Isolation, identification and investigation some biochemical properties of lactic acid bacteria from "Mam sac chua" and "Com me" in Tien Giang province

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ABSTRACT

The objective of this study was isolate, identify and investigate some biochemical properties of strains of lactic acid bacteria from "com me" and "mam sac chua" in Tien Giang province. Nineteen strains of LAB were isolated from four "com me" and three "mam sac chua" samples. They have characterized of lactic acid bacteria such as: halo rings in MRS agar environment added 0,85% $CaCO_3$, rod-shaped cells, Gram positive, catalate and oxidase negative. All 19 strains of LAB were able to produce lactic acid in MRS broth (1,01 - 2,23mg/mL after 24 hours). Three strains of LAB were isolated from "com me" were able to produce lactic acid in MRS broth at salt concentration of 0, 2, 4 and 6% (0,57 - 1,29mg/mL after 24 hours). In particular, strains of LAB were coded ML3 and ML4 produced the highest lactic acid and VB strain was the most salinity tolerance. Therefore, these three srains were choosed to identify species by molecular biology technique. The results of identification were *Staphylococcus piscifermentans* VB, *Lactobacillus plantarum* ML3 and *Lactobacillus plantarum* ML4 because they are 99% homologous to *S. piscifermentans* and *L. plantarum.*

1. Introduction

Lactic acid bacteria (LAB) important in the food industry, especially traditional fermented foods. LAB is considered a food-safety microorganism because it is safe to use. In addition, reducing the pH in the product also helps inhibit microorganisms that cause spoilage. LAB-generated substances such as lactic acid, carbon dioxide and bacteriocin, ... are considered as substances capable of preventing pathogenic microorganisms and microorganisms from infecting products (Mahantesh, Ajay, Anand, & Ramana, 2010).

"Mam sac chua" and "Com me" are traditional and popular sour fermented food in Viet Nam. The most special feature of these 2 products are they can be used to eat without cooking. In the process of making "com me" and "mam sac chua", lactic acid bacterias are the main factors, that help the sour fermentation take place. The lactic acid produced sour and characteristic aroma for these 2 foods. The lactic acid reduced the pH of the culture, inhibited the growth of rot-causing bacteria, prolonged the shelf life of the product without using any other food additives. In order to enrich the studies of lactic acid bacteria and as a basis for improving the processing of traditional fermented foods (add bacteria to materials), this study was done.

2. Materials and methods

2.1. Materials

Samples: Three samples of "mam sac chua" and four samples of "com me" were collected from traditional markets in Tien Giang province.

Figure 1. "Mam sac chua" (A) and "Com me" (B) samples from traditional markets in Tien Giang province

Chemicals: Environment MRS broth (India), Agar (Merck), $CaCO_3$ (China), KOH (China), H_2O_2 (China), NaOH 0,1 N (China), phenolphtalein (China); Chemicals for conducting PCR: Use chemicals in the PCR kit, including: Pr Taga's Go Taq Green Master Mix 2X, pair of primer 27F/1492R. Chemicals for electrophoresis of PCR products: Agarose 1 – 1,5% (Merck), TAE buffer 1X, loading buffer (Bio-Rad), Ethidium bromide (fluorescent indicator-EtBr), 1kb DNA ladder (Promega).

2.2. Methods

2.2.1. Isolation lacid acid bacteria from "mam sac chua" and "com me" samples

Samples handling: Each sample of "mam sac chua" and "com me" was finely ground, mixed well and weighed 10 g into flasks containing 90mL of sterilized physiological saline, shake 150rpm at 37℃ for 1,5 hours.

Isolation of lactic acid bacteria: Lactic acid bacterias from samples of "mam sac chua" and "com me" were isolated on MRS agar environment added 0,85% $CaCO_3$ and incubated at 37℃ for 48 hours. Typical colonies dissolved $CaCO_3$ (halo rings) were selected inoculated repeatedly on MRS agar until homogeneous colonies were identified and briefly identified as lactic acid bacteria by some biochemical tests: morphology of isolates (Axelsson, 2004), gram characteristics (Suslow, Matsubara, & Davies, 1987), ability to produce catalase and oxidase enzymes (Reiner, 2010; Shields & Cathcart, 2010).

2.2.2. Investigation of lactic acid production ability of isolated bacterial strains

Each strain of bacteria was cultured in 15mL MRS broth at 37°C for 24 hours and the lactic acid content was determined by the Therner titration method.

All 15 mL of bacterial solution after 24 hours of culture were used to centrifuge. After that, 10mL of clear solution were draw into a flask, add 20mL of distilled water and 1 - 2 of phenolphthalein drops at concentration of 1%. That mixture was titrated with NaOH solution of 0,1N until light pink solution appeared about 30 seconds, stopped titration and recorded the volume of NaOH used.

The acidity is calculated in Therner degrees.

$$° T = V_{NaOH\ consumed} \times 10 \quad (1)$$

$$\text{Lactic acid} = ° T \times 0.009 \quad (2)$$

In which: ° T is Therner degree, 1 ° T corresponds to 9 mg of lactic acid

2.2.3. Investigation of salinity tolerance of bacterial strains were isolated from "mam sac chua"

Each strain of bacteria was cultured in 15mL of MRS broth at salt concentrations of 0, 2, 4 and 6% at 37°C for 24 hours. Salinity tolerance of bacterial strains were expressed through the lactic acid content and it was determined by the Therner titration method.

2.2.4. Identification of species of bacterial strains, that were isolated by molecular biology technique

The most potential bacterial strains were identified based on the 16S rRNA gene sequence. The results were analyzed by sequencing analysis 5.3 software and compared on Gene NCBI bank (U.S. National Library of Medicine - National Center for Biotechnology Information, n.d.) to find closely species.

2.2.5. Methods of analysis and data processing

The data were processed using Microsoft Excel, Minitab 16 Statistical Software to analyze statistics and ANOVA.

3. Results and discussion

3.1. Results

3.1.1. Isolation lacid acid bacteria from "mam sac chua" and "com me" samples

Nineteen strains of bacteria, that were able to produce lacid acid on the MRS agar added $CaCO_3$ 0,85% were isolated from 3 samples of "mam sac chua" were coded TT, VB, GC and 4 samples of "com me" were coded CC, HC, CP, ML in Tien Giang province. Colony morphological characteristics of a typical strain of bacteria was cultured after 2-3 days was shown in Figure 2.

All colonies of 19 bacterial strains were isolated had round colonies, milky white to ivory white, whole cover or serrated. All 19 bacterial strains were Gram positive, catalase and oxydase negative. Almost the cell shape of bacteria strains were short rods beside one strain was globular. These are also prominent features phenotype of the lactic acid bacteria, that were described by (Kandler & Weiss, 1986).

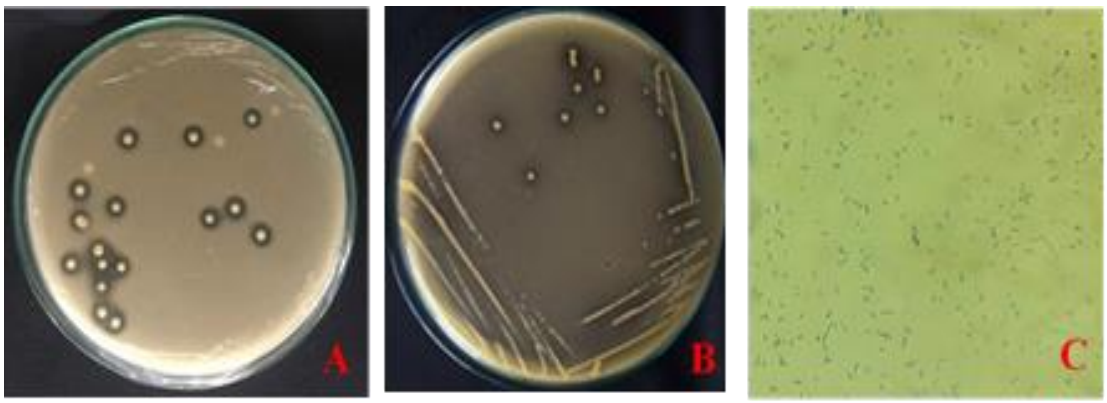

Figure 2. Colony morphology (A, B) and cell morphology (C) of ML3 strain was isolated from "com me" samples

Table 1

The morphological characterization of 19 bacterial strains

Strain of bacteria	The colony	The cells shape	Gram	Catalase	Oxydase
CC1	Round, whole cover, milky white	Short rods	(+)	(-)	(-)
CC2	Round, serrated, milky white	Short rods	(+)	(-)	(-)
CC3	Roud, serrated, ivory white	Short rods	(+)	(-)	(-)
CC4	Roud, serrated, ivory white	Short rods	(+)	(-)	(-)
HC1	Round, whole cover, milky white	Short rods	(+)	(-)	(-)
HC2	Roud, serrated, ivory white	Short rods	(+)	(-)	(-)
HC3	Round, serrated, milky white	Short rods	(+)	(-)	(-)
HC4	Roud, serrated, ivory white	Short rods	(+)	(-)	(-)
CP1	Round, whole cover, ivory white	Short rods	(+)	(-)	(-)
CP2	Roud, serrated, ivory white	Short rods	(+)	(-)	(-)
CP3	Roud, serrated, ivory white	Short rods	(+)	(-)	(-)
CP4	Round, whole cover, milky white	Short rods	(+)	(-)	(-)
ML1	Round, whole cover, milky white	Short rods	(+)	(-)	(-)
ML2	Roud, serrated, ivory white	Short rods	(+)	(-)	(-)
ML3	Round, whole cover, milky white	Short rods	(+)	(-)	(-)
ML4	Round, whole cover, milky white	Short rods	(+)	(-)	(-)
TT	Round, whole cover, ivory white	Short rods	(+)	(-)	(-)
VB	Round, whole cover, ivory white	Globular	(+)	(-)	(-)
GC	Round, serrated, milky white	Short rods	(+)	(-)	(-)

**Note: positive (+), negetive (-)*

Source: Data analysis result of the research

3.1.2. Investigation of lactic acid production ability of isolated bacterial strains

The amount of lactic acid were produced by 19 bacterial strains in MRS broth after 24 hours were shown in Figure 3.

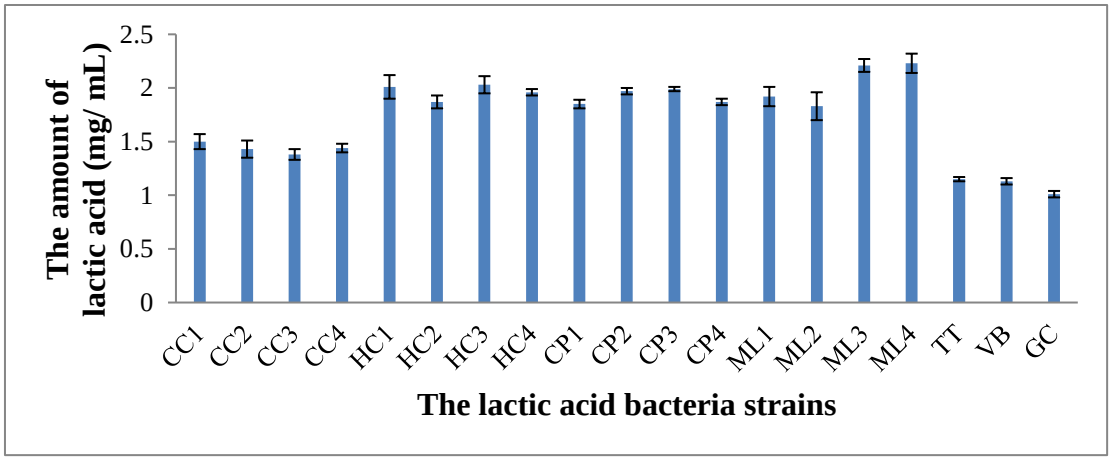

Figure 3. The latic acid production capacity of 19 bacterial strains

Through the Figure 3, all 19 bacteria strains were able to produce lactic acid in MRS broth after 24 hours with relatively high acid content ranged from 1,01 to 2,23mg/mL. In particular, the strain of ML4 bacteria produced the highest amount of lactic acid (2,23mg/mL after 24 hours) and statistically significant difference from the remaining bacterial strains. The results of lactic acid contents were produced in this study similared to lactic acid contents, that were produced by bacteria strains from "Tom chua" (1,12 - 2,19mg/mL after 24 hours (Truong, Nguyen, Nguyen, Nguyen, & Nguyen, 2019).

3.1.3. Investigation of salinity tolerance of bacterial strains were isolated from "mam sac chua"

In the process of producing "mam sac chua", salt in high concentrations was added to inhibit the bacteria causing rot and moderate the fermentation speed to create a specific flavor for the product. Therefore, the strains of bacteria from samples of "mam sac chua" could salinity tolerance. Salinity tolerance of them was assessed by growing in MRS broth added NaCl salts at concentrations of 0, 2, 4 and 6%. The latic acid production capacity of 3 bacteria strains at 4 different salt concentrations after 24 hours were determined by Therner titration. The results of lactic acid production capacity of bacterial strains in MRS broth medium are shown in Figure 4.

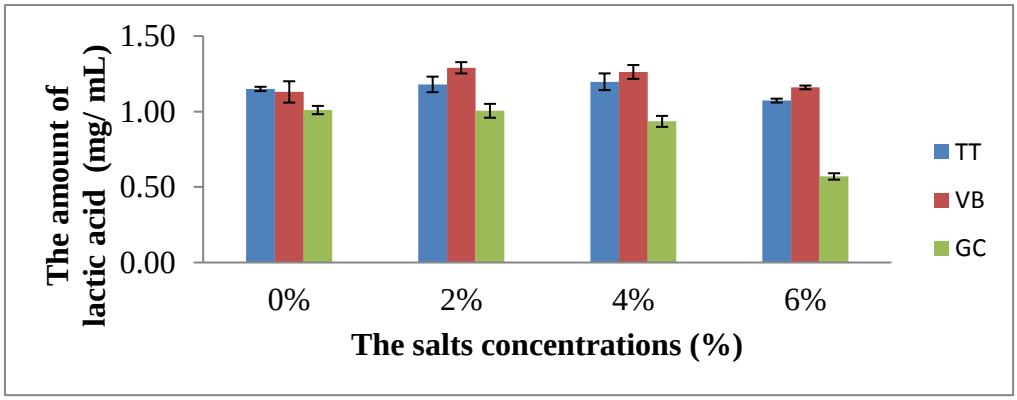

Figure 4. The salinity tolerance of 3 bacterial strains, that were isolated from samples of "Mam sac chua"

Through Figure 4, all 3 strains of the bacteria were able to survive and produce lactic acid in MRS broth added salt at concentration of 0, 2, 4 and 6%, the lactic acid contents ranged from 0,57 to 1,29mg/mL after 24 hours. In particular, the strain of VB produced the highest amount of lactic acid and was statistically different from the 2 remaining strains in all 4 salt concentrations. The salinity tolerance of 3 bacterial strains in this study is lower than the salinity tolerance of the bacterial strains from "Tom chua" (0,38 - 2,19mg/mL after 24 hours) (Truong et al., 2019).

3.1.4. Identification of species of bacterial strains, that were isolated by molecular biology technique

The DNA of 3 potential bacterial strains VB, ML3 and ML4 were successfully extracted with good quality PCR products, with a single clear band for each strain of bacteria on gel electrophoresis with a gene segment size of about 1500 bp (Figure 5). The sequencing of 16S rRNA gene of 3 bacterial strains were shown in Table 2.

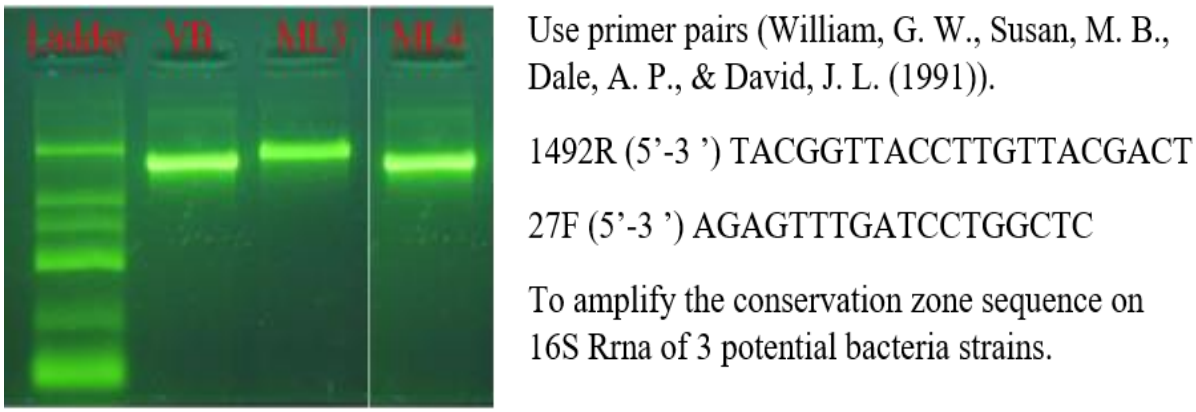

Figure 5. Spectroscopy of PCR gene product 16S rRNA of 3 bacteria strains VB, ML3 and ML4

Table 2

Results of gene sequencing 16S rRNA of 3 bacteria strains VB, ML3 and ML4

Strains	The gene sequencing 16S rRNA
VB	CTATAATGCAAGTCGAGCGAACAGACGAGGAGCTTGCTCCTCTGACGTA GCGGCGGACGGGTGAGTAACACGTGGGTAACCTACCTATAAGCTGGAATAAC TCCGGGAAACCGGGGCTAATGCCGGATAATATGCGAACGCATGGTTCCGCAA TGAAAGACGGTTTTGCTGTCACTTATAGATGGCCCGCGCCGTATTAGCTAGTT GGTAAGGTAACGGCTTACCAAGGCAACGATACGTAGCCGACCTGAGAGGGTG ATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAG TAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGT GATGAAGGTCTTCGGATCGTAAAACTCTGTTATTAGGGAAGAACAAGTGCGT AGGTAACTATGCGCACCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTA CGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATT GGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCACGG CTCAACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGAGGAA AGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCA GTGGCGAAGGCGACTTTCTGGTCTGCAACTGACGCTGATGTGCGAAAGCGTG GGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTG CTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCAC TCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGA CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCT

Strains	The gene sequencing 16S rRNA
	TACCAAATCTTGACATCCTTTGACCGCTCTAGAGATAGAGTCTTCCCCTTCGG GGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATG TTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCAGCATTAA GTTGGGCACTCTAAGTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATG ACGTCAAATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTACAATGGA CAGTACAAAGGGCAGCGAAACCGCGAGGTCAAGCAAATCCCATAAAGCTGTT CTCAGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGT AATCGTAGATCAGCATGCTACGGTGAATACGTTCCCGGGTCTTGTACACACCG CCCGTCACACCACGAGAGTTCGTAACACCCGAAGCCGGTGGAGTAACCTTTTA GGAGCTAGCCGTCGAA
ML3	TAATACATGCAAGTCGAACGAACTCTGGTATTGATTGGTGCTTGCATCAT GATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGC CCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACCGCATAACAACT TGGACCGCATGGTCCGAGCTTGAAAGATGGCTTCGGCTATCACTTTTGGATGG TCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGAT ACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCC AAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCT GATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGT TGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAA CCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGG CAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAG TCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGGGAA ACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGT AGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGA CGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAGTC CATACCGTAAACGATGAATGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCT GCAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAA CTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT CGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATACTATGCAAATCTAAG AGATTAGACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGGTTGTCGTC AGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATT ATCAGTTGCCAGCATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAACC GGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTA CACACGTGCTACAATGGATGGTACAACGAGTTGCGAACTCGCGAGAGTAAGC TAATCTCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACAT GAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTC CCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAG TCGGTGGGGTAACCTTTTAGGAACCAGCCGCCTAA
ML4	TGCCTATAATGCAAGTCGAACGAACTCTGGTATTGATTGGTGCTTGCATC ATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCT GCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACCGCATAACAA CTTGGACCGCATGGTCCGAGCTTGAAAGATGGCTTCGGCTATCACTTTTGGAT GGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATG ATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGC CCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGT CTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCT GTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTT AACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGT GGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTA AGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGGG AAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGC GTAGATATATGGAAGAACACCAGTGGCGGAGGCGGCTGTCTGGTCTGTAACT

Strains	The gene sequencing 16S rRNA
	GACGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAG TCCATACCGTAAACGATGAATGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTG CTGCAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGCCGCAAGGCTGA AACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAA TTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATACTATGCAAATCTAA GAGATTAGACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGGTTGTCGT CAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAT TATCAGTTGCCAGCATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAAC CGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCT ACACACGTGCTACAATGGATGGTACAACGAGTTGCGAACTCGCGAGAGTAAG CTAATCTCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACA TGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTT CCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAA GTCGGTGGGGTAACCTTTTAGGAACCAGCCGCCTAAGGGG

Source: Data analysis result of the research

PCR products of 3 potential bacterial strains VB, ML3 and ML4 were sequenced and identified based on combination of biochemical characteristics and comparing nucleotide sequence in 16S-rRNA gene with corresponding genes on the basis of NCBI data by BlastN. The results showed that the bacteria strains VB, ML3 and ML4 had nucleotide sequences of 16S rRNA genes similar to 99% with the genes of *Staphylococcus piscifermentans* and *Lactobacillus plantarum*. Therefore, the bacteria strains VB, ML3 and ML4 were isolated as *S. piscifermentans* VB, *L. plantarum* ML3 and *L. plantarum* ML4.

Through molecular biology techniques, two strains bacteria ML3 and ML4 were identified as LAB, the strain bacteria VB were not as LAB. The isolated results of this study are consistent with the LAB isolated results from "mam sac chua" in Can Tho city (Do, Nguyen, & Nguyen, 2014) and from traditional fermented products (fish, meat, vegetables,...) of Thailand, in addition to common isolates such as: *Lactobacillus spp.* and *Pediococcus acidilactici*, sometimes isolated *Staphylococcus* (Tanasupawat, Okada, & Komagata, 1998).

According to research of (Antonio et al., 2008) on determining the kinetics of the microbial population present in cheese products from fermented sheep milk at the maturing stage. The results showed that *Lactobacillus paracasei* is the dominant species, and other *Lactobacillus* species were found at an early stage of ripening. Non-LAB strains such as *Kocuria* and *Staphylococcus* are detected at the end of fermentation.

4. Conclusions and suggestions

4.1. Conclusion

Nineteen bacteria strains with characteristic of LAB were isolated from 3 samples of "mam sac chua" and 4 samples of "com me". All of them were able to produce lactic acid in MRS broth from1,01 to 2,23mg/mL after 24 hours.

Three bacteria strains were isolated from samples of "mam sac chua" were able to survive and produce lactic acid in MRS broth added NaCl salt at concentration of 0, 2, 4 and 6% from 0,57 to 1,29mg/mL after 24 hours.

This study was identified *S. piscifermentans* VB bacteria from samples of "mam sac chua", *L. plantarum* ML3 and *L. plantarum* ML4 bacterias from samples of "com me".

4.2. Suggestions

Investigation of probiotic viability of bacteria strains, that were isolated.

Investigation of substrate usability: glucose, saccarose and starch of bacterial strains, that were isolated as a basis for the trial of biosafety "mam sac chua" and "com me" products by adding bacteria strains into these products at fermentation.

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