dependent on the number of viable microorganisms (Mattila-Sandholm et al. 2002; Saldanha 2008). It has been suggested that probiotic bacteria should be present in food products at a minimum level of 10^5-10^9 CFU/ml (5-9 log CFU/ml) (Lourens et al. 2000). According to the product labels, A and C should contain 6 to 7 log CFU/ml minimum viable probiotic bacteria. Meanwhile, the

bacteria counts recovered from A and C were 9.3±6.9 and 9.0±6.9 log CFU/ml, respectively. The recovered bacterial counts not only fell in the range of the minimum level (~9 log CFU/ml) but were approximately 2 to 3 log CFU/ml more than the labelled amounts. It is therefore believed that A and C are of better capability in providing health benefits to consumers.

TABLE 1. Probiotic contents of drinks A, B and C

Probiotic drinks	Probiotic strains	Minimum viable bacterial count stated on the product labels (log CFU/ml)	Recovered bacterial count (log CFU/ml)*
A	*Lactobacillus casei*	7	9.3 ± 6.9[a]
B	*Lactobacillus acidophilus, Bifidobacterium lactis* DR10	Not mentioned	5.2 ± 3.5
C	*Lactobacillus casei, Lactobacillus acidophilus*	6	9.0 ± 6.9[b]

* The experiment was performed in triplicate and the bacterial count is expressed as log CFU/ml + log standard deviation

[a] The bacterial count recovered from A was significantly different from that recovered from B, p < 0.043

[b] The bacterial count recovered from C was significantly different from that recovered from B, p < 0.046

There was no minimum viable bacteria count mentioned on the drink B label (Table 1). The probiotic bacteria were therefore revived and enumerated on MRS agar. The amount was found to be 4 log CFU/ml lower than A and C. The statistical significant values were p < 0.043 (comparison between A and B) and 0.046 (comparison between B and C). Despite the differences, the number was still within the recommended level. According to Shah (2000), the amount of viable probiotic microorganisms in a probiotic product and storage condition are contributing factors to a relatively low bacterial count recovered from a product. However, drinks A, B and C were obtained from the same location and stored under the same condition. The amounts of viable probiotic bacteria recovered from A and C were in accordance to the product labels and the recommended level. As a result, storage condition is less likely to cause the lower bacterial count recovered from B. It is likely that the number of viable probiotic bacteria added to drink B was less than A and C and thus resulted in low bacterial recovery.

GRAM STAINING AND CATALASE TEST

Four colonies were selected randomly from each of the probiotic cultures on the MRS agar. The characteristics of the bacteria were determined using crystal violet and tested with hydrogen peroxide. The bacterial isolates appeared as purplish-blue (gram positive) and rod-shaped bacilli (data not shown) under light microscopic observation. They were either single rods or arranged in pairs and chains. This finding was consistent with that reported by Settanni and Moschetti (2010). The high peptidoglycan content in gram-positive bacterial cell walls causes retention of crystal violet and therefore LAB appear as purple-bluish rods.

On the other hand, exposure of the isolates to hydrogen peroxide did not result in bubble formation and therefore resulted in negative catalase test (data not shown). This observation was attributable to LAB as anaerobes that are unable to produce catalase without the presence of heme (König & Fröhlich 2009). Due to the absence of catalase, LAB is unable to catalyze the conversion of hydrogen peroxide to water and oxygen and thus results in a negative catalase test. Altogether, the positive gram-staining and negative catalase test proved that the isolates were LAB.

pH TOLERANCE

It is particularly crucial for probiotic bacteria to survive acidic environment in the stomach and bile acids in the upper small intestine in order to render optimal benefits to hosts (Mattila-Sandholm et al. 2002). The pH tolerance test was carried out *in vitro* to predict the viability of probiotic bacteria after an exposure to the gastric and bile acid pH (Rönkä et al. 2009). The twelve isolates were subjected to pH3-6 treatments for 2 h. Two-hour incubation was performed because the duration represented the retention time for bacteria in the gut (Botić et al. 2007). Generally, all isolates survived pH4-6 treatments with a minor declination at pH6 (Figure 1). The results were similar to that reported by Voravuthikunchai et al. (2006) in which most of the *Lactobacillus* strains survived pH4 and 5 *in vitro*. There were only A1, A2, B4 and C1 surviving pH 3 which represented the gastric pH. This shows that these four isolates possessed better pH tolerance than the other isolates. According to Vinderola and Reiheimer (2003), tolerance to acidic pH is very strain specific. In addition, Lin et al. (2006) reported that the majority of LAB isolated from commercial products only grew moderately at pH3.2.
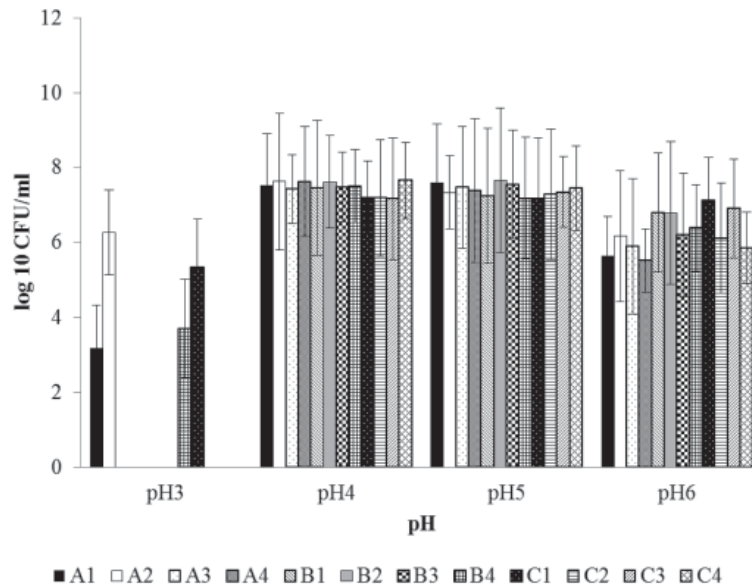
FIGURE 1. Viable bacterial counts (log CFU/ml) after pH 3-6 treatments. There were only isolates A1, A2, B4 and C1 survived pH 3-6. The test was performed in triplicate. The results were recorded as log mean CFU/ml ± s.d.

This scenario explains why there were few isolates survived pH3.0 in this study. The pH tolerance of probiotic bacteria to the gastrointestinal pH can be possibly enhanced by co-ingestion of milk (Gardiner et al. 2000).

ANTIMICROBIAL TEST

LAB have long been associated with highly competent antimicrobial capabilities in preventing the growth of pathogenic bacteria (Settanni & Moschetti 2010; Winkelströter et al. 2011). LAB produce and secrete organic acids, bacteriosins and nisin into the surroundings, thereby inhibit the growth of pathogenic microorganisms (Carr et al. 2002; Hartmann et al. 2011). A number of secreted antimicrobials have been identified in LAB. For examples, organic acids, hydrogen peroxide and bacteriocins. Organic acids such as lactic acid and acetic acid lower the local pH which in turn retards the growth of pH-sensitive pathogens (De Keersmaecker et al. 2006). LAB also produce hydrogen peroxide as a strong oxidizing agent in inhibiting the growth of pathogenic bacteria (Monteagudo-Mera et al. 2012). On the other hand, bacteriocins inhibit the growth of gram-positive and gram-negative bacteria by permeabilizing the cell membrane and interfering with the activities of essential enzymes.

Since isolates A1, A2, B4 and C1 showed better tolerance to pH3-6, these isolates were therefore expected to colonize the gastrointestinal tract and exert antimicrobial effects better. As previously described, the four isolates were also believed to produce and secrete antimicrobial substances into the growth media. As a result, the bacterial growth media was filtered and tested for antimicrobial effects against two indicator bacteria, namely *E. coli* and *S. aureus*. Table 2 summarizes the diameters of inhibition

zones formed by antimicrobial substances secreted by isolates A1, A2, B4 and C1 against the two indicator bacteria. The positive control, ampicillin produced inhibition zones of 17.50 ± 0.71 and 19.50 ± 0.71 mm in response to *E. coli* and *S. aureus*, respectively. These readings were in accordance to that recommended by EUCAST (Matuschek et al. 2013) and thus validated the reliability of the test results.

The sizes of inhibition zones formed varied among the isolates regardless of the indicator bacteria. This is speculated to be attributable to different levels of antimicrobial substances secreted into the growth media by the isolates since the level of antimicrobial substances produced by probiotic bacteria has been shown to be strain-dependent by Hartmann et al. (2011). Some isolates (A2 and B4) also formed larger inhibition zones against *E. coli* than *S. aureus*. This observation could be explained by differences in membrane compositions of *S. aureus* (gram positive) and *E. coli* (gram negative). The membranes of gram negative bacteria are exposed directly to the exteriors whereas the gram positive bacterial membranes are surrounded by thick cell walls which lead to slightly lower sensitivity of *S. aureus* to LAB antimicrobial substances (Fimland et al. 2002). Among the four isolates, C1 showed the highest inhibition against *E. coli* (17.50 ± 0.71 mm) and *S. aureus* (17.85 ± 0.21 mm). The inhibition zone produced by C1 in response to *E. coli* was indifferent from that produced by ampicillin ($p > 0.05$, 17.50 ± 0.71 mm) The inhibition zone diameter produced by C1 against *S. aureus*, on the other hand, was smaller than that of ampicillin ($p < 0.05$, 19.50 ± 0.71 mm). Despite forming smaller inhibition zones against *E. coli* and *S. aureus* than C1, isolates A1, A2 and B4 were still effective in preventing the growth of *E. coli* and *S. aureus*

TABLE 2. Growth inhibition of isolates A1, A2, B4 and C1 against *E. coli* and *S. aureus*

Isolates	Inhibition zone diameters (mm)	
	E. coli	*S. aureus*
A1	14.25 ± 0.35	14.25 ± 1.1
A2	14.15 ± 0.21	12.50 ± 0.71
B4	16.25 ± 0.35	14.25 ± 0.35
C1	17.50 ± 0.71[a]	17.85 ± 0.21b
Ampicillin	17.50 ± 0.71	19.50 ± 0.71

The test was performed in triplicate. The results were stated as mean inhibitory zone ± s.d

[a] The inhibition zone diameter was insignificantly different (p > 0.05) from that produced by ampicillin against
E. coli

[b] The inhibition zone diameters were significantly different (p < 0.05) from that produced by ampicillin against
S. aureus

with inhibition zone diameters ranging from 12.50+0.71 to 16.25 ± 0.35 mm (Table 2). Overall, isolates A1, A2, B4 and C1 were capable of producing antimicrobial substances to prevent the growth of *E. coli* and *S. aureus* despite variations in the inhibition zone diameters.

16s rDNA SEQUENCING AND PHYLOGENETIC TREE RECONSTRUCTION

Coeuret et al. (2004) reported that there are a number of probiotic products containing different probiotic strains than claimed. Since health benefits conferred by probiotic microorganisms are strain-dependent, it is therefore crucial to authenticate the bacterial strains added to probiotic products. 16S rDNA sequencing was employed to identify the probiotic strains in this study. This method has been proven to be effective in detecting and identifying a wide range of organisms in cultures or clinical specimens (Rampini et al. 2011). The 16s rDNA was amplified and sequenced and the results were compared with the existing sequences in the GenBank database and tabulated in Table 3. The results showed that all of the isolates were *Lactobacillus* with 98-99% homology except C1 (96%) (Table 3). The identified LAB were also compared with the

probiotic strains claimed by manufacturers on the product labels (Table 1). Drink A was claimed to contain only *Lactobacillus casei* (*L. casei*) and this was largely agreeable to that identified in this study in which isolates A1-A4 were *L. casei*. However, the bacteria isolated from drink B were different from the claimed ones. They constituted mostly of *L. casei* except isolate B1 (*L. paracasei*) while *L. acidophilus* and *Bifidobacterium lactis* (*B. lactis*) DR10 were claimed to be added to drink B. This incompatibility was similar to that reported by Coeuret et al. (2004) in which many probiotic products were found to contain probiotic strains that were different from the claimed ones. Hence, it emphasizes the importance of carrying out this study in order to authenticate the probiotic strains used to manufacture the selected probiotic drinks in Malaysia.

L. casei and *L. acidophilus* were claimed to be added to drink C; however, there was only *L. casei* isolated in this study. This is largely due a relatively lower proportion of *L. acidophilus* added to drink C as compared to *L. casei* which in turn lowered the successful rate for *L. acidophilus* isolation (Bailey et al. 1979). Furthermore, Temmerman et al. (2002) also suggested that a higher successful rate for microorganism isolation could also be achieved by using a larger number of isolates. The isolation of *L. acidophilus*

TABLE 3. Bacterial strains of the isolates and their sequence homologies (%) compared to that in the GeneBank Database

Isolates	Bacterial strains	Homology (%)+
A1	*Lactobacillus casei*	99
A2	*Lactobacillus casei*	98
A3	*Lactobacillus casei*	99
A4	*Lactobacillus casei*	99
B1	*Lactobacillus paracasei*	99
B2	*Lactobacillus casei*	99
B3	*Lactobacillus casei*	99
B4	*Lactobacillus casei*	99
C1	*Lactobacillus casei*	96
C2	*Lactobacillus casei*	98
C3	*Lactobacillus casei*	99
C4	*Lactobacillus casei*	99

+Homology was recorded according to that shown in the BLAST

can hence be improved by selecting greater numbers of LAB colonies.

A phylogenetic tree (Figure 2) was also reconstructed in order to further illustrate the closeness of the isolates to *Lactobacillus*. Phylogeny fr. was employed in the phylogenetic tree reconstruction due to its robustness, reliability and less-laboriousness (Dereeper et al. 2008). Two outgroups, namely *L. acidophilus* and *Bifidobacterium longum* (*B. longum*) subsp. *infantis* ATCC 15697 were included in the phylogenetic tree. These two outgroups were selected based on, (i) B is claimed to contain *L. acidophilus* and *Bifidobacterium*, (ii) C is claimed to contain *L. acidophilus* and *L. casei* and (iii) they are commonly used probiotic strains (Settanni and Moschetti, 2010). Figure 2 illustrates that A1-C4 were closely related to *L. casei* and *L. paracasei* but not the outgroups. The genetic change score was relatively lower (0.081) than the outgroups (0.116 for *L acidophilus* and 0.308 for *B. longum* subsp. *infantis* ATCC 15697). The results were also in accordance to the gene sequencing results in which the 16s rDNA sequences of A1-C4 were highly homologous to that of *L. casei* and *L. paracasei*. It is therefore confirmed that the isolates were *L. casei* (A1-A4, B2-B4 and C1-C4) or *L. paracasei* (B1).
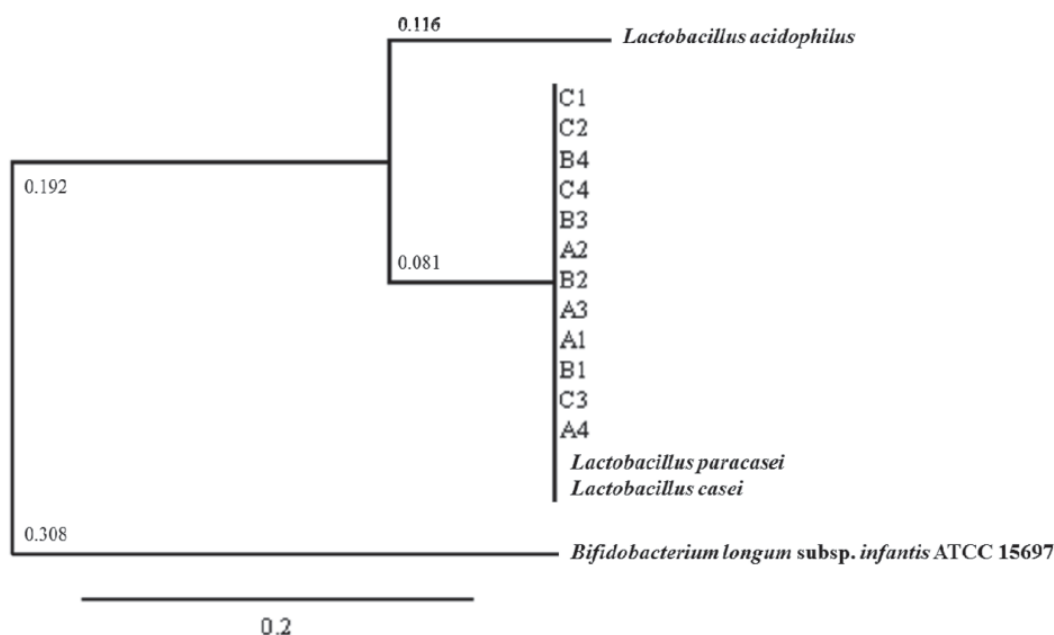

FIGURE 2. The phylogenetic tree of the twelve isolates (*L. casei* and *L. paracasei*), *L. acidophilus* and *Bifidobacterium longum* subsp. *infantis* ATCC 15697. The line segment labeled with '0.2' indicates an amount genetic change of 0.2 (the number of nucleotide changes divided by the length of sequence). Isolates A1 to C4 were closely related to the *Lactobacillus* genus, especially *L. casei* and *L. paracasei*

CONCLUSION

Validation of probiotic product content is essential to ensure the quality and reliability of the products. This study has shown that probiotic drinks are possible to contain different probiotic counts and strains than claimed as observed in isolates obtained from drink B. The recovered bacterial count was 4 log CFU/ml lower than drinks A and C. Among the isolates, there were only A1, A2, B4 and C1 surviving the gastric pH besides showing effective antimicrobial activities. In conclusion, probiotic food contents maybe differ from that of labelled and the differences are possible to affect probiotic health benefits to consumers. Hence a stringent and precise labeling policy should be reinforced in the production of probiotic products.

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