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Identification of predominant *Lactobacillus* species in liquid sourdough fermentation

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Abstract

Two high protein wheat flour samples of Red Horse (RH) and Bake with Yen (BY) were examined for predominant *Lactobacillus* spp. in fermented liquid sourdough. The identification of *Lactobacillus* spp. was based on biochemical tests of catalase test, gas carbon dioxide production, arginine test, the ability to grow at temperature of 15°C and 45°C and carbohydrate fermentation using API50CH kit. Those strains were identified as *Lactobacillus* spp. and confirmed using polymerase chain reaction (PCR) of 16S rRNA partial sequencing analysis. In the present study, we successfully isolated and identified the *Lactobacillus plantarum* and *L. fermentum* which were predominant bacteria in liquid sourdough of the sample RH and BY brand, respectively.

Introduction

Lactic acid bacteria (LAB) are fundamental for sourdough properties and these include lactic fermentation, proteolysis, generation of volatile compounds, anti-mould and anti-ropiness, which are important activities found during dough leavening (Gobbetti, 1998; Hammes and Gänzle, 1998). The leavening process in dough preparation was originally used to produce the light spongy loaf but now it is also known that this process also improved the flavour of the bread.

Since the liquid sourdough are obtained through the process of fermentation, the microorganisms involved are a mixture of lactic acid bacteria (LAB) and yeasts. *Lactobacillus* spp. is among important microbes in sourdough fermentation and are also known as generally recognised as safe (GRAS) bacteria (Gobbetii, 1998; Corsetti and Settanni, 2007). They belong to either two groups of obligate and facultative heterofermentative or obligate homofermentative species (Hammes and Vogel, 1995). The presence of LAB in sourdough leads to the production of exopolysaccharides (EPS) which increase the fibre content, viscosity and improve the product texture. An increase in fibre content offers beneficial effects to consumers by lowering the cholesterol level. Levan and dextran are the most studied EPS, which can be produced in the dough by LAB under certain cultural conditions (Tieking and Ganzle, 2005). The most frequently isolated species of LAB in sourdoughs reported are *Lactobacillus sanfransicensis*, *L. reuteri*, *L. rossiae*, *L. delbrueckii* ssp., *L. casei*, *L. plantarum*, *L. brevis*, *L. alimentarius*, *L. fermentum* (Ottogalli *et al*., 1996).

Only a few strains of LAB were found to be suitable for liquid fermentation or in association with yeasts (Carnevali *et al*., 2007). In the present study, we isolated and identified the *Lactobacillus* spp. from fermented liquid sourdough of wheat flour as potential starter culture for liquid sourdough fermentation in bread.

Methods and Materials

Wheat flour samples

Two high protein wheat flour samples of Red Horse (RH) and Bake with Yen (BY) were supplied by Prestasi Flour Mill Sdn. Bhd., Selangor and Federal Flour Mill Bhd., Pahang respectively. Both are the major flour suppliers in Malaysia. Both wheat flour samples from these two suppliers contain characteristics including : moisture ≤ 14g / 100g, protein 12-13g / 100 g and ash content ≤ 0.57g / 100 g.

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Sourdough preparation

Liquid sourdough was prepared by adding wheat flour to distilled water (pH 7.0) at a ratio of 1:1 w/w, giving a dough yield of 200. After mixing manually for 5-10 min, mixture was fermented at 30˚C for 48 h in incubator.

pH measurement

The pH values of the sourdough were determined by a pH meter (PHM210-MeterLab).

Total Titratable Acidity (TTA)

Total titratable acidity (TTA) was determined by suspending 10 g sample in 90 ml sterile distilled water, mixed in a stomacher (Stomacher 400, USA) for 1 min and titrated to final pH 8.5 using 0.1 M NaOH. The TTA was expressed in ml 0.1 M NaOH. All analyses were performed in duplicate.

Enumeration of Lactic acid bacteria (LAB)

Enumeration of LAB was conducted by pour plate method as described by Robert and Greenwood (2003). A 10 g of sourdough sample was homogenized with 90 ml of 0.85% (w/v) sterilized peptone water (Merck) solution by blender (Waring Commercial Blender, USA). Serial dilutions were prepared and poured into MRS agar (Merck). A total of 10 ppm of cycloheximide (Merck) was added to the MRS agar for prevention of the growth yeasts and moulds (Okada *et al*., 1992). The plates were then incubated at 37˚C for 3 days in an anaerobic jar.

Isolation and identification

A serial dilution was made before isolation of Lactic acid bacteria by spreading 0.1 ml of sourdough samples as described above onto de Man, Rogosa and Sharp (MRS) agar (Merck). All plates were then incubated at 37˚C for 3 days. The tentative LAB isolates grown onto MRS agar were randomly selected and inoculated onto MRS slant agar for biochemical tests. The tentative LAB isolates were examined for their morphology cells and colonies, Gram test, catalase, CO_2 production from glucose, ability to grow at temperature of 15°C and 45°C, hydrolysis of arginine, sugar fermentations and carbohydrate fermentation profiles using the commercial API® 50CH test kit (BioMérieux, Germany).

Bacterial growth and chromosomal DNA extraction

All isolates were grown in MRS broth at 37°C with shaking at 200 rpm overnight. Total genomic DNA of the *Lactobacillus* isolates were extracted by phenol-chloroform-isoamyl method as described by Sambrook *et al*., (1989). The PCR amplification was conducted using a pair of primer as described by Corsetti *et al*., (2006). The primer sequences are LacbF 5'-TGCCTAATACATGCAAGT-3' and LacbR 5'-CTTGTTACGACTTCACCC-3'. The primers were supplied from First Base Laboratories (Selangor, Malaysia).

Amplification of the 16S rDNA gene was performed in a final volume of 50 µl. Each reaction mixture contained 50 µl volume containing 25 µl of PCR Master Mix (Qiagen), 2.5 µl of 1.0 µM each primer (Forward and reverse), and 3 µl of 100 ng DNA template and 17 µl of nucleas free water (NFW). A negative-DNA control was performed by adding 3 µl of NFW, a positive control was performed by adding 3 µl of the DNA sample. PCR was carried out in Eppendorf thermal-cycler (Eppendorf, Germany) with a temperature program consisting of the initial denaturation at 94°C for 1 min to complete denaturation of the DNA template, followed by 35 cycles of denaturation at 94°C for 15 seconds, annealing for 1 min at 35.6°C, polymerization at 72°C for 1 min and final elongation at 72°C for 10 min.

The amplification PCR products were analyzed by electrophoresis using 1.0% (w/v) agarose gel in 1X TAE buffer (40 mM Tris-OH, 20mM acetic acid and 1mM of EDTA; pH 7.6) at 90 V for 40 min and stained by ethidium bromide. A 1 kb DNA ladder (Vivantis, Malaysia) was used as size reference. The gels were visualized using UV transilluminator (Fujifilm LAS 2000). The amplification PCR products were purified by the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) as instructed by the manufacturer.

DNA sequencing and analysis of sequenced data

The amplified 16S rDNA partial sequences were performed by First Base Laboratories (Selangor, Malaysia). Sequence similarities were determined by comparing to 16S rDNA sequences available in the nucleotide databases of the GenBank (*http://www.ncbi.nlm.nih.gov/BLAST*), using the Basic Local Alignment Search Tool (BLAST) program from the genus identification

Results and Discussions

pH value and total titratable Acid (TTA)

The pH value and total titratable acid (TTA) of both flour brands were tabulated in Table 1. As shown in Table 1, The pH value of both sourdough decreased after 48 hours fermentation. The decline of pH was concomitant with the increase of acid production in both fermented sourdough. This was supported by the increasing value of TTA value in both fermented

Table 2. Biochemical test and carbohydrate profile using API® 50CH test kit of tentative *Lactobacillus* spp. in liquid sourdough Red Horse and Bake with Yen

Brand	Red Horse		Bake with Yen		
Strains identified	*L. plantarum*	*L. brevis*	*L. fermentum*	*L. pentosus*	*L. buchneri*
Number of strains identified by API® 50CH test	24	1	18	1	6
CO_2 from Glucose	-	-	+	+	+
Growth at :					
15°C	+	+	-	-	-
45°C	-	-	+	+	+
$NH_3$from Arginine	-	-	-	-	-

samples (Table 1) which indicated the presence of organic acids in liquid sourdough after 48 hours fermentation. The formation of organic acids (lactic and acetic), alcohols (acetoin, aldehydes, ketones) and various carbonyl compounds during fermentation are due to the presence of LAB and yeasts which contributed to the decrease of pH and enhanced the aroma of the bread. These findings are in agreement with Gobbetti *et al*. (1995a; 1995b) who reported that non-volatile compounds including organic acids produced by homo and heterofermentative LAB would decrease pH and contribute to a pleasant aroma to the bread dough.

Enumeration of Lactic acid bacteria (LAB)

The tentative *Lactobacillus* spp. colonies forming in MRS agar were 1 x 10^6 cfu/g and 4.8 x 10^9 cfu/g for liquid sourdough Red Horse and Bake with Yen, respectively. The presence of *Lactobacillus* spp. in flour dough was expected. According to Rehman *et al*. (2006), in good bakery practice, a sponge should contain metabolically active LAB at 10^8 – 10^9 cfu/g and yeasts at 10^6 – 10^7 cfu/g. The LAB is primarily responsible for acidification and leavening of the dough. However, the LAB may either originate from natural flour sources or from fermented dairy products (De Vuyst and Neysens, 2005).

Isolation and identification of Lactic acid bacteria (LAB)

A total of 50 *Lactobacillus* spp. strains were isolated from sourdough Red Horse (RH) and Bake with Yen (BY). All *Lactobacillus* spp. strains were identified using biochemical tests and API® 50CH test kit. Table 2 tabulated the biochemical test and carbohydrate profile using API® 50CH test kit of tentative *Lactobacillus* spp. in liquid sourdough Red Horse and Bake with Yen.

Colonies which appeared in rod shape under microscope, Gram positive and catalase negative and negative in arginine hydrolysis test were further evaluated using API® 50CH test kit. Among 50 *Lactobacillus* strains that were both isolated from liquid sourdough RH and BY, 24 *Lactobacillus* strains isolated from liquid sourdough RH were *Lactobacillus plantarum* and a single isolate was *L. brevis*. While, *Lactobacillus* strains isolated from liquid sourdough BY comprised 18 strains of *L. fermentum*, 6 strains of *L. buchneri* and a single strain of *L. pentosus* (Table 2).

The *L. plantarum* and *L. brevis* strains from

Table 3. Identification of *Lactobacillus* strains isolated from fermented liquid sourdough Red Horse (RH) and Bake with Yen (BY) using 16S rDNA partial sequences and API® 50CH test kit

Sample	Strain designation	API® 50CH test		16s rRNA partial sequences	
		Species	ID (%)	Species	ID (%)
RH	RH1	*Lb. plantarum*	94.0	*Lb. plantarum*	100
RH	RH2	*Lb. plantarum*	94.0	*Lb. plantarum*	100
RH	RH3	*Lb. plantarum*	97.9	*Lb. plantarum*	99
RH	RH4	*Lb. plantarum*	97.9	*Lb. plantarum*	100
RH	RH5	*Lb. plantarum*	93.8	*Lb. plantarum*	99
RH	RH6	*Lb. plantarum*	93.7	*Lb. plantarum*	100
RH	RH7	*Lb. plantarum*	98.0	*Lb. plantarum*	99
RH	RH8	*Lb. plantarum*	93.8	*Lb. plantarum*	99
RH	RH9	*Lb. plantarum*	98.0	*Lb. plantarum*	100
RH	RH10	*Lb. plantarum*	94.0	*Lb. plantarum*	100
RH	RH11	*Lb. plantarum*	97.9	*Lb. plantarum*	99
RH	RH12	*Lb. plantarum*	97.9	*Lb. plantarum*	99
RH	RH13	*Lb. plantarum*	98.0	*Lb. plantarum*	99
RH	RH14	*Lb. plantarum*	97.9	*Lb. plantarum*	100
RH	RH16	*Lb. plantarum*	98.0	*Lb. plantarum*	100
RH	RH15	*Lb. brevis*	93.0	*Lb. plantarum*	100
RH	RH16	*Lb. plantarum*	94.0	*Lb. plantarum*	99
RH	RH17	*Lb. plantarum*	94.0	*Lb. plantarum*	100
RH	RH18	*Lb. plantarum*	94.0	*Lb. plantarum*	99
RH	RH19	*Lb. plantarum*	94.0	*Lb. plantarum*	99
RH	RH20	*Lb. plantarum*	82.0	*Lb. plantarum*	99
RH	RH21	*Lb. plantarum*	97.9	*Lb. plantarum*	99
RH	RH22	*Lb. plantarum*	97.9	*Lb. plantarum*	99
RH	RH23	*Lb. plantarum*	98.0	*Lb. plantarum*	99
RH	RH24	*Lb. plantarum*	92.6	*Lb. plantarum*	99
RH	RH25	*Lb. fermentum*	96.0	*Lb. plantarum*	100
BY	BY1	*Lb. fermentum*	90.2	*Lb. fermentum*	100
BY	BY 2	*Lb. fermentum*	78.6	*Lb. fermentum*	99
BY	BY 3	*Lb. fermentum*	79.9	*Lb. fermentum*	99
BY	BY 4	*Lb. fermentum*	76.1	*Lb. fermentum*	99
BY	BY 5	*Lb. fermentum*	79.9	*Lb. fermentum*	99
BY	BY 6	*Lb. fermentum*	90.2	*Lb. fermentum*	99
BY	BY 7	*Lb. fermentum*	76.1	*Lb. fermentum*	99
BY	BY 8	*Lb. pentosus*	75.2	*Lb. fermentum*	98
BY	BY 9	*Lb. fermentum*	78.0	*Lb. fermentum*	99
BY	BY10	*Lb. fermentum*	70.0	*Lb. fermentum*	100
BY	BY11	*Lb. fermentum*	70.0	*Lb. fermentum*	100
BY	BY 12	*Lb. fermentum*	48.5	*Lb. fermentum*	99
BY	BY 13	*Lb. fermentum*	91.3	*Lb. fermentum*	100
BY	BY 14	*Lb. fermentum*	76.1	*Lb. fermentum*	100
BY	BY 15	*Lb. fermentum*	90.2	*Lb. fermentum*	99
BY	BY 16	*Lb. buchneri*	46.2	*Lb. fermentum*	99
BY	BY 17	*Lb. buchneri*	51.4	*Lb. fermentum*	99
BY	BY 18	*Lb. fermentum*	79.9	*Lb. fermentum*	99
BY	BY 19	*Lb. buchneri*	43.2	*Lb. fermentum*	99
BY	BY 20	*Lb. buchneri*	49.6	*Lb. fermentum*	100
BY	BY 21	*Lb. buchneri*	50.1	*Lb. fermentum*	99
BY	BY 22	*Lb. buchneri*	43.6	*Lb. fermentum*	99
BY	BY 23	*Lb. fermentum*	91.3	*Lb. fermentum*	98
BY	BY 24	*Lb. fermentum*	78.6	*Lb. fermentum*	100
BY	BY 25	*Lb. fermentum*	78.6	*Lb. fermentum*	100

RH-Red Horse; and BY- Bake with Yen

RH sourdough were not able to produce CO_2 from glucose, indicating they were in homofermentative LAB groups. This LAB group was only able to ferment glucose to lactic acid. Whereas, L. *fermentum*, *L. buchneri* and *L. pentosus* were in the group of heterofermentative LAB which they were able to ferment glucose to lactic acid, ethanol/acetic acid, and CO_2 (Sharpe, 1979; Axelsson, 1993). *L. plantarum* and *L. brevis* strains from RH sourdough were able to grow at 15°C, but no growth at 45°C. While, *L. fermentum*, *L. buchneri* and *L. pentosus* in liquid sourdough BY were able to grow at 45°C, but none of the colonies grow at 15°C.

As shown in Table 2, the results indicated L. plantarum was the predominant species in liquid sourdough RH followed by *L. brevis*. While, *L. fermentum* was found predominating BY sourdough sample, followed by *L. buchneri* and *L. pentosus*. Corsetti and Settani (2007) reported *L. sanfrancisensis*, *L. brevis* and *L. plantarum* are the lactobacilli that are most frequently isolated from sourdough. While Stolz (2003) reported that the dominat LAB of fermented sourdoughs are homofermentative lactobacilli and pediococci which includes *L. casei*, *L. delbrueckii*, *L. farciminis*, *L. plantarum*, *Pediococci acidilactici* and *P. pentosaceus*.

16S rDNA partial sequences

All *Lactobacillus* strains (n=50) which were analyzed using API® 50CH test kit were confirmed by molecular approaches of 16S rDNA partial sequences. Figure 1 shows amplified PCR products using 16S rDNA primers which produced a single band of 1500 bp in size. Surprisingly, using molecular techniques all *Lactobacillus* strains belong to *L. plantarum* (Table 3). This results was in contrast with the API® 50CH test kit where 24 *Lactobacillus* strains belong to *L. plantarum* and a single isolate belong to *L. brevis* from liquid sourdough Red Horse (RH). The percentage of identification (ID) through 16S rDNA partial sequences showed more than 99 % (Table 3). Similar observation to *Lactobacillus* strains isolated from liquid sourdough Bake with Yen (BY) where all *Lactobacillus* strains examined, belong to *L. fermentum* with ID percentage more than 98% (Table 3).

In the present study, results obtained from 16s rDNA partial sequences were slightly different with API® 50CH test kit tested in both *Lactobacillus* strains isolated from liquid sourdough RH and BY samples. Temmerman et al. (2004) and Ehrmann and Vogel (2005) reported identification of sourdough Lactic acid bacteria (LAB) by morphological and biochemical characteristics using carbohydrate fermentation patterns are less convincing for taxonomic resolution. However, combination of those techniques with molecular approaches will offer higher accuracy results (Ehrmann and Vogel, 2005).

Conclusion

In conclusion, liquid sourdough prepared from two different brands of RH and BY, comprised different LAB group species. The predominant LAB strains in RH liquid sourdough were *Lactobacillus plantarum* which belongs to LAB homofermentative group. While *L. fermentum* was found predominant in BY liquid sourdough which belongs to LAB heterofermentative group. Both of these species are commonly listed as probiotic strains, which are also generally recognized as safe (GRAS) bacteria. The results of the study also demonstrated the potential of both bacteria species (*L. plantarum* and *L. fermentum*) to be used as homogenous starter culture of liquid sourdough fermentation in bread.

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