Probiotic characteristics of *Bifidobacterium infantis* isolated in Vietnam

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Abstract

Bifidobacterium is anerobic bacterium which is popular in human and animal intestine. *B. infantis* was successfully isolated from a new-born child's fecal sample previously. In this study, the probiotic characteristics of this strain such as: extracellular enzyme production ability; antibiotic activity against food-born pathogen like *E. coli*, *S. aureus*; organic acid production and bile-salt sufferance were evaluated. The results showed that the isolated can produce amylase and protease enzyme, do not produce cellulase enzyme; the organic acids also were produced during development of bacterium. The bacterium can against *E. coli* and *S. aureus* and can grow in the media containing up to 1% of bile salt. Results of this study are the basis for further studies to apply these bacteria as a source of probiotic beneficial to human health in Vietnam.

Keywords: *Bifidobacterium infantis*, probiotic, *E. coli*, exoenzymes, antibiotic activity.

1. INTRODUCTION

Understanding the importance of healthy living, scientists always find methods and create healthy products to ensure that. Therefore, the introduction of probiotics to the human life has been done in which probiotic was described as "a live microbial supplement that beneficially affects the host by improving the balance of gut bacteria" [1]. Since their discovery, Bifidobacteria are a group of bacteria present predominantly in the infant gut as demonstrated by Turroni et al. in 2012 through the method of sequencing the genes of the bacterial source taken from the stool samples of infants [6]. *Bifidobacterium* are mentioned in many reports that they bring many benefits such as: against experimental periodontitis (EP) in rats [5], rapid release of acute diarrhea episodes in German shepherd dogs and Larador hybrid dogs [3], or even decrease in the symptoms of periodontitis in humans [2].

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Bifidobacterium has been discovered with characteristics that meet the criteria for becoming a probiotic such as: tolerance to bile salts, ability to adhere to gastric mucosa, competition with harmful bacteria, participate in immune regulation [4]. In this report, the evaluation for probiotic characterization of *Bifidobacterium infantis* which isolated in Vietnam was performed. The isolated strain can produce extracellular amylase and protease as well as organic acid during development of bacterium. The bacterium can against *E. coli* and suffer from bile salt at 0.5% concentration.

2. MATERIALS AND METHODS

2.1. Materials

Bacterial strain: *Bifidobacterium infantis* (*B. infantis*) strain PSHN-T2 was isolated in laboratory of DNA Identification Center, Institute of Biotechnology (IBT), Vietnam Academy of Science and Technology (VAST) from a stool sample of an infant (7 days) which was born in Hanoi Obstetrics and Gynecology Hospital [20]; Other bacterial strains which used as intestinal pathogenic bacteria such as *Escherichia coli* (*E. coli*) strain TBĐV-E1, *Staphylococcus aureus* (*S. aureus*) strain TBĐV-S5 and control experiment strain such as *Bifidobacterium longum* (*B. longum*) strain SCJP-Mei2 were obtained from IBT's bacterial collection.

Chemicals used in experiment were obtained from Sigma-Aldrich, Merck (Germany) or from Vietnam and China; Oxoid gas box and gas-pak was obtained from Mitsubishi Gas Chemical company, Japan

Culture medium: MRSc medium (Man, Rogosa and Sharpe) included: Glucose 20 g/L, Peptone 10 g/L, Meat extract 10 g/L, Yeast extract 5 g/L, K_2HPO_4 2 g/L, $CH_3COONa.3H_2O$ 5 g/L, $(NH_4)_3C_6H_5O_7$ 2 g/L, $MgSO_4.7H_2O$ 0,2 g/L, $MnSO_4.5H_20$ 0,05 g/L, Tween 80 1,08 g/L, L-cysteine 0,5 g/L, Agar 12 g/L, pH 6,5; LB (Luria Bertani broth) medium: Peptone 10 g/L, Yeast extract 5 g/L, NaCl 10 g/L, Agar 10 g/L.

2.2. Methods

2.2.1. Agar Diffusion test

The evaluation of ability to produce extracellular enzymes of bacterial strain had been done by agar diffusion method. *B. infantis* and control *B. longum* strains were cultured in aqueous MRSc medium at 37°C for 24 h in Oxoid gas box under anerobic condition. After overnight culture, the culture was collected by centrifuge at 10,000 rpm for 10 min and transfer the supernatant into the new tubes.

For the agar diffusion test, the following medium was used: 1% carboxymethyl cellulose (CMC); or 1% skim milk; or 1% starch in 2% agar plate. 0.2 mL of cultured mediums were added into agar holes (8 mm diameter) and the plates were incubated for 24 h at room temperature. Triplicate sets of plates were prepared for each culture medium. After

incubation, the plates were overlayered with lugol's solution for starch plate, Congo red solution for CMC plate, and for the skim milk plate, observed directly without staining solution. The radius of the degradation zones was measured [7-8]. Results were calculated by subtracting of total diameter of clear zone from hole diameter with 3 times average.

2.2.2. Organic Acids production test

Organic Acid quantification based on titration method with NaOH [21]. Two bacterial strains were initiated culture for 24 h in MRSc medium in an Oxoid gas box under anerobic condition at 37°C. 10% starter culture was inoculated into new MRSc aqueous medium and incubated further for 24 h at above condition. The culture was centrifuged at 12,000 rpm for 10 min to remove biomass. 10 mL of supernatant of each bacterial strain were added into 20 mL of autoclaved distilled water, added 1 - 2 drops of phenolphthalein. Titrate with 0.1 N NaOH until a faint pink color appears, persist for 30s, then stop. Record the volume of NaOH (mL) used in this titration. Negative control is medium without culture of bacteria. Result was performed in triplicate. Acidity is calculated according to the formula:

$$\textbf{Acid content (mg/mL)} = V_{\textbf{NaOH consumed}} \textbf{ *10 *0.009}$$

2.2.3. Resistance to intestinal food-borne pathogens

Bifidobacterium strains were grown in aqueous MRSc medium in an Oxoid gas box under anerobic condition at 37°C for two days. The culture solution was centrifuged for 10,000 rpm at 10 min to remove the biomass. The agar diffusion test was applied in this experiment to check the resistance of *Bifidobacterium* against intestinal food-born *E. coli* and *S. aureus.*

E. coli and *S. aureus* bacterial strains were grown in LB medium at 37°C for 24h in shaking incubator. The bacterial strains were spread evenly on LB agar plates and allowed to completely dry. Holes with 8 mm in diameter were punch in the above prepared LB plates of *E. coli* and *S. aureus*. 0.2 mL of cultured medium of *Bifidobacterium* after removed cell biomass were added into each agar holes. Incubated the plates at 37°C for 24 hours. The results were recorded by observe the sterile ring surround the agar hole, measure the sterile ring [7-8]. The experiment had been done in triplicated.

2.2.4. Tolerance to bile salts

MRSc agar was divided each 20 mL/tube into falcon tubes and allowed to cool down before added bile salts (stock solution at 50%). The final bile salt concentration reached 0.1, 0.3, and 0.5%, mixed well. Added 10 µL of *Bifidobacterium* (OD_{600nm} about 1.8) mixed well and poured quickly onto a sterile plate to avoid bacteria from prolonged exposure to the air. Incubate the plate in an Oxoid gas box under anerobic condition at 37°C for 24h ~ 48h, observe and evaluate the growth rate [22].

3. RESULTS AND DISCUSSIONS

3.1. Extracellular enzymes production ability

Agar Diffusion Test is an easy and simple method to perform for determination of the ability to produce extracellular enzymes of isolated *B. infantis* bacteria. The cultured medium of *B. infantis* should contain the enzymes if the bacteria would produce those enzymes as extracellular enzymes. In these experiments, protease, cellulase and amylase enzymes were proposed to present in the culture medium and tested. The efficiency of resolving 3 types of substrates (Skim milk for protease, CMC for cellulase, and Starch for amylase) of bacterial strains was evaluated through the clear zone around the agar hole supplemented with bacterial cultured medium. The results are in Figure 1.

1 **2** **3**

Figure 1. *The clear zone of Bifidobacterium strains with agar diffusion test*

Skim milk plate (1), CMC plate (2), Starch plate (3). B. infantis (T2) và B. longum (Mei 2), control is autoclaved distilled water (H_2O)

According to Figure 1, *B. infantis* (T2) strain was capable of breaking down skim milk with a clear zone of 8 mm in radius, unable to degrade CMC, and digesting starch with a clear zone of 2 mm in radius. Similarly, the *Bifidobacterium* used as control, *B. longum* (Mei 2) was able to degrade skim milk which produced a clear zone of 3 mm in radius, 2 mm in radius with starch, and was unable to degrade CMC (Table 1). From these results, the ability to produce extracellular protease and amylase were presented in both *Bifidobacterium* strains but extracellular cellulase production were not found. *Bifidobacterium* lived dominantly in human gut. In many reports, *Bifidobacterium* play a role in breaking down the larger molecule to smaller for their energy as well as easy absorb by human gut. The enzyme producing by *B. infantis* hydrolyzed the active glycolipid directly to lactosylceramide [11]. In Motomitsu report, *B. bifidum* posed extracellular enzyme to liberate lacto-N-biose from human milk oligosaccharide (HMO); *B. longum* subsp. *infantis* imports intact HMO to be hydrolyzed by intracellular enzyme [12]. *B. infantis* also contributed in degradation of red

seaweed agarose by the action of two agarolytic β-galactosidases and produced neoagarobiose [13]. Thus, further experiment may need to determine the composition of extracellular enzymes which secreted by *B. infantis* and *B. longum* in our experiment.

3.2. Organic acids production ability

Titration results using NaOH showed that the two bacterial strains were able to produce organic acid with amount of 1,018 g/L in medium by *Bifidobacterium infantis* and 0.775 g/L in medium by *Bifidobacterium longum*, respectively (Table 1).

Table 1. *Substrate degradation results of two strains of Bifidobacterium*

(radius of clear zone in mm)*

No.	***Bacterial strain***	***Substrates****			***Acid concentration (g/L)***
		Skim milk (mm)	***CMC (mm)***	***Starch (mm)***	
1	*B. infantis*	8	0	2	1.018
2	*B. longum*	3	0	2	0.775

The ability to produce short chain fatty acids (SCFA) was presented in gut bacteria [14] including formate, acetate, propionate and butyrate. SCFA play important roles in gut such as: integrity of gut, glucose homeostasis, lipid metabolism, appetite regulation and immune function [14]. The butyrate displays a key role in gut health. Bifidobacteria are acetate-producing microorganisms and also affect to butyrate production even they are not directly producing butyrate [15]. The organic acid produced by two strains of *Bifidobacterium* in our experiment could be acetate compound.

3.3. Resistance to intestinal pathogen bacteria

Test bacterium chosen in this experiment were a gram-negative bacteria strain *E. coli* and a gram-positive bacteria strain *S. aureus*. The experiment had been performed by exposing the test bacterium to the culture broth that has been centrifuged to remove the biomass of *Bifidobacterium* strains using the agar diffusion method. Resistance to the test microorganism was shown to vary among bacterial strains. *B. infantis* can inhibit both tested bacteria *E. coli* and *S. aureus* with a radius of inhibition zone of approximately 3 mm. On the other hand, *B. longum* was not resistant to the gram-negative bacteria *E. coli* but inhibit the gram-positive bacteria *S. aureus* from growth with a radius of clear zone approximately 2.5 mm (Figure 2, Table 2). Interestingly, our result was opposite to Fanglei et al report in which they showed that their *B. longum* can inhibit the growth of *S. aureus* but not inhibit the growth of *E. coli* [16]. This can be explained by their strains of bacteria may be different from our strains [19]. Different strain of *B. infantis* may have different strength of

antimicrobial activity against *E. coli* and other antagonistic strains [19]. According to Meiqian et al, *B. infantis* protected against intestinal inflammation and tissue damage in newborn mice in vivo when exposed to *Cronobacter sakazakii*. And they suggested that factors released from *B. infantis* bring that protective effect [17]. This factor may be a 5 - 10 kDa molecule that is heat and acid stable and resistance to DNAse, RNAse, and protease was found in the culture medium of these organisms which already reduced the inflammatory response in a primary human fetal enterocyte cell line [16, 18]. Isolation and characterization of this molecule is necessary to develop a new antibiotic from biological source.

Figure 2. *Inhibition ability of Bifidobacterium to gram negative bacteria E. coli (4) and gram positive bacteria S.aureus (5). Bifidobacterium infantis (T2), Bifidobacterium longum (Mei 2), control is autoclaved distilled water (H_2O)*

3.4. Tolerance to bile salts

From Figure 3, the bacterium could survive and colonies appeared in all concentrations from 0 to 1% of bile salt. This means that *B. infantis* could tolerate at high concentrations of bile salts up to 1%. While in case of *B. longum* strain, the bacteria could survive and colonies appeared in only 0.1% of bile salt concentration. The higher concentration of bile salts from 0.3 to 0.7% which tested in this experiment did not appear any colony (Figure 4). The summary of result was showed in Table 2 also. In this experiment, our *B. infantis* strain could be tolerant to bile salt better than *B. longum* strain.

Table 2. *Summary result of resistant of Bifidobacterium to intestinal pathogen bacteria and the ability of bile salt tolerant of Bifidobacterium*

No.	Bacterial strain	Resistant to food-born pathogen (*mm)		Bile salts (#)				
		E. coli	*S. aureus*	0.1%	0.3%	0.5%	0.7%	1%
1	*B. infantis*	3	3	+++	+++	+++	nd	++
2	*B. longum*	0	2.5	+	-	-	-	nd

*Note: *Radius of clear zone; # minus indicated that no tolerant to bile salt, number of plus indicated the intensity of tolerant to bile salt; nd: not determine*

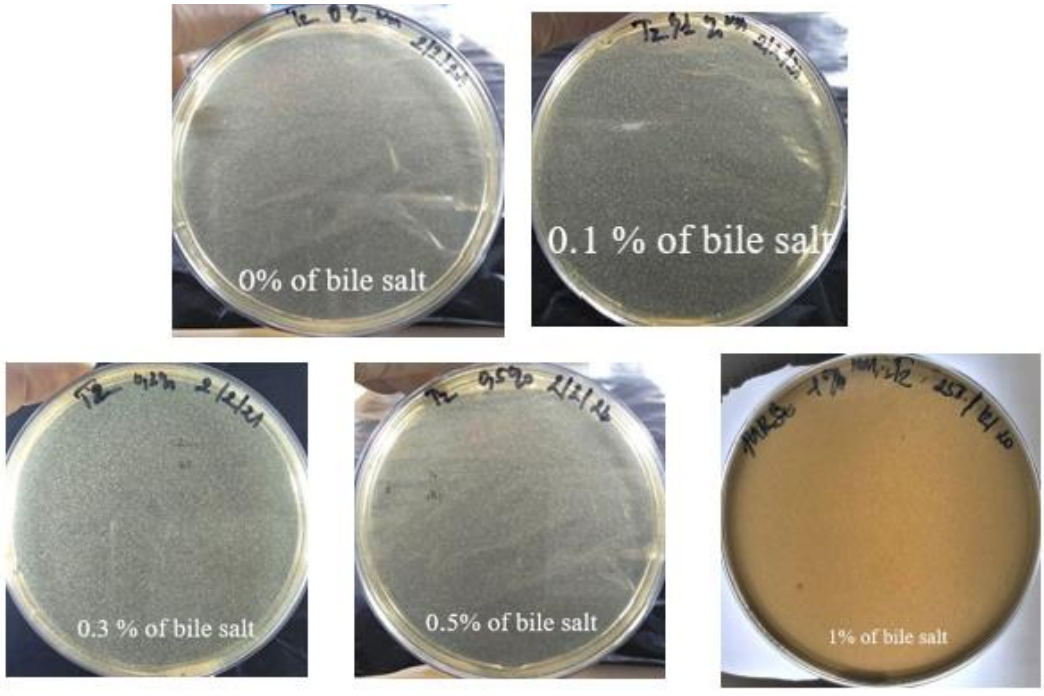

Figure 3. *Ability to tolerant to bile salt of Bifidobacterium infantis (T2)*

The *B. infantis* showed stronger ability to resistance to bile salt than *B. longum* in this experiment as well as in comparison with other experiment. Similarly, Andrea et al. found only 10 of 40 strains of *Bifidobacterium* were resistant to 0.5% bile salt [9]. In Andrea experiment, there were no any strain of *B. longum* survive in 0.5% of bile salt. His result is suitable with our result in which *B. longum* strain can survive with only 0.1% of bile salt. However, *B. longum* can survive to 0.3% bile salt in experiment of Kim and Lee [10]. But it was not clear in our result at this concentration. In the case of *B. infantis*, Andrea's results

showed 2 among 4 strain of *B. infantis* were resistance to 0.5% of bile salt. Therefore, our isolated strain is stronger which still can survive in 1% of bile salt.

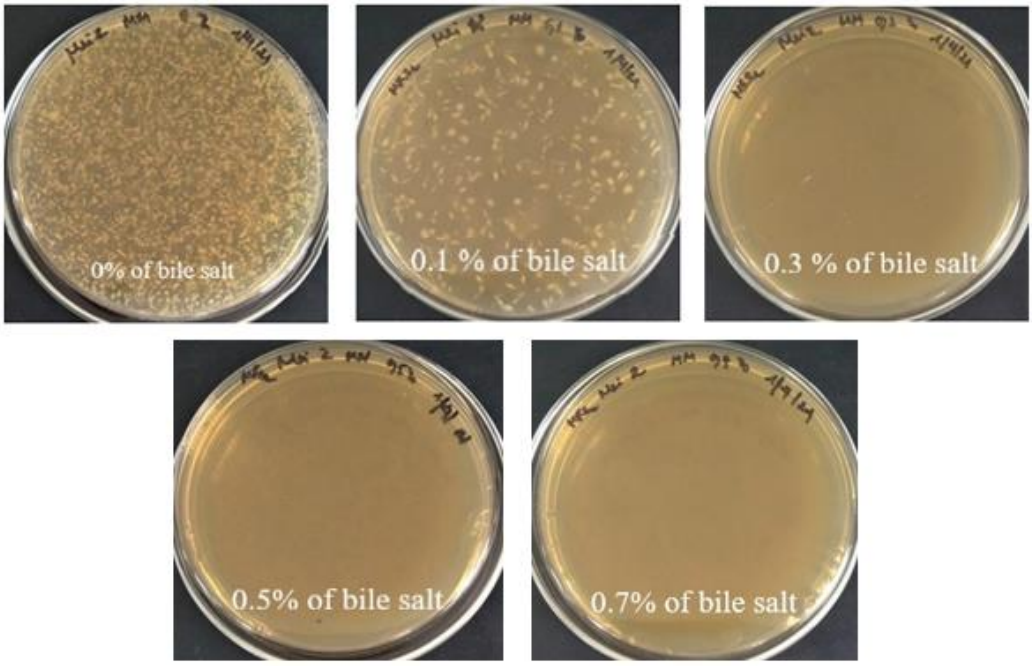

Figure 4. *Ability to tolerant to bile salt of Bifidobacterium longum (Mei 2)*

4. CONCLUSION

With the high potential of using *Bifidobacterium* as probiotic and many benefits to human health, culture of the bacteria should be developed in Vietnam. Even the strictly anerobic characteristics of these strains may make difficulty for the high scale culture, it is necessary to study further for the application of using *Bifidobacterium* as probiotic. We first showed the isolation of *B. infantis* in Vietnam [20] and in this report, some of probiotic characterization of the strain were presented and showed to be good for further application.

REFERENCES

[1]. R. Fuller, "History and development of probiotics," *Probiotics*., pp 1-8, 1992.

[2]. M. M. Invernici, S. L. Salvador , P. H. F. Silva , M. S. M. Soares, R. Casarin, D. B. Palioto, S. L. S. Souza, M. Taba Jr, A. B. Novaes Jr, F. A. C. Furlaneto, and Michel R Messora, "Effects of *Bifidobacterium* probiotic on the treatment of chronic periodontitis: a randomized clinical trial," *Journal of Clinical Periodontology,* vol. 45, no. 10, pp. 1198-1210, 2018.

[3]. R L Kelley, D. Minikhiem, B. Kiely, L. O'Mahony, D. O'Sullivan, T. Boileau, and J. Soon Park, "Clinical benefits of probiotic canine-derived *Bifidobacterium animalis* strain AHC7 in dogs with acute idiopathic diarrhea," *Veterinary*

Therapeutics:Reseearch in applied veterinary medcine, vol. 10, no.3, pp 121-130, 2009.

[4]. A. M. O Leite , M. A. L. Miguel, R. S. Peixoto, P. Ruas-Madiedo , V. M. F. Paschoalin , B. Mayo, and S. Delgado, "Probiotic potential of selected lactic acid bacteria strains isolated from Brazilian kefir grains," *Journal of Dairy Science*, vol. 98, no. 6, pp 3622-3632, 2015.

[5]. L. F. F. Oliveira, S. L Salvador, P. H. F. Silva, F. A. C. Furlaneto , L. Figueiredo, R. Casarin, E. Ervolino, D. B. Palioto, S. L. S. Souza, M. Taba. Jr , A. B. Novaes Jr , and M. R. Messora , "Benefits of *Bifidobacterium animalis* subsp. *lactis* Probiotic in Experimental Periodontitis," *Journal of Periodontology,* vol. 88, no. 2, pp 197-208, 2017.

[6]. F. Turroni, C. Peano, D. A. Pass, E. Foroni, M. Severgnini, M. J. Claesson, C. Kerr, J. Hourihane, D. Murray, F. Fuligni, M. Gueimonde, A. Margolles, G. De Bellis, P. W O'Toole, D. van Sinderen, J. R. Marchesi, and M. Ventura, "Diversity of bifidobacteria within the infant gut microbiota," *Plos One*, vol. 7, no. 5, pp e36957, 2012.

[7]. S Magaldi, S. Mata-Essayag, C. Hartung de Capriles, C. Perez, M. T. Colella, C. Olaizola, and Y. Ontiveros, "Well diffusion for antifungal susceptibility testing," *International Journal of Infectious Diseases*, vol. 8, no. 1, pp 39-45, 2004.

[8]. H. T. Nguyen, "Isolation and Selectation of bifidobacteria for application in probiotics production," Master of Science thesis dissertation, University of Science, Hanoi, 2017.

[9]. A. Gómez Zavaglia, G. Kociubinski, P. Pérez, and G. De Antoni, "Isolation and Characterization of Bifidobacterium Strains for Probiotic Formulation," *Journal of Food Protection*, vol. 61, no.7, pp. 865-873, 1998.

[10]. G.-B. Kim and B. H. Lee, "Genetic analysis of a bile salt hydrolase in Bifidobacterium animalis subsp. lactis KL612," *Journal of Applied Microbiology*, vol. 105, pp. 778-790, 2008.

[11]. G. Larson, P. Falk, and L. C. Hoskins, "Degradation of Human Intestinal Glycosphingolipids by Extracellular Glycosidases from Mucin-degrading Bacteria of the Human Fecal Flora," *The Journal of Biological Chemistry*, vol. 263, no. 22, pp. 10790-10798,1988.

[12]. M. Kitaoka, "Bifidobacterial Enzymes Involved in the Metabolism of Human Milk Oligosaccharides[1–3]," Advances in Nutrition (American Society for Nutrition), vol. 3, no. 3. pp 422S–429S, 2012.

[13]. E. J. Yun , S. Yu, N. J. Park, Y. Cho, N. R. Han, Y.-S.Jin, and K. H. Kim , "Metabolic and enzymatic elucidation of cooperative degradation of red seaweed agarose by two human gut bacteria," *Scientifc Reports*, vol. 11, pp13955, 2021.

[14]. D. J. Morrison and T. Preston, "Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism," *Gut Microbes*, vol. 7, no. 3, pp. 189-200, 2016.

[15]. G. Alessandri, M. C. Ossiprandi, J. MacSharry, D. van Sinderen, and M. Ventura, "Bifidobacterial Dialogue with Its Human Host and Consequent Modulation of the Immune System," *Frontiers in Immunology*, vol. 10 (2348), pp. 1-12, 2019.

[16]. F.Zuo. R. Yu, X. Feng, and L. Chen, "Characterization and in vitro properties of potential probiotic Bifidobacterium strains isolated from breast-fed infant feces," *Annals of Microbiology*, vol. 66, no. 3, pp1027-1037, 2016.

[17]. M. Weng, K. Ganguli, W. Zhu, H. N..Shi, and W. A. Walker"Conditioned medium from Bifidobacteria infantis protects against Cronobacter sakazakii-induced intestinal inflammation in newborn mice," *American Journal of Physiology Gastrointestinal and Liver Physiology*, vol. 306, no. 9, pp G779-G787, 2014.

[18]. K. Ganguli, D. Meng, S. Rautava, L. Lu, W. A.Walker, and N. Nanthakumar "Probiotics prevent necrotizing enterocolitis by modulating enterocyte genes that regulate innate immune-mediated inflammation," *American Journal of Physiology Gastrointestinal and Liver Physiology*, vol. 304, no. 2, pp G132-G141, 2013.

[19]. I. Aloisio, C. Santini, B. Biavati, G. Dinelli, A. Cencič, W. Chingwaru, L. Mogna, and D. Di Gioia, ''Characterization of Bifidobacterium spp. Strains for the treatment of enteric disorders in newborns," *Applied Microbiology and Biotechnology*, vol. 96, pp. 1561–1576, 2012.

[20]. D. T. Men, P. T. Le, T. V. Tuan, N. T. T. Lan, N. T. M. Huyen, "Isolation and identification of Bifidobacterium spp. from infant intestinal tract," *Vietnamese Journal of Food Control*, vol. 3, no. 2, pp. 125-132, 2020.

[21]. E. Vamanu, A. Vamanu, and C. Gheorghe, "Isolation of a Lactobacillus plantarum strain used for obtaining a product for the preservation of fodders," *African Journal of Biotechnology*, vol. 4, no. 5, pp. 403-408, 2005.

[22]. A. M. O. Leite, M. A. L. Miguel, R. S. Peixoto, P. Ruas-Madiedo, V. M. F. Paschoalin, B. Mayo, and S. Delgado, "Probiotic potential of selected lactic acid bacteria strains isolated from Brazilian kefir grains," *Journal of Dairy Science*, vol. 98, no. 6, pp 3622-3632, 2015.

Đặc tính probiotic của Bifidobacterium infantis phân lập tại Việt nam

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Tóm tắt

Bifidobacterium là vi khuẩn kỵ khí thường thấy trong ruột người và động vật. B. *infantis* được phân lập từ mẫu phân của một trẻ sơ sinh tại Hà Nội. Trong nghiên cứu này, các đặc tính probiotic của chủng B. *infantis* đã phân lập như: khả năng tạo các enzyme ngoại bào; các hoạt tính kháng khuẩn chống lại các vi khuẩn gây ngộ độc thực phẩm như *E. coli*, *S. aureus*; khả năng sinh acid hữu cơ và chịu muối mật được đánh giá. Kết quả cho thấy chủng vi khuẩn đã phân lập có thể tạo enzyme amylase và protease, không tạo enzyme cellulase; acid hữu cơ cũng được tạo ra trong quá trình sinh trưởng của vi khuẩn. Vi khuẩn đã phân lập có thể kháng lại *E. coli* và *S. aureus* và phát triển trong môi trường chứa tới 1% muối mật. Kết quả nghiên cứu này là cơ sở cho các nghiên cứu tiếp theo nhằm ứng dụng vi khuẩn này như một nguồn probiotic có ích cho sức khỏe con người tại Việt Nam.

Từ khóa: *Bifidobacterium infantis, probiotic, E. coli, exoenzymes, đặc tính probiotic.*