

Isolated from Fresh Orange Juice

การคัดแยก การศึกษาคุณสมบัติและการบ่งชี้ชนิดของแบคทีเรียแลคติกจากน้ำส้มคั้นสด

Sureeporn Wichiansri (สุรีย์พร วิเชียรศรี)* Dr.Surasak Siripornadulsil (ดร.สุรศักดิ์ ศิริพรอดุลศิลป์)**

Dr.Wilailak Siripornadulsil (ดร.วิไลลักษณ์ ศิริพรอดุลศิลป์)***

ABSTRACT

Fruit juice contains high sugar content, low pH, high acid and vitamin, the conditions in which lactic acid bacteria (LAB) can grow. LABs are industrially important microorganisms and have probiotic properties. The aims of this study were to isolate, characterize and identify LAB from orange juice purchased from street market. LAB were screened by spread plate on de Man, Rogosa and Sharpe (MRS) agar supplemented with 1% calcium carbonate and 0.004% bromocresol purple then selected and characterized. The 3 isolates were tolerant to a high sugar concentration, non-hemolytic, and molecularly identified using 16S rDNA as *Weissella confusa* JU2 and *Lactobacillus plantarum* JU3 and JU4. *W. confusa* have been reported that they were able to causes sepsis and infections in humans and animals while *L. plantarum* species are considered as beneficial LAB and often used as probiotic supplements in food products.

บทคัดย่อ

น้ำผลไม้ประกอบไปด้วยน้ำตาลสูง, มีค่า pH ต่ำ, มีกรดและวิตามินสูง ซึ่งเป็นคุณสมบัติที่เหมาะสมต่อการเจริญของแบคทีเรียแลคติก ซึ่งเป็นจุลินทรีย์ที่มีความสำคัญในอุตสาหกรรมและมีคุณสมบัติเป็นโพรไบโอติก วัตถุประสงค์ของการศึกษานี้คือ คัดแยก ศึกษาคุณสมบัติ และบ่งชี้ชนิดของแบคทีเรียแลคติกที่พบในน้ำส้มคั้นสดที่ซื้อจากตลาดสด โดยแบคทีเรียแลคติกจะถูกคัดแยกโดยการเพาะเลี้ยงบนอาหารเลี้ยงเชื้อ de Man, Rogosa and Sharpe (MRS) ที่ผสม 1% แคลเซียมคาร์บอเนตและ 0.004% bromocresol purple พบว่ามี 3 ไอโซเลท ที่มีคุณสมบัติเป็นแบคทีเรียแลคติก, ทนต่อสภาวะที่มีความเข้มข้นของน้ำตาลสูง, ไม่ย่อยสลายเม็ดเลือดแดง และเมื่อระบุชนิดโดยการวิเคราะห์ลำดับนิวคลีโอไทด์ของ 16S rDNA พบว่า JU2 มีความคล้ายคลึงกับ *Weissella confusa* และ JU3 และ JU4 มีความคล้ายคลึงกับ *Lactobacillus plantarum* ซึ่งในปัจจุบันพบว่า *W. confusa* ถูกรายงานว่าเป็นสาเหตุของการเกิดโรคและการติดเชื้อในคนและสัตว์ ในขณะที่ *L. plantarum* เป็นชนิดที่ถูกนำมาใช้เป็นจุลินทรีย์โพรไบโอติกในอาหาร

Keywords: Lactic acid bacteria, Probiotic, Orange juice

คำสำคัญ: จุลินทรีย์แลคติก โพรไบโอติก น้ำส้มคั้น

* Student, Master of Science Program in Microbiology, Faculty of Science, Khon Kaen University

** Assistant Professor, Department of Microbiology, Faculty of Science, Khon Kaen University

*** Assistant Professor, Department of Microbiology, Faculty of Science, Khon Kaen University

Introduction

Lactic acid bacteria (LAB) are Gram-positive, non-motile, non-spore forming, rod- and coccus-shaped bacteria that can ferment carbohydrates and alcohols to produce lactic acid and other end products which give the typical flavor and can also preserve nutrients and vitamins of fruits and vegetables (Okcu et al., 2016). LAB are industrially important organisms due to their GRAS (Generally Recognized as Safe) status and play the role in the production of fermented food, and dairy product (Herreros et al., 2005; Siripornadulsil et al., 2014).

Juice is a liquid (drink) that contains a fruit and vegetable. It can also refer to liquids that are taste with these or other biological food sources. It's intending for direct consumption products prepared mechanically from ripen fruits and preserved exclusively by physical means (Bates et al., 2001). Fresh juice is a product that has a short shelf-life because it is normally non-sterilized after processing and storage in refrigerator. Microorganisms are the main cause of juice spoilage but it is difficult to detect the beginning of juice spoilage because the origin and source of infection is often unknown. Juice products are contaminated by ingredients and/or during juice processing. Recently, several studies have reported the detection of LAB in several juice products such *Lactobacillus casei* DN 114 001, *L. rhamnosus* GG, *L. paracasei* NFBC 43338, and *Bifidobacterium lactis* Bb-12 found in orange juice and pine apple juice and *L. plantarum* C3 found in tomato juice (Martins et al., 2013) suggesting that LAB are naturally existing and could be isolated from fruit juices.

Nowadays, LAB are known as microorganisms that have a significant role as probiotics and used as supplements in many food products such as synbiotic products containing a combination of probiotic and prebiotic (Nematollahi et al., 2016). Probiotic properties of LAB on human health are such as aid digestion, improve bowel movement and prevent enteric pathogen infection. The most common probiotic products are dairy products and the probiotic consumers are usually vegetarians, individuals with lactose intolerance and cholesterol-restricted diets (De Palencia et al., 2008). Selection and characterization of LAB derived from natural fruit juice could offer the potential LAB that can be applied in the fruit juice products.

Objective of the study

The aims of this study were isolation, characterization and identification LAB from fresh orange juice.

Materials and Methods

Isolation of LAB from orange juice

Fresh orange juices were purchased from Mor-Din-Daeng street market and Complex KKU center. The samples were spread on de Man, Rogosa and Sharpe (MRS) agar supplemented with 1% calcium carbonated and 0.004% bromocresol purple and incubated in candle jar at 37 °C for 2-5 days (Barbu, 2008; Yang et al., 2016). The yellow colonies surrounding with clear zone were selected and characterized. The single colony was smeared on a microscope slide, stained with Gram staining solutions and observed under light microscope with 100X oil immersion

lens (Ketsawaddiwong et al., 2016). These strains were simple selected by catalase and oxidase tests. For catalase test, the bacterial colony was transferred to a microscopic slide and dropped with 3% H₂O₂ solution. Gas production was observed as catalase positive. For oxidase test, bacterial culture was streaked on the filter paper and dropped with oxidase reagent. The appearance of blue color is oxidase positive (Zanirati et al., 2015)

Characterization of LAB isolated from orange juice

Carbohydrate utilization

The bacterial cultures were grown in MRS broth for 24 h and adjusted to OD₆₀₀ = 0.6. These starter cultures were centrifuged to obtain cell pellet and washed twice and re-suspended with 0.85% NaCl solution. The cell suspension was dropped onto medium agar containing 0.5% (w/v) peptone, 0.3% (w/v) yeast extract, 1.5% (w/v) agar, 0.004 % (w/v) bromocresol purple supplemented with 5, 10, 20 and 30% (w/v) carbohydrate and incubated at 37 °C for 24 h. The carbohydrates tested in this experiment were 4 types of sugar including glucose, lactose, sucrose, and fructose. The sugar utilization was indicated by measuring the ratio between clear zone and colony diameters (Kadere, Kutima, 2012).

Total acidity

LAB cultures (OD₆₀₀ = 0.6) were inoculated into MRS broth containing 5% (v/v) glucose (pH=6.5) and incubated at 37°C under static condition for 2 days and LAB culture was collected at 0, 1, 2 days. The viable cells (i) were counted using 10-fold serial dilution and drop plate method on MRS agar after incubated at 37°C for 2 days. The cell culture was centrifuged at 10,000 rpm, 15 minutes at 4°C to obtain cell free supernatant (CFS) for determination of pH (ii) and total acidity (iii) by added 2 ml of CFS into 100 ml of deionizing water and 1 ml of phenolphthalein (0.5% in 50% alcohol) as indicator. The percentage of lactic acid was determined by titration with 0.1N NaOH and calculated according to the following formula (AOAC, 1990).

$$\% \text{ total acidity} = \frac{(\text{ml of NaOH used}) \times (\text{conc. NaOH}) \times (\text{equivalent weight of lactic acid}) \times (100)}{(\text{ml of sample used})}$$

Antibiotic susceptibility

LAB were grown in MRS broth and incubated at 37°C for 24 h, adjusted to 0.6 (OD₆₀₀) and swabbed on MRS agar. The antimicrobial disks were placed on the agar and incubated at 37°C for 24 h. The clear zone surrounding diameters were measured (Buahom et al., 2018).

Hemolytic activity

LAB were incubated at 37°C for 24 h in MRS broth to obtained starter and adjusted to OD₆₀₀ = 0.6. Then, LAB cell suspension was dropped on blood agar and incubated at 37 °C. After 24 h, the agar around colony was observed. Hemolysis was classified into 3 types. (1) β -hemolysis, a clear zone surrounding the colonies; (2) α -hemolysis, dark and greenish under colony and (3) γ -hemolysis or non-hemolysis, no change under and around the colony (Punya et al., 2014).

Antimicrobial activity by double layer method

LAB were incubated at 37°C for 24 h in MRS broth, adjusted 0.6 (OD₆₀₀) and dropped on MRS agar and air dried. The plate was overlaid with 10 mL LB agar on the surface agar and allowed till the agar was solidified. Then, the pathogenic bacteria at the age of 18 h (OD₆₀₀ = 0.6) were swabbed on the surface agar and incubated at 30°C for 24 h. The bacterial pathogens used in this study included *Bacillus cereus*, *Escherichia coli*, *Listeria innocuae*, *Staphylococcus aureus* and *Salmonella* Typhimurium. The antibacterial activity was indicated by measuring the clear zone and colony diameters and the inhibition ratio was calculated by the clear zone divided by the colony zone (Buahom et al., 2018).

Molecular identification of LAB strains

LAB strains were identified by 16S rRNA partial sequence analysis. The chromosomal DNA of LAB was prepared by phenol chloroform extraction. The universal primers 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1500R (5'-CTACGGCTACCT TGTTACGA-3') were used for 16S rDNA gene amplification (Tajabadi et al., 2011). Each 50 µl reaction mixture contains 5 µl of 10x buffer, 0.2 mM dNTPs, 1.25U *Taq* DNA polymerase, 0.3 µM of each primer and 1 µl genomic DNA. The reaction mixtures were first incubated for 2 min at 94°C, and then amplified for 35 cycles according to the following temperature profiles: denaturing at 94°C for 30 s, annealing at 55-60°C for 30 s, extension at 72°C for 1.5 min, and followed by final extension at 72°C for 10 min. Aliquots of 3 µl PCR products were analyzed on 1% agarose gel electrophoresis. The amplified PCR products were purified and sequenced directly, BLAST search of these sequences against the NCBI database was. The phylogenetic tree was constructed using MEGA 7.0.26 program.

Results

The whole 3 orange juice samples were found 3 isolates with yellow colony and clear zone were observed from only fresh orange juice sample from Mor-Din-Daeng street market. They were Gram positive, short rod and non-spore forming and showed negative activity on catalase and oxidase test. These bacterial strains were suggested as LAB. They were tested for carbohydrate utilization by dropping the starter culture on MRS agar supplemented with bromocresol purple and carbohydrates including mono- or di- saccharides. The result exhibited all LAB strains were resistant in high sugar concentration but types and concentration of carbohydrates have effects on bacterial growth (Tables 1). They also well grew and produced acid as shown in Table 2. The viable cell were increased more than 5 log after 24 h and slightly increased when longer incubation time except JU4 was exhibited viable cell over 11 log CFU/mL after 48 hour. Moreover, the total acidity in CFS was determined by titration. The percent acidity was increases in longer incubation time. JU3 and JU4 were exhibited high percent acidity when compared with JU2. This result was similar to pH values of CFS. The pH value of JU2- and JU3-CFS were lower than that of JU2 after 48 h.

In addition, antibiotic susceptibility, hemolysis activity and antimicrobial activity of the three LAB strains were also investigated. The result of antibiotic susceptibility of LAB strains were shown in Table 4. All LAB strains

were susceptible to ampicillin, erythromycin and bacitracin but resistant to tetracycline, streptomycin, vancomycin and gentamycin. JU2 was more sensitive to antibiotics than JU3 and JU4. For the test of hemolysis activity, all isolates were dropped on blood agar to observe hemolysis type compare with *B. cereus* as positive control. All LAB strains were non-hemolytic. The results of antimicrobial activity against pathogenic bacterial of LAB obtained by double layer method were shown in Table 4. All isolates were grown under microaerobic condition and able to produce the antibacterial agents that can inhibit growth of both Gram positive and negative pathogens. The inhibition ratio demonstrated that JU3 and JU4 were more effective than JU2 against *B. cereus*, *E. coli* and *L. innocuae* but JU2 showed higher antibacterial activity than JU3 against *S. Typhimurium*.

LAB were molecularly identified by 16S ribosomal DNA. LAB strains were amplified by PCR using 27F and 1500R primers and BLAST search analysis also confirmed the identity to LAB. The phylogenetic tree was shown in Figure 1. The JU2 strain was identified as *W. confusa* with bootstrap support 100% while JU3 and JU4 strains were closely related to *L. plantarum* bootstrap support 99%.

Table 1 Carbohydrate fermentation of lactic acid bacteria at 37°C

Isolate	Non	Glucose (% w/v)				Fructose (% w/v)				Sucrose (% w/v)				Lactose (% w/v)				
		5	10	20	30	5	10	20	30	5	10	20	30	5	10	20	30	
JU2	NC	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
JU3	NC	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+	++	+++	+++	+++	+++
JU4	NC	+++	++	+	++	+++	+++	+++	+++	+++	+++	++	++	+	++	++	++	++

Ratio between clear zone/colony: + 1-1.99; ++ 2-2.99; +++ >3

Table 2 Lactic acid production of LAB fermentation

Isolate	Viable cell (Log CFU/mL)		pH		% acidity	
	24 H	48 H	24 H	48 H	24 H	48 H
JU2	8.82±1.72	8.46±1.72	4.5	4.5	0.77±0.00	0.91±0.06
JU3	9.29±1.34	9.39±1.79	4.5	4	1.23±0.06	1.77±0.19
JU4	9.81±1.49	11.54±1.53	4.5	4	1.27±0.13	2.00±0.13

Table 3 Antibiotic susceptibility test of lactic acid bacteria

Isolate	Erythromycin	Tetracycline	Gentamicin	Ciprofloxacin	Ampicillin	Bacitracin	Streptomycin	Rifampicin	Enrofloxacin	Vancomycin	% susceptibility
JU2	S	R	R	I	S	S	R	R	R	R	30
JU3	S	R	R	R	S	S	R	S	R	R	40
JU4	S	R	R	R	S	S	R	R	R	R	30

S Susceptible; I Intermediate; R Resistant

Table 4 Antimicrobial activity of LAB against pathogenic bacterial by double layer method

Isolate	Gram Positive			Gram Negative	
	<i>B. cereus</i>	<i>L. innocuae</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. Typhimurium</i>
JU2	+	+	+	+	++
JU3	++	++	+	++	+
JU4	++	+	+	+	+

+ Inhibition ratio 1-1.5 ; ++ Inhibition ratio >1.5-2

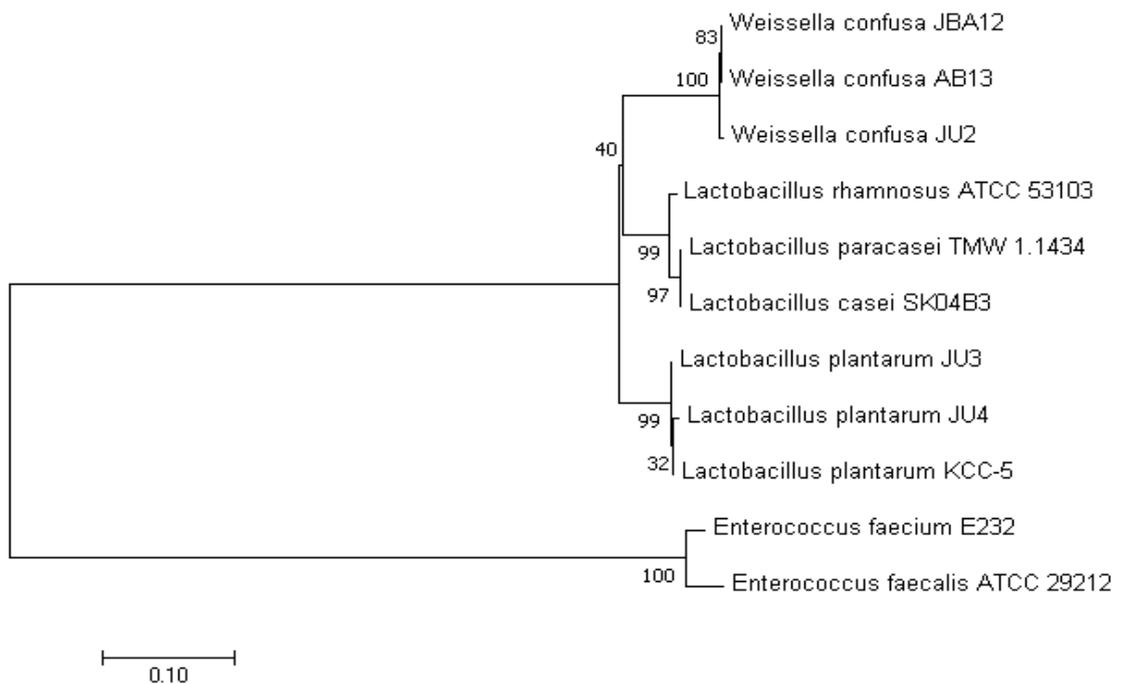


Figure 1 Phylogenetic tree of LAB isolated from orange juice perform by MEGA 7.0.26 program with Neighbor-joining Tree

Discussion

All 3 strains isolated from orange juice exhibited LAB characteristics including Gram positive, non-spore forming, non-motile, γ -hemolysis and catalase and oxidase negative (Halder et al., 2017). LAB can ferment several types of sugar to produce lactic acid and various end products. Four types of sugars used as carbon source in this study consisted of mono-saccharides (glucose, fructose and sucrose) and di-saccharide (lactose). All LAB isolates were able to ferment all sugars and tolerate up to 30% sugar. LAB can normally convert sugar and produce organic acids via fermentation and make the culture low pH (Ishola, Adebayo-Tayo, 2012). The susceptibility of LAB isolates tested against 10 antibiotics indicated that they were susceptible to the β -lactam antibiotic group such as ampicillin which has a broad spectrum on antibacterial activity (Liasi et al., 2009). The LAB isolates showed a strong inhibitory activity against many food borne pathogens of both Gram positive (*B. cereus*, *L. innocua*, and *S. aureus*) and Gram negative (*S. Typhimurium* and *E. coli*) suggesting that they were able to produce some metabolites with a broad spectrum antibacterial activity.

The LAB strains were molecularly identified as *W. confusa* JU2 and *L. plantarum* JU3 and JU4. The *Weissella* species (formerly *L. confusus*) is *Lactobacillus*-like microorganism with vancomycin resistance. It can be found in fermented foods, sugar cane and human feces and also has been suggested as a probiotic. However, the previous reports suggested that this species can cause sepsis and other serious infections in humans and animals such as causal agent of abscess, bacteraemia and endocarditis (Kumar et al., 2011; Fairfax et al., 2014). In the other hand, *L. plantarum* is accepted as a bacterial probiotic. It is also known as a normal flora microorganism in human and animal intestine that shows many beneficial properties such as inhibitory effect on pathogen, reduction the effect and duration of several types of diarrhea and ability to modulate the immune system. Nowadays, *Lactobacillus* spp. are most often used as probiotic supplements in foods because of their GRAS status (Verdenelli et al., 2009; Zago et al., 2011).

Conclusions

LAB strains were isolated from orange juice demonstrating their naturally existing in plant. Due to their ability to ferment many types of sugar and convert them to many organic acids including lactic acid, the LAB cultures are usually low in pH. Thus, many LAB have been used as starter cultures in several foods since the low pH can help preserving food and prolong their shelf-life. The tolerance to high sugar concentration of isolated LAB make them suitable for adding into high-sugar foods. The susceptibility to only some antibiotics is relatively acceptable to apply in food products. *W. confusa* JU2 was recognized as pathogen and will be discarded for further study. In contrast, *L. plantarum* JU3 and JU4 were regarded as safe species. Therefore, these two strains will be additionally investigated for their probiotic properties and/or for their application as supplement in orange or other fruit juices.

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