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Research Article
Confirmation of the Identity of *Lactobacillus* Species using Carbohydrate Fermentation Test (API 50 CHL) Identification System

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Abstract

Background and Objective: The word 'probiotic' in greek, means 'for life'. Probiotics are defined as live microbial feed supplement which have several health benefits. The objective of this study was to screen and identify 20 bacterial strains provided by School of Industrial Technology, USM, Malaysia. **Materials and Methods:** Twenty bacteria culture was screened using morphological and biochemical studies. From the 20 bacteria cultures, 9 of those exhibited viable count of above 9.0 $\log_{10}$ CFU mL^{-1} are selected for acid and bile tolerance tests. From 9 bacteria, five bacteria culture were able to tolerate acid and bile which exhibited viable counts of more than 9.0 $\log_{10}$. **Results:** All the 20 bacteria were confirmed as probiotics *Lactobacillus* using morphological and biochemical studies. The identity of the five lactobacilli, FTDC 0582, FTDC 0785, FTDC 2916, FTDC 4462 and FTDC 4793 were confirmed to be *L. brevis*, *L. plantarum*, *L. plantarum*, *L. casei* and *L. plantarum*, respectively. **Conclusion:** It can be concluded that, a carbon utilization microplate assay system developed by API CHL 50 has the potential to simplify the identification scheme of probiotic bacteria to the genus level.

Key words: *Lactobacillus*, probiotic bacteria, industrial technology, microbial feed

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The concept of probiotics was introduced by Metchnikoff[1]. The term "probiotics" was coined by Lilly and Stillwell[2] to describe substances produced by micro-organisms that stimulate the growth of another. The word "substances" in Parker's definition of probiotics were resulted in a wide connotation that included antibiotics[3]. *Lactobacillus* and *Bifidobacterium* are two major groups of micro-organisms which are well known for their probiotic properties[4-11]. Probiotics produce antimicrobial agents such as organic acids, hydrogen peroxide and bacteriocins which are antagonistic to pathogenic micro-organisms[12-16].

Probiotics are also defined as "a live microbial feed supplement which beneficially affects the host by improving its intestinal microbial balance"[17]. The definition of probiotics with respect to host and habitat of the microflora is given as "a viable mono-or mixed culture of micro-organisms which, when applied to animal or man, beneficially affects the host by improving the properties of the indigenous microflora"[18]. It was further investigated by Schrezenmeir and de Vrese[19] who proposed probiotics as "a preparation of a product containing viable, defined micro-organisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in this host". According to the World Health Organization (WHO)[20], probiotics are living micro-organisms which when administered in adequate numbers confer a health benefit on the host.

The safety of *Lactobacillus* sp. and *Bifidobacterium* sp. as probiotics has been extensively reviewed[21-26]. Probiotics are treated as Generally Recognized As Safe (GRAS) micro-organism by the Federal Food and Drug Administration[27-30]. The identification of *Lactobacillus* depends mainly on physiological and biochemical criteria[31-33]. The prescreening of different *Lactobacillus* sp. was performed using various methods, such as conventional biochemical and physiological tests[34-36]. It can also be identified by more complex technique such as carbohydrate fermentation patterns using commercially available kits[37-38].

Twenty probiotics culture bacteria of *Lactobacillus* used in the present study were provided by the School of Industrial Technology, Universiti Sains Malaysia. It was essential to confirm the identity of the probiotics culture bacteria. Therefore, the objective of this part of study was to identify the twenty bacterial strains provided by School of Industrial Technology, USM, Malaysia.

MATERIALS AND METHODS

The study was conducted in the School of Pharmaceutical Sciences, University Sains Malaysia, Penang, Malaysia from July-December, 2017.

Bacteria cultures: Twenty probiotics bacteria cultures given by the School of Industrial Technology, Universiti Sains Malaysia. The cultures were FTDC 9393, FTDC 0582, FTDC 0785, FTDC 4462, FTDC 5127, FTDC 5030, FTDC 8891, FTDC 4793, FTDC 1295, FTDC 8133, FTDC 8264, FTDC 2916, FTDC 2804, FTDC 1960, FTDC 8592, FTDC 3666, FTDC 3871, FTDC 1903, FTDC 1861 and FTDC 8033.

Enumeration of lactobacilli: For the enumeration of lactobacilli, 10.0 mL of bacterial culture was inoculated into 90.0 mL of MRS broth and incubated at 37°C for 24 h (Memmert, Germany). The enumeration of viable cells was conducted after ten-fold serial dilution of fresh bacterial culture. About 1.0 mL of each dilution was cultivated in MRS agar using pour plate method. The plates were incubated at 37°C for 48 h.

Morphological and biochemical evaluation: The morphological and biochemical characteristics of the bacteria were determined after 48 h of incubation on MRS agar. The evaluation included shape, size, margin, colour and Gram-staining test.

Analytical profile index (API 50 CHL) identification kit: API 50 CHL carbohydrate identification kit is comprised of 50 biochemical tests. The API 50 CHL strip is made up of 5 smaller strips. Each smaller strip consists of 10 wells of different carbohydrate substrates. Briefly, all the strips were removed from their packaging and placed in the tray.

The inoculum was prepared according to the manufacturer's instruction by aseptically transferring pure culture of lactobacilli from MRS agar into the API Suspension Medium ampoule (2.0 mL) using sterile swab. The suspension was mixed and 350 µL was transferred to a second API Suspension Medium ampoule (5.0 mL) to reach turbidity equivalent to McFarland standard # 2. The final inoculum was prepared by transferring 700 µL from the initial bacterial suspension (API Suspension Medium ampoule, 2.0 mL) into an API 50 CHL Medium (10.0 mL). The suspension was mixed and 150 µL (inoculated API 50 CHL

medium suspensions) was measured into the well using sterilized micropipette and covered with 50 µL mineral oil. The strips were incubated (Memmert, Germany) at 37°C for 48 h.

After the incubation of 48 h (manufacturer instruction), each well was observed for colour changes. The positive result was confirmed by the change of colour of bromocresol purple indicator from purple to yellow, except for well # 26 (for esculin hydrolysis test), by the change in colour from purple to a darker colour or black. The first well on the strip was used as a control. No change in the colour indicated negative result. The result was analyzed using api-web™ identification software database (Biomérieux, France, V 5.1) to identify *Lactobacillus* species.

RESULTS AND DISCUSSION

Morphological characteristics: The bacteria were creamy white colonies, rod-shaped with sharp margins. The colony diameter was in the range of 2.0-5.0 mm (Fig. 1). All the strains are Gram-positive, non-spore forming and non-motile (Table 1). The results were consistent with the findings of Tabatabaei-Yazdi *et al.*[39] and cross reference information provided in Bergey's Manual of Determinative Bacteriology[36] on *Lactobacillus*.

Identification using API 50 CHL kit: The results of carbohydrate fermentation test of API 50 CHL identification kit were shown in Table 2. The results obtained coincided with the characteristics of *Lactobacillus*[36,40]. The results in Table 3 provided the identity of lactobacilli species based on carbohydrate fermentation profiles using API 50 CHL database. FTDC 0785, 2916 and 4793 showed a 99.9% identity of *Lactobacillus plantarum*. FTDC 0582 was identified as *Lactobacillus brevis* with 99.0% identity. On the other hand, FTDC 4462 showed 99.8% identity with *Lactobacillus paracasei* 3 and *Lactobacillus paracasei* 1. The result was in good agreement with the findings of other researchers[23,31,41,42]. API 50 CHL identification kit was reported as an important tool for lactobacilli identification[43-45]. It can be used for taxonomic identification which is based on phenotypical characteristics to identify the different species of *Lactobacillus*[45,46].

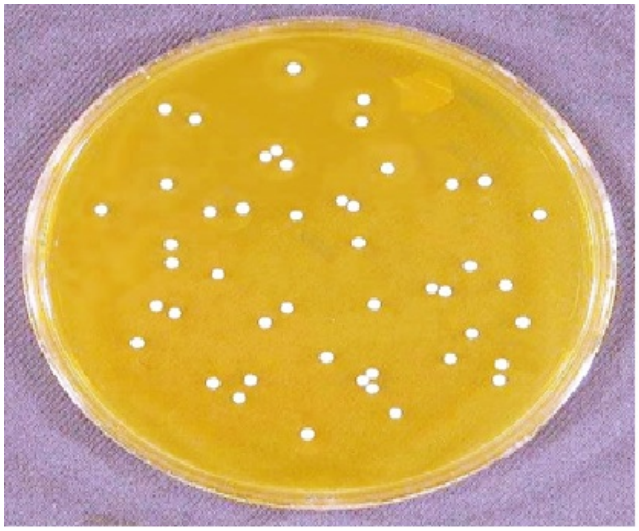

Fig. 1: Morphological characteristic of probiotics colonies grown on MRS agar

Table 1: Morphological and biochemical tests for probiotics

Test parameters	Bacteria culture																			
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
Gram-reaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Spore forming	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Motility test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+: Positive result (Gram-positive in case of Gram-staining and capability to motile in case of motility), -: Negative result (Gram-negative in case of Gram-staining and inability to motile in case motility). A: FTDC 9393, B: FTDC 0582, C: FTDC 0785, D: FTDC 4462, E: FTDC 5127, F: FTDC 5030,G: FTDC 8891, H: FTDC 4793, I: FTDC 1295, J: FTDC 8133, K: FTDC 8264, L: FTDC 2916, M: FTDC 2804, N: FTDC 1960, O: FTDC 8592, P: FTDC 3666, Q: FTDC 3871, R: FTDC 1903,S: FTDC 1861, T: FTDC 8033

Table 2: Carbohydrate fermentation profiles of *Lactobacillus* species

	Lactobacillus species				
Carbohydrate fermentation	FTDC 0582	FTDC 0785	FTDC 2916	FTDC 4462	FTDC 4793
Control	Neg	Neg	Neg	Neg	Neg
Glycerol	Neg	Pos	Neg	Neg	Pos
Erythritol	Neg	Neg	Neg	Neg	Neg
D-Arabinose	Neg	Neg	Neg	Neg	Neg
L-Arabinose	Pos	Pos	Neg	Neg	Neg
Ribose	Pos	Pos	Pos	Pos	Pos
D-Xylose	Pos	Neg	Neg	Neg	Neg
L-Xylose	Neg	Neg	Neg	Neg	Neg
Adonitol	Neg	Neg	Neg	Neg	Neg
β-Metil-D-xiloside	Neg	Neg	Neg	Neg	Neg
Galactose	Neg	Pos	Pos	Pos	Pos
D-Glucose	Pos	Pos	Pos	Pos	Pos
D-Fructose	Neg	Pos	Pos	Pos	Pos
D-Mannose	Neg	Pos	Pos	Pos	Pos
L-Sorbose	Neg	Neg	Neg	Neg	Neg
Rhamnose	Neg	Pos	Neg	Neg	Pos
Dulcitol	Neg	Neg	Neg	Neg	Neg
Inositol	Neg	Neg	Neg	Neg	Pos
Mannitol	Neg	Pos	Pos	Pos	Pos
Sorbitol	Neg	Pos	Neg	Pos	Pos
α-Methyl-D-mannoside	Neg	Pos	Pos	Neg	Neg
α-Methyl-D-glucoside	Neg	Neg	Pos	Neg	Neg
N-Acetyl-glucosamine	Neg	Pos	Pos	Pos	Pos
Amygdalin	Neg	Pos	Pos	Pos	Pos
Arbutin	Neg	Pos	Pos	Pos	Pos
Esculin	Neg	Pos	Pos	Pos	Pos
Salicin	Neg	Pos	Pos	Pos	Pos
Cellobiose	Neg	Pos	Pos	Pos	Pos
Maltose	Neg	Pos	Pos	Pos	Pos
Lactose	Neg	Pos	Neg	Pos	Pos
Melibiose	Neg	Pos	Pos	Neg	Pos
Saccharose	Neg	Pos	Pos	Pos	Pos
Trehalose	Neg	Pos	Pos	Pos	Pos
Inulin	Neg	Neg	Neg	Neg	Neg
Melezitose	Neg	Pos	Pos	Pos	Pos
D-Raffinose	Neg	Pos	Pos	Neg	Pos
Amidon	Neg	Neg	Neg	Neg	Neg
Glycogen	Neg	Neg	Neg	Neg	Neg
Xylitol	Neg	Neg	Neg	Neg	Neg
â-Gentiobiose	Neg	Pos	Pos	Pos	Pos
D-Turanose	Neg	Neg	Pos	Neg	Neg
D-Lyxose	Neg	Neg	Neg	Neg	Neg
D-Tagatose	Neg	Neg	Neg	Pos	Neg
D-Fucose	Neg	Neg	Neg	Neg	Neg
L-Fucose	Neg	Neg	Neg	Neg	Neg
D-Arabitol	Neg	Pos	Pos	Neg	Pos
L-Arabitol	Neg	Neg	Neg	Neg	Neg
Gluconate	Neg	Pos	Pos	Neg	Pos
2-Keto-gluconate	Neg	Neg	Neg	Neg	Neg
5-Keto-gluconate	Pos	Neg	Neg	Neg	Neg

Pos: Positive reaction: Color changed and Neg: Negative reaction: Color not changed

Table 3: Identification of *Lactobacillus* species based on carbohydrate fermentation profiles using API 50 CHL database

Lactobacillus sp.	API identification	ID (%)
FTDC 0582	*Lactobacillus brevis*	99.0
	Lactobacillus buchneri	0.7
FTDC 0785	*Lactobacillus plantarum*	99.9
	Lactococcus pentosus	0.1
FTDC 2916	*Lactobacillus plantarum*	99.9
	Lactobacillus brevis	0.1
FTDC 4462	*Lactobacillus paracasei* subsp. *paracasei* 3	84.4
	Lactobacillus paracasei subsp. *paracasei* 1	15.4
	Lactobacillus plantarum	0.2
FTDC 4793	*Lactobacillus plantarum*	99.9
	Lactobacillus rhamnosus	0.1

ID: Identity (%), the percentages following the scientific names of species represent the similarities from the computer-aided database of the API-web™ API 50 CHL V5.1 software

CONCLUSION

The identity of 20 probiotic culture was confirmed as *Lactobacillus* using morphological and biochemical studies prior to the commencement of the study. From the 20 bacteria cultures, five bacteria culture, FTDC 0582, FTDC 0785, FTDC 2916, FTDC4793 and FTDC 4462 were able to tolerate acid and bile which exhibited viable counts of above 9.0 $\log_{10}$ CFU mL^{-1} were selected for subsequent study. The identity of the five lactobacilli, FTDC 0582, FTDC 0785, FTDC 2916, FTDC 4462 and FTDC 4793 were confirmed as *L. brevis*, *L. plantarum*, *L. plantarum*, *L. casei* and *L. plantarum*, respectively using API 50 CHL identification kit.

SIGNIFICANCE STATEMENT

This study discovered the isolation and identification of unknown probiotics strains from Malaysian fermented foods that can be beneficial for use. This study will help the researchers to uncover the critical areas of the identification for research and commercial purposes.

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