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CHARACTERISTICS OF *LACTOBACILLUS* STRAINS ISOLATED FROM VIETNAMESE PATIENTS WITH TYPE 2 DIABETES

Hoang The Hung[1,4], Hoa Thi Minh Tu[2], Pham Thi Thu Uyen[3], Le Thi Thanh Huyen[5], Vu Thi Hien[2], Ha Thi Thu[2], Le Hoang Duc[1,2], Nguyen Trung Nam[1,2], Nguyen Thi Tuyet Nhung[3,✉]

[1]*Graduate University of Science and Technology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Cau Giay District, Hanoi, Vietnam*
[2]*Institute of Biotechnology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Cau Giay District, Hanoi, Vietnam*
[3]*R&D Department, Kenubio Company, D47-LK5, Duong Noi, Ha Dong City, Hanoi, Vietnam*
[4]*Military Academy of Logistics, Ministry of National Defence, Ngoc Thuy, Long Bien District, Hanoi, Vietnam*
[5]*Transport Hospital Joint Stock Company, Chua Lang, Lang Thuong, Dong Da District, Hanoi, Vietnam*

✉To whom correspondence should be addressed. E-mail: nttnhung@gmail.com

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SUMMARY

Many studies show that hyperglycemia can lead to injury to the intestinal mucosal barrier, increasing the likelihood of infection, leading to intestinal inflammation in people with type 2 diabetes. Recently, investigations indicate that the gut microbiome plays a very important role in this disease in which *Lactobacillus* in the gut has been shown to regulate glucose and lipid metabolism, improving oxidative stress, and inhibiting inflammatory responses. In this study, by the method of oriented isolation, the number of *Lactobacillus* bacteria in the subgroup of healthy people was determined to be (3,9 ± 1.2) x 10^3 CFU/g and in the group of diabetic patients was (2.7 ± 0.8) x 10^3 CFU/g. The study identified 17/68 selected *Lactobacillus* strains that were resistant to both *Staphylococcus aureus* ATCC13709 and *Escherichia coli* ATCC 11105. Moreover, these strains were also resistant to acid pH 2. Besides that, 16/17 strains tested were able to survive over 90% in 0.3% bile salt environment. Among the 17 strains studied, 3 strains of *Lactobacillus* 13, *Lactobacillus* 16, *Lactobacillus* 17 showed probiotic characteristics such as antibacterial ability, acid tolerance and bile salt tolerance. All three strains of *Lactobacillus* 13, *Lactobacillus* 16, and *Lactobacillus* 17 belong to species of *Lactobacillus plantarum*. This result makes a useful contribution to guide the application of *Lactobacillus* strain in creating dietary supplements for people with type 2 diabetes.

Keywords: Acid and bile salts tolerance, antibacterial, *Lactobacillus*, probiotic features, type 2 diabetes.

INTRODUCTION

Type 2 diabetes is a major challenge to the medical profession worldwide, being a major cause of many diseases such as hypertension, stroke, coronary heart disease, kidney failure, and diabetic retinopathy (Martín-Timón, 2014). According to the International Diabetes Federation, the worldwide prevalence of diabetes was 8.8% in 2015 and by 2040 is projected to increase to 10.4%. In Vietnam, there were 3.5 million people suffer from diabetes in 2015 and this number is forecasted to increase to 6.1 million by 2040 (Ogurtsova *et al*., 2017).

Lactic acid bacteria are a safe group of bacteria, Gram-positive, morphology LAB are rods and cocci, non-spore-forming, non-motility, catalase-negative (Alkema *et al.*, 2016). Lactic acid bacteria can survive growing conditions in a wide temperature range from 15 to 45°C, pH from 3 to 11, including the main genera *Lactobacillus, Lactococcus, Carnobacterium, Enterococcus, Lactosphaera, Leuconostoc, Melissococcus, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus, and Weissella* (Walter, 2008; Khalisanni, 2011).

Species such as *L. acidophilus, L. plantarum, L. casei, L. casei rhamnosus, L. bulgaricus, L. fermentum, L. reuteri, Lactococcus lactis, L. cremoris, Bifidobacterium bifidum, B. infantis, B. adolecentis, B. longum, B. breve, Enterococcus faecalis*, and *E. faecium* are the most common species widely used as probiotics for humans, and in fermentation and food preservation (Larsen *et al*., 2010). In addition to the ability to produce organic acids, some strains of lactic acid bacteria are also capable of producing substances that inhibit pathogenic microorganisms, such as diacetyl, hydroperoxide, acetaldehyde, and bacteriocin (Axelsson, 2004).

The main cause of obesity is an imbalance of calories, stemming from lifestyle changes and diets high in fat (Turnbaugh, Gordon, 2009). Currently, there is a growing concern about obesity-induced obesity and its effect on the gut microbiota. Recent studies have shown that alterations in the gut microbiota are strongly associated with diabetes. In obese and diabetic individuals, the gut microbiota was different from the healthy microbiome (Harsch and Konturek, 2018). Firmicutes counts were lower, while Bacteroides and Proteobacteria counts were higher than those without diabetes (Larsen *et al*., 2010). According to the study of Larsen and others (2010), Bacteriodes and Firmicutes species are proportional to the reduction of insulin resistance. However, this correlation has not been elucidated.

There is still no cure for diabetes, patients often have to take synthetic inhibitors to maintain blood sugar control, but these drugs have many side effects such as diarrhea and bloating. Therefore, the search for new drugs that are effective and safe is essential. Recent studies suggest that several species of the gut microbiota may be effective in preventing and managing type 2 diabetes (Behera *et al.*, 2018). Among them, *Lactobacillus* is one of the probiotics that are effective in supporting the treatment of diabetes. Animal and human research suggest that probiotic *Lactobacillus* supplements can help restore gut microbiota composition, help prevent certain metabolic disorders such as obesity (Mazloom *et al*., 2019), type 2 diabetes, and help improve or prevent inflammatory bowel disease in obese people (Hemarajata, Versalovic, 2013; (Wiciński *et al.*, 2020). In this study, we investigated changes in *Lactobacillus* in obese subjects with type 2 diabetes for the first time. Research on some valuable probiotic properties of native strains. This result makes a useful contribution to orient the application of strains in creating supplement foods to help enhance immunity in preventing and treating of type 2 diabetes.

MATERIALS AND METHODS

Materials

All participants provided written consent prior to investigation. All methods were performed in accordance with the Declaration of Helsinki. Research subjects: *Lactobacillus* were isolated from the feces of people with diabetes type 2 and healthy humans. Criteria of patients participating in the research: i) just got diabetes type 2, ii) glycemic index > 7,0, iii) obese person, BMI >27, iv) aged 25-65 years old, v) no antibiotics were used within 1 month before sampling. Blood sugar testing and stool sample collection took place on the same day at Hanoi Transport Hospital Joint Stock Company. Stool samples were collected and transferred to a sterile box. Samples were transported to the laboratory on ice.

Indicator microorganism strains: *S. aureus* ATCC13709 and *E. coli* ATCC 11105 from Collection of Biomaterials Technology Department - The Institute of Biotechnology (IBT) of the Vietnam Academy of Science and Technology (VAST).

LB medium (g/L) for culture of test bacteria: peptone 5 g; yeast extract: 5g; NaCl 3 g; agar 2%; H_2O 1 L; pH 6.5 – 7.

MRS medium for culture and isolation of lactic acid bacteria: peptone 10g; meat extract 10 g; yeast extract 5 g; ammonium citrate 2 g; K_2HPO_4 2 g; $MgSO_4.4H_2O$ 0.04 g; glucose 20 g; $MgSO_4.7H_2O$ 0.2 g; CH_3COONa 5 g; Tween 80 mL; agar 2%; pH 6.5 – 6.8.

Methods

Isolation of lactic acid bacteria

Isolation of lactic acid bacteria was performed according Abbasiliasi and others (2012) with improvements according to Hoa Thi Minh Tu (Hoa Minh Tu *et al*., 2013). MRS agar medium for isolation of lactic acid bacteria supplemented with 0.5% $CaCO_3$ to distinguish lactic acid bacteria from other groups of bacteria and used 0.01% (w/v) sodium azide to inhibit Gram-negative bacteria. One gram of stool was dissolved in 9 ml of sterile saline. The solution was then diluted 10 times in succession until 10^{-7}. Each dilution from 10^{-4} to 10^{-7} was spread on MRS agar plates, repeated 3 times, and incubated under anaerobic conditions at 37ºC for 48 to 72 h. Lactic acid bacteria are identified by the $CaCO_3$ solubilizing ring around the colony in the presence of lactic acid. Confirmation of the isolated bacterial strains is based on cell morphology, colony morphology, Gram staining pattern and negative catalase. Selected lactic acid bacteria colonies are cleaned and cultured to prepare for the next experiment.

Determination of antibacterial ability by agar well diffusion

Antibacterial activity was detected by agar well diffusion (Jacobsen *et al.*, 1999): i) the isolated strains were cultivated in MRS broth at 37ºC for 14-16 hours, centrifuge the fermentation broth and collect the supernatant liquid; ii) indicator microorganisms were cultured overnight at 37ºC in LB broth. Indicator bacterial suspension (100 µL) was spread on LB agar plates and holes were made on the plates. Then, 75 µL supernatant liquid in step i was added to each hole, kept at 4ºC for 4 hours, incubated at 37ºC for about 24 hours. The antimicrobial activity was determined by measuring the diameter of the inhibition zone around the holes.

Acid tolerance properties

Lactobacillus strains were cultured overnight on MRS medium. Take 2 tubes, each with 2 mL of the broth cultured in step 1, centrifuge (10 min at 5000 rpm) to collect the biomass. The collected pellets were resuspended in 2 mL MRS medium preadjusted to pH 2.0 and incubated at 37°C for 3 h. The control was used to count the number at 0 hours. Viability was determined using the plate count method. The dilutions were plated on MRS agar and incubated anaerobically at 37°C for 24h.

Bile salts tolerance properties

Lactobacillus strains were cultured overnight on MRS medium. The bile salts solution is dissolved and then sterilized by a bacterial filter. Add 0.3% bile salts to MRS medium. To determine the tolerance to bile salts, 4 mL of the overnight culture of *Lactobacillus* was taken, divided into 2 tubes, and centrifuged 10 min at 5000 rpm to collect the biomass. Cell suspensions were suspended in 2 mL of MRS containing bile salts and MRS medium without bile salts was used as a control, then incubated at 37ºC for 3 h. Viability was determined using the plate count method. The dilutions were plated on MRS agar and incubated anaerobically at 37°C for 24h.

Classification of bacterial strains based on 16S rRNA gene sequencing

Lactobacillus strains were grown overnight in 5 ml of MRS broth, centrifuged to collect

biomass. The strain DNA was separated using the ThermoFisher kit according to the manufacturer's instructions. PCR reaction was performed with ThermoFisher master mix, template DNA, and primer 27F/1492R with the following sequences: 27F: 5'-AGAGTTTGATCCTGGCTCAG-3', 1492R: 5'-GGTTACCTTGTTACGACTT-3'. The 16S rRNA gene sequences were analyzed automatically by PRISM@3700 Genetic Analyzer (ThermoFisherScientific, USA), analyzed by BioEdit software (Hall, 1999), compared with NCBI data by BLAST program. The phylogenetic tree was built using MEGA 7 software (Kumar, Stecher and Tamura, 2016).

RESULTS AND DISCUSSION

Total *Lactobacillus* in the intestinal tract of healthy and diabetic human

Normally, the human gastrointestinal tract contains many types of bacteria, so the process of determining the presence of *Lactobacillus* in stool samples is often difficult. To obtain the best results, we isolated *Lactobacillus* using the isolation method of the lactic acid bacteria as described in the methods section. After 36 hours of culture at 37ºC, many colonies appeared with relatively uniform size and shape, mainly smooth round milky white colonies and transparent color rings around the colonies due to $CaCO_3$ being degraded by lactic acid (Fig.1B). By preliminary research (data not shown here) 68 strains of *Lactobacillus* were isolated, with some characteristics such as gram-positive bacteria, rod shape, non-spore-forming, inability to motility, catalase-negative, ferment glucose and decompose $CaCO_3$. According to Axelsson (2004), lactic acid bacteria include 12 genera, but only *Lactobacillus* and *Carnobacterium* have rod shape, the other is globular. The genus *Lactobacillus* differs from the genus *Carnobacterium*, which is present only in meat and meat products, is pH 9 tolerant, is not tolerant to low pH, and is not found in the intestinal tract of humans and animals, while *Lactobacillus* is the opposite (Axelsson, 2004).

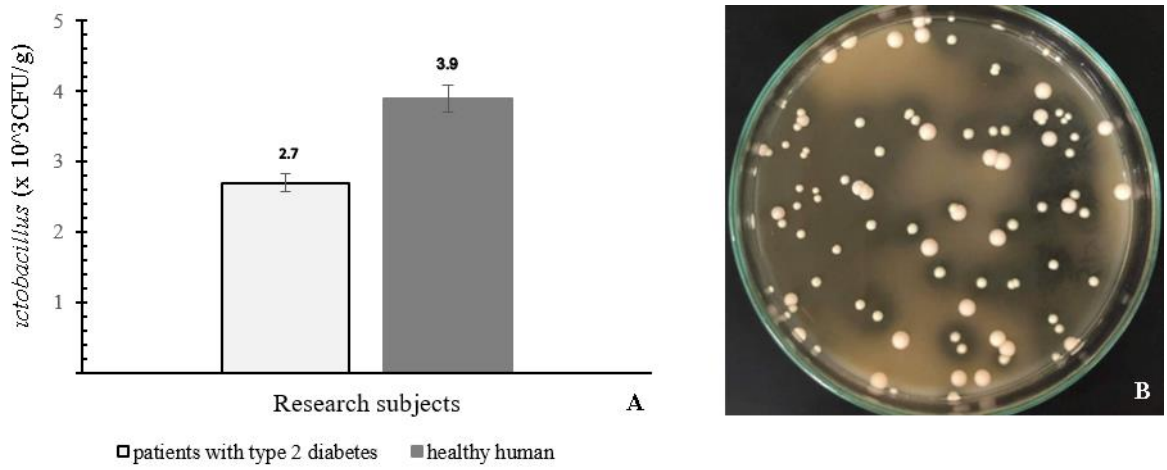

Figure 1. *Lactobacillus* isolated on MRS medium supplemented $CaCO_3$.

From the obtained results, the isolated strains belong to the genus *Lactobacillus*. The number of *Lactobacillus* bacteria in the healthy people subgroup was (3.9 ± 1.2) x 10^3 CFU/g and (2.7 ± 0.8) x 10^3 CFU/g in the diabetic patient group. The number of *Lactobacillus* genus in the group of healthy people was significant higher 1.2 x 10^3 CFU/g compared to the bacteria in the group of patients with type 2 diabetes (Fig.1A). Research by Larsen (2010)

has shown that in the group of patients, the composition of Firmicutes increased, whereas the composition of Bacteroidetes decreased compared to healthy people. Thus in this study, 68 strains of *Lactobacillus* isolated from feces were selected based on the criteria of Axelsson (2004) and had a large and clear $CaCO_3$ soluble ring around the colonies for subsequent studies.

Inhibition of pathogenic bacteria

One of the probiotic properties of human health is its ability to resist pathogenic bacteria. *E. coli* and *S. aureus* are common causes of community- and hospital-acquired infections, leading to death if not treated promptly and adequately (Matta *et al.*, 2018). Therefore, in this study, two control strains *E. coli* ATCC 11105 and *S. aureus* ATCC13709 were used to evaluate the preliminary antibacterial ability of selected *Lactobacillus* strains. Of the 68 strains of *Lactobacillus*, 17 strains were identified to resist to both indicator strains with inhibition zone ranging from 5.2 to 20.3 mm. In which, 5/17 *Lactobacillus* strains have strong resistance to both *S. aureus* ATCC13709 and *E. coli* ATCC 11105 with large inhibition zone from 16.3 mm to 20.3 mm, including *Lactobacillus* 02, 13, 15, 16, 17, (Fig. 2). The results of this study are similar to the study of Rao (2015) on the ability of 8 *Lactobacillus* strains to resist *S. aureus*, in which the *Lactobacillus* COORG-3 inhibits *S. aureus* the most with an inhibition zone is 15 mm (Rao *et al*., 2015).

In another study by Zhang (2016) on the ability to inhibit pathogenic bacteria of 20 *Lactobacillus* strains, 3 strains, including *Lactobacillus* 1089, 1115, and 1141 had the best resistance to *S. aureus* with an inhibition zone up to more than 20 mm. Shim and others (2016) evaluated the inhibition of *E. coli* by 6 *Lactobacillus* strains including *L. gasseri, L. rhamnosus, L. acidophilus* SNUL, *L. plantarum, L. paracasei, L. acidophilus* (Antibio300). This group of authors concluded that the average inhibition zone of each strain was different, but the difference was not significant, the diameter of the inhibition zone ranged from 10.5 mm to 20.5 mm. Notably, all 17 strains selected in this study have good resistance to both tested strains, and they are always stable after many tests. Therefore, these 17 strains were further studied to find some valuable characteristics of the strain in the next experiments.

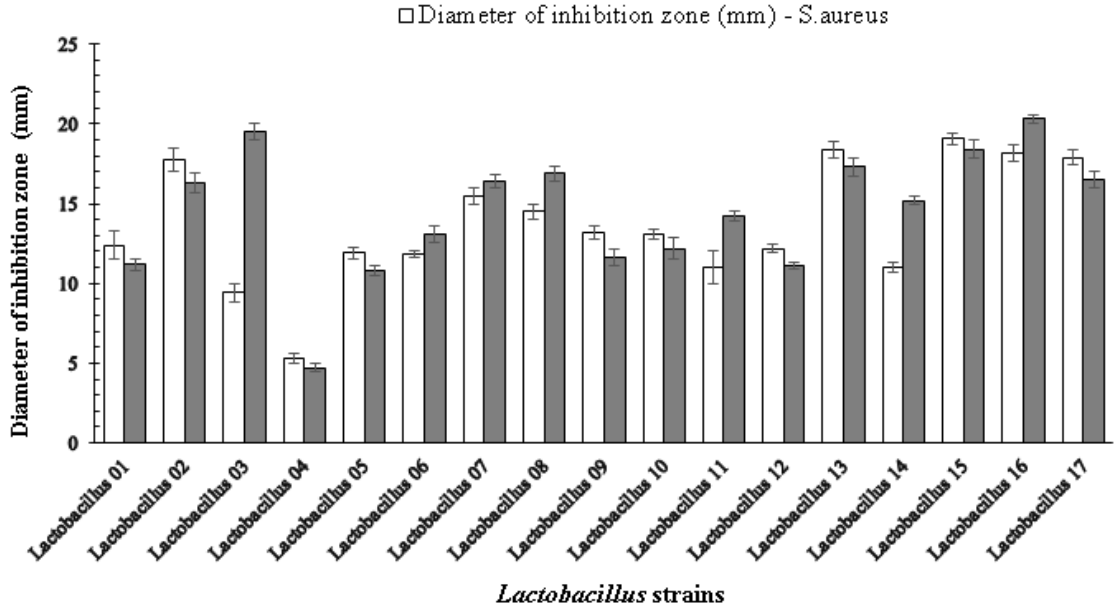

Figure 2. Antimicrobial activity of selected *Lactobacillus* strains.

Acid tolerance properties

Pathogen-inhibiting strains of *Lactobacillus* are only valid as a probiotic if they can tolerate pH and bile salts in the human gastrointestinal tract. Before reaching the intestinal tract, probiotic bacteria must first survive in the environment with stomach acid for about 3 hours. Normally, gastric pH varies from person to person and ranges from 2.0 to 4.0 (Rao *et al*., 2015; Zhang *et al.*, 2016). In pH 2.0, the viability of 17 strains ranged from 21 to 94%. There are 8 strains, including *Lactobacillus* 01, 02, 03, 04, 13, 15, 16, 17, with a relatively high number of viable cells ranging from 82 to 96%. *Lactobacillus* strains 05, 06, 07, 08, 09,11, 14 had low viability at pH 2.0 ranging from 21 to 50%.

The results of this study are similar to those of Jacobsen and others (1999) when the authors examined the survival after 3 hours in the environment with pH 2.5 (Shim *et al*., 2016). In a study by Ha and others (2020), after 3 hours of culture at pH 3.0, all 3 tested *Lactobacillus* strains had high survival rates with 95.27%, 94.15%, and 98.54%, respectively (Ha *et al*., 2020). Bao and others (2010) when studying the viability of 90 *Lactobacillus* strains isolated from traditional fermented milk of ethnic minorities in Mongolia and China in pH 3, they found that 35/90 strains grew well in acidic conditions (pH 3.0) (Bao *et al*., 2010). Eleven strains were further screened from 35 strains with high tolerance to simulated gastric juice (pH 2.5, 3 hours incubation). Of these, only strain F6 (isolated from traditional Mongolian dairy products) was able to survive with 53.7% in simulated gastric juice (pH 2.0) (Bao *et al*., 2010). In our study, of the 17 strains tested, the viability fluctuated between 21 and 96%, different from the study of Bao *et al.* (2010). This result can also be explained by the origin of the studied strains. Specifically, these strains were isolated in the human intestinal tract thus these strains were also tolerated to low pH in the host stomach so they became dominant over other species, possibly as a result of adaptation to a particular environment, giving them an advantage over *Lactobacillus* in fermented products.

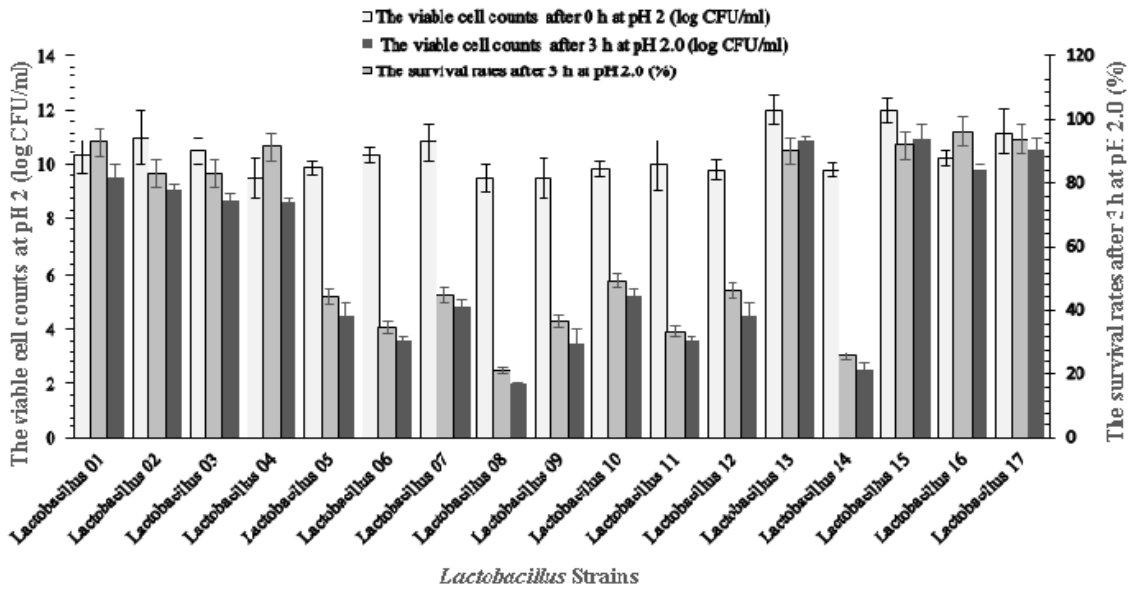

Figure 3. Acid tolerance of *Lactobacillus* strains at pH 2.0.

Bile salts tolerance properties

Another challenge to microbial survival in the human gut is the presence of bile in the large intestine. Therefore, the ability to tolerate bile is considered an important property of

Lactobacillus to survive. The average bile salts concentration in the human intestine is 0.3%, so this is commonly used to select probiotic strains resistant to bile. In this experiment, after 24 h incubation in MRS medium supplemented with 0.3% (w/v) bile salts, all 17 tested strains showed high and relatively uniform survival rates among strains. There were 16/17 strains with viability ranging from 90 to 97.3%, and only *Lactobacillus* 06 had a lower number of viable cells with 65.5% (Fig. 4). Strains with a high number of cells above 95% were *Lactobacillus* 04, *Lactobacillus* 09, *Lactobacillus* 16, and *Lactobacillus* 17. Strains with the lowest number of cells in bile salts were *Lactobacillus* 08, *Lactobacillus* 06, and *Lactobacillus* 02. This tolerance to bile salts represents an advantage for the survival of these bacteria in the intestinal tract. Some proofs to confirm this hypothesis have been proved by Song *et al.* (2015), Ha *et al.* (2020). With the same purpose of research subjects but different in the origin of isolates, Song *et al.* (2015) investigated the tolerance to bile salts of 10 *Lactobacillus* strains isolated from different sources. It showed that 5/10 strains (50%) were resistant to 0.3% bile salts, including *Lactobacillus.* sp. JNU 8829, *L. casei* MB3, *L. sakei* MA9, *L. sakei* CH8, and *L. acidophilus*. Another study by Hassanzadazar *et al.* (2014), when studying the bile tolerance of 28 *Lactobacillus* strains, belonging to 3 species of *L. plantarum*, *L. casei*, and *L. delbruki* isolated from Koozeh cheese, they found that 27/28 isolates could not tolerate bile salts and only one strain, *L. casei*, was able to survive in 0.3% bile salts. While Ha *et al.* (2020) showed that most strains isolated from human intestinal tract can grow well in the medium supplemented with 0.3% bile salts, the survival rates of these strains ranged from 90 to 99%.

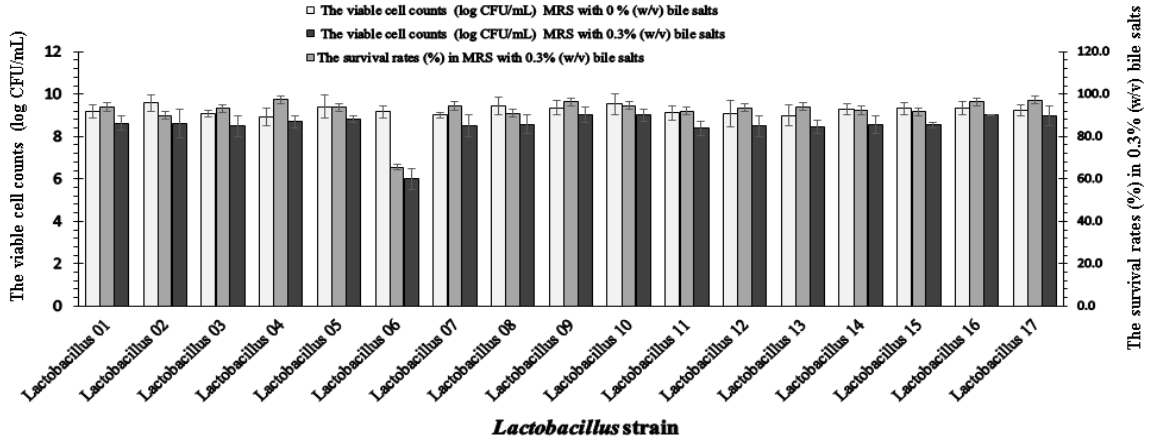

Figure 4. Ability of *Lactobacillus* strains to tolerate 0.3% (w/v) bile.

Classification to strains with potential applications

Among 17 strains studied for their antibacterial ability, tolerance to pH 2, and bile salts concentration of 0.3%, we have identified 3 strains of *Lactobacillus* 17, *Lactobacillus* 16, *Lactobacillus* 13 that converge all 3 properties of precious probiotics. Therefore, the strained were classified by 16S rRNA gene. Using the primer 27F/1492R, a specific 16S RNA gene fragment of about 1500 bp in size was amplified (Fig. 5A). After sequencing, the gene sequences were compared with genetic data and analyzed by BLAST on GeneBank. The results of all 3 strains of *Lactobacillus* 17, *Lactobacillus* 16, and *Lactobacillus* 13 had a high similarity of 99.93%, 99.93% and 100%, respectively, with species *L. plantarum* on GenBank. The phylogenetic tree was built using MEGA7 software to compare and analyze the nucleotide sequences from the three strains with species that are the most

similar in species composition on GenBank (Fig. 5B). The results showed that all 3 strains of *Lactobacillus* 13, *Lactobacillus* 16, *Lactobacillus* 17 belonged to the *L. plantarum* species so were signified *L. plantarum* 13, *L. plantarum* 16, and *L. plantarum* 17.

L. plantarum, a beneficial lactic acid bacteria, is found in fermented foods and in the intestinal tract of humans and animals (Behera *et al*., 2018). Currently, *L. plantarum* is commonly used as an adjunct probiotic in the treatment of antibiotic-associated gastrointestinal disorders in humans and animals (Hickson, 2011). Notably, strains of *L. plantarum* 13, *L. plantarum* 16, *L. plantarum* 17 were isolated from type 2 diabetes patients in Vietnam. These strains have the good antibacterial ability with the two indicator strains and have a high survival rate of over 90% in low acid pH 2 and 0.3% bile salts after 3 hours of culture. This result makes a useful contribution to guide the application of strains in creating dietary supplements to enhance immunity in the prevention and treatment of diabetes. However, further studies are needed before it can be applied in practice.

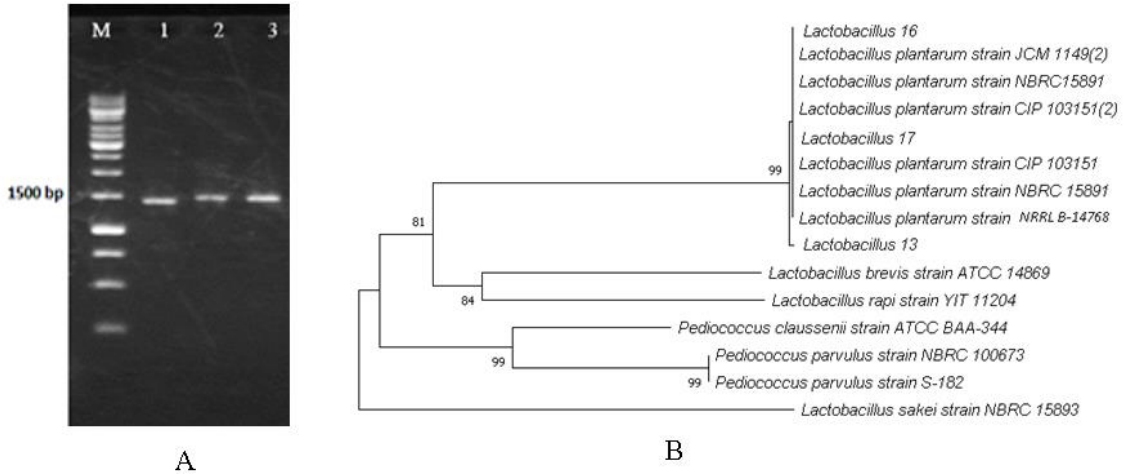

Figure 5. A. PCR products of 3 strains: M: Maker, 1: *Lactobacillus 13; 2: Lactobacillus 16; 3. Lactobacillus 17.* B. Phylogenetic tree of *Lactobacillus* species. The bootstrap confidence values were generated using 100 permutations.

CONCLUSIONS

The number of *Lactobacillus* in the feces of the subgroup of healthy people was (3.9 ± 1.2) x 10^3 CFU/g and in the group of diabetic patients was (2.7 ± 0.8) x 10^3 CFU/g. The number of *Lactobacillus* in the group of healthy people was 1.2 times higher than that from the feces of patients with diabetes. Of the 68 isolated strains, 17 strains *S. aureus* ATCC13709 and *E. coli* ATCC 11105 with inhibition zone from 5.2 to 20.3 mm. All 17 strains were resistant to acid pH 2 and bile salts 0.3%. We have identified 3 strains of *Lactobacillus* 17, *Lactobacillus* 16, *Lactobacillus* 13 that converge all 3 properties precious probiotics and by analyzing 16S RNA gene sequencing, those strains have been identified, all belonging to the species *L. plantarum*. This result makes a useful contribution to guide the application of *Lactobacillus* strain in creating supplement foods for patients with type 2 diabetes.

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