ISOLATION, IDENTIFICATION AND SELECTION OF *ENTEROCOCCUS* STRAINS WITH HIGH POTENTIAL PROBIOTIC FROM INFANT FAECES

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SUMMARY

Bacteria of the genus *Enterococcus* are found in a wide variety of habitats such as soil, water, plants, fermented foods and in the human digestive tract. From the perspective of metabolic, *Enterococcus* spp. produces lactic acid as the main product of carbohydrate fermentation. With the purpose of researching and studying the capacity applying of this group in the field of probiotic, the present study was carried out isolations and selection the *Enterococcus* -types having applicability to use for probiotic micro-organisms from origin of healthy infant faeces. We isolated and identified *Enterococcus* spp. by combining physiology tests and biological chemistry tests with sequenced amplification of the intermediate ITS - PCR. As a result, we selected 31 bacterial strains belong to *Enterococcus* spp. Then we continued to survey probiotic properties such as durable capability and survival in the low pH condition, digestive enzyme, adhering to intestinal epithelial cell in *in-vitro* condition. In addition, we still survey the antagonism to pathogenic bacteria in the intestine, the capability of resistance to some types of antibiotic products and the produce bacteriocin initially. Through the research, we have success isolation and selection potential strains of *Enterococcus*, through the survival capability in inclement condition and may live well in intestinal condition. Besides, they can resist and compete to some pathogenic bacteria strains. Finally, the research initially help us choose 6 strains belong to *Enterococcus* for further research and application as probiotics.

Keywords: Bacteriocin, *Enterococcus*, isolation, identification, lactic acid bacteria, probiotic.

INTRODUCTION

Probiotic therapy is a diet added live bacteria to have useful influence on physiological of consumer by modulating mucous membrane and immune system as well as improving nutrition and balancing intestinal microbiological system. In the human's intestine, there are about 10 billion bacteria in one gram of excrement. It consists of a couple hundreds of species bacteria creating intestinal microbiological system abundantly. Enterococci and streptococci group D create a part of importance in local microbiology system in human's intestine and some animals species, popularly in *E. faecalis* and *E. faecium*. Especially, *E. faecalis* is usually present in people's large intestine, but it is rarely found in another animals.

Hardie and Whiley (1997) described general features of *Enterococcus* spp., and these features are applied for most of species belong to *Enterococcus* genus. This is discretionary anaerobic organism group, single globular form or combined string, positive gram, negative catalase. They appear everywhere in environment, but they are popular in the products of milk and especially in people's intestine and a number kinds of mammals. Enterococci can live and develop in wide ranges of temperature, from 10^{o}C to 45^{o}C, optimum in 35^{o}C to 37^{o}C, survival at least thirty minutes at 60^{o}C. Thank to sodium - ATPase pump on cytoplasm - membrane, they can resist NaCl up to 6.5% medium and they can grow in wide pH range medium 4.6 - 9.6, optimum in 6.5 - 7.5. Most of *Enterococcus* spp. can hydrolyze esculin with the present of bile salt 40%.

According to FAO (2002), the selection and applying for probiotic purpose usually use acid lactic bacteria (LAB). Standards of selection include some following importance factors are the origin of bacteria is clear and safety, survivable in acid condition and high bile salt concentration in small intestine, contribution in to adjustment intestine function, producing vitamin and releasing enzyme help improving food digestion, well adhesive ability help bacteria settling a long time in the intestine environment and increase yield, produce enzyme and bacteriocin can inhibit the development pathogenic bacteria.

With probiotic property, they were added into dairy product that they help remarkable improve food qualities and improve consumer's health. *Enterococcus* strain also belongs to acid lactic bacteria. Furthermore, they are microbial flora in human and some domestic animals. However, they were not still studying and applying in a suitable way. To know further these bacteria, we carried out studying *Enterococcus* strains and we gain some initial achievements.

METERIAL AND METHOD

Isolation and Identification: Bacteria resources: bacteria were isolated from the first month infant faeces without illness nor with symptoms of diarrhea being collected at Tu Du hospital and Gia Dinh hospital, Ho Chi Minh city, Vietnam. It is diluted and spread on BEA (Bile Esculin Azid) medium, incubated at 37°C (Domig *et al*., 2003, Falkam, 1970). After two days, we select separate colonies that have 0.5 - 1mm diameter, milky color or pure, and have black - brown halo surrounding colonies, then isolate them to become purebred and store them in MRS broth medium (adding 20% glycerol). Some collected bacteria species will be Gram dyed, tested catalyses, and study glucose fermentation particularity, survival ability and growth in MRS broth medium that has NaCl 6.5% and medium containing bile solution 40%, growth at temperature 10°C, 45°C from 2 - 7 days and survival at 60°C in 30 minutes. After that using PCR technique to amplify intermediate region order rDNA 16S - 23S that is preserved well in ITS region of *Enterococcus* to help identifying *Enterococcus* spp by using two primer pS1490, 5'- TGC GGC TGG ATC CCC TCC TT-3' and pL132, 5'- CCG GGT TTC CCC ATT CGG-3' (Alves *et al*., 2004).

Studying of probiotic characteristics: Effects of Enzyme at low pH: using synthetic gastric juice that has ingredient similar to gastric juice of stomach to observe ability withstanding pH at low level (pH 1, pH 2, pH 3). *Effects of Trypsin at pH 8:* Survival of these bacteria species is effected by alkaline pH and digestive enzyme in small intestine. Using salt water contains trypsin 0.01% and controlling pH 8 by NaOH 0.1N by Collins *et al* (2004).

Adhesive Ability: A well adhesive ability will help bacteria settling a long time in the intestine environment. Agglomeration in ammonium sulfate: using method from Lankova *et al* (2004).

Adhesiveness to solvent: according to the method of Rosenberg *et al* (1990), adhesiveness of cells reflects the hydrophilic or hydrophobic property of cells.

Studying ability to antagonism pathogenic bacteria: To study bacteria resistance ability, we used some common pathogenic bacteria in intestine such as *E. coli, Klebsialla, Salmonella, Shigella* and *Staphylococcus.* 3 µL *Enterococcus* solution that is activated in Petri dishes that contain MRS medium, kept at 37°C in 24 hours. After that, covered these dishes by 10 ml LB medium 0.75% agar that is boiled and mixed with 1 ml pathogenic bacteria solution (activated one night), kept at 37°C in 24 hours. And then they are tested the formation of bacteria resistance rings. If the diameter of these rings is more widen, it is clear that studying species has ability to resist to correlative pathogenic bacteria strongly.

Resistance to antibiotics: To test this ability, we use the method that Petri dishes have a paper containing antibiotic according to criterions (Amoxillin/Clavulanic, Penicillin, Vancomycine, Tetracycline, Erythromycine, Sulfamethoxacol/Trimethoprim, Rifampicin, Norfloxacine). Studied bacteria species are cultured to activate in MRS liquid medium at 37°C in 24 hours. Covered the face of agar that is prepared before (Petri dishes contain 15ml MRS-agar medium) by 7ml medium that added 200µl activated cell solution (cell concentration is about 10^6 - 10^7 cells/ml), kept at room temperature in 15 minutes, putting antibiotic dishes on the face of agar, kept at 37°C in 24 hours. After that, we may have a result from observing and measuring diameter of antibacterial rings and compare with the criteria table (resistant, intermediate or sensitive) (Marcinakova *et al*., 2004).

Studying ability to produce bacteriocin: Using diffuseness over agar-wells method is described first time by Tagg and McGiven (1971). *Enterococcus* species are cultured in MRS liquid medium 1% Glucose at 37°C in 48 hours. They are rejected their mass by centrifugal machine 13,000 round/minute and controlled pH of float solution to 6.5 by sterilize NaOH 1M. Then we use floating rough bacteriocin test resistance to control bacteria.

RESULTS AND DISCUSSION

Isolation of Enterococci from infant faeces: After 24 - 48h incubation at 37°C on BEA agar, we obtained 34 bacterial strains satisfied these following characteristics: diplococcal bacteria gram - positive, negative

Table 1. Survey of physiology characteristics of obtained 34 *Enterococi* strains

Strains	NaCl 6.5%	Bile salt 40%	pH 9.6	10°C	45°C	60°C/30'	Glucose fermented Into acid	Hydrolysis arginine
E. faecalis	+	+	+	+	+	+	+	+
TD1 - TD13	+	+	+	+	+	+	+	+
GD1 - GD7; GD9; GD10; GD11; GD14; GD15; GD20; GD21	+	+	+	+	+	+	+	+
GD8	–	+	+	–	–	+	+	+
GD12	+	+	+	–	+	+	+	+
GD13	+	+	+	–	–	+	+	+
GD16; GD17; GD18; GD19	–	+	+	+	+	+	+	+

(+): Bacteria can resistance and growth; (-): Bacteria can not survive.

catalase, hydrolysis esculine and arginine. In there, there are 13 strains from infant faeces samples at Tu Du Hospital was signed from TD1 to TD13, 21 strains from infant faeces samples at Gia Dinh Hospital was signed from GD1 to GD21.

Identification of physiological and biochemical characteristics: After testing physiological and biochemical characteristics, we obtained these following results (Tab 1).

Almost strains are able to grow in 6.5% NaCl, 40% bile salt, 9.6 pH medium. They grow at 10^0C to 45^0 C, and they were also heat resistant 30 minutes at 60^0C. Exception of some strains such as GD8, GD16, GD17, GD18 and GD19 did not grow in 6.5% NaCl. GD8, GD13, GD15 strains did not grow at temperature both 10^0C and 45^oC. Biochemical characteristics test: all 34 strains fermented glucose into lactic acid, not give gas and hydrolysis arginine (Data not show).

Almost isolated strains have physiological characteristics being suitable with characteristic of *Enterococcus* in animals. However, GD8, GD13, GD15, GD16, GD17, GD18, GD19 strains were not reliable. Therefore, they need to combine PCR technique amplifying 280-620bp ITS regions to give more precise results.

We used ITS-PCR methods to amplify ITS intergenic spacer (16S and 23S rDNA) to identify *Enterococcus* species promptly by primers pS1490 and pL132. The length of amplification is from 280 - 620 bp and may be more than one replicon unit. It depends on the presence or absence of different tRNA genes (Tyrrel *et al* 1997).

From electrophoresis of ITS-PCR products, we found that 31 isolates have positive and the length of these bands were correlative to positive control band ladder (1, *Enterococcus faecalis* ATCC 29212) (Fig 1). The results were regconized that there were 31 strains giving two main bands have size from 370-500bp, 3 strains gave negative PCR results in well 22, 27 and 29 (GD8, GD13, GD15, respectively).

Therefore, through the survey about physiological and biochemical characteristics, and PCR technique amplifying ITS sequence to identify 31 *Enterococcus strains*, simultaneously eliminated 3 strains not belong to *Enterococcus* species is GD8, GD13, GD15. Isolated strains belong to the *Enterococcus* species will be surveyed to select the most potential probiotic strains.

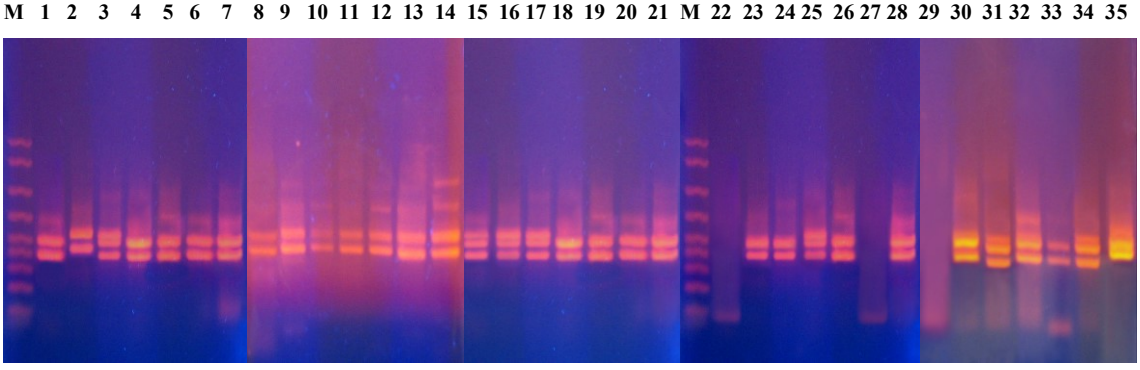

Figure 1. ITS-PCR products of 34 *Enterococcus* isolated strains. M, 50 - 2000bp ladder; 1, *Enterococcus faecalis* ATCC 29212; from well 2 to 14 corresponding to TD1 - TD13; from well 15 to 35 corresponding to GD1 - GD21

Spectrum of probiotic characteristics *in vitro* (*Presence in low* pH): The results were shown that at pH 3, almost strains can be resistant more than 180 minutes, at pH 2, there were some differences in resistance abilities to this pH value. TD3, TD4, TD5, TD6, GD10, GD11, GD12, GD20 trains can survive in 30min, TD2, GD3, GD21 strains survived in 60min, TD1, GD4, GD7 strains survived in 90min. There are no strains can survived at 180min. At pH 1 TD6 can survive in 30min, TD5, GD4, GD7, GD21 survived in 60min, there were no strains survived from 90 - 180min.

Resistance of trypsin at alkaline pH: With medium contain trypsin 0.01% at pH 8 around 0 to 180 minutes. We realize that at this trypsin concentration and this pH were not affected to the survival of 31 survey trains. Therefore, with resistance abilities to bile salt 40%, they can survive in small intestine condition well.

Adherence property of 31 survey strains: The high percentage of agglomeration the high adherence that bacteria cell can gain. Thought the experiments about agglomeration in ammonium sulfate and adherence with solvent, we realize that these strains adhere well in solvent and agglomerate well in ammonium sulfate concentration from 2M to 4M. The result showed the high correlation between two tests. As a result, we detected some strains that have adherence abilities well such as, Ef, TD10, TD12, TD13, GD1, GD5, GD6, GD9, GD10, GD17, GD19.

Resistance to pathogenic bacteria: All 31 survey strains resistance well to *E. coli*, *Salmonella* but resistance weakly to *Staphilococcus*. Remarkably, there are 13 strains resistance well to 5 types pathogenic bacteria, such as: TD3, TD10, TD11, GD5, GD6, GD7.

Table 2. Results in remainder strains

Strains	pH low	Trypsin	Adherence	Bacteria resistance	Vancomycin	Bacteriocin
TD1	+++	+	+	++++	S	++
TD3	++	+	++	++++	S	++
TD7	++	+	+	++	S	++
TD8	++	+	++	+++	S	++
TD9	++	+	++	++	S	++
TD10	++	+	+++	++++	S	++
TD13	++	+	+++	+++	S	++
GD3	++	+	+++	++	S	++
GD10	++	+	++	++	S	++
GD11	++	+	+	+++	S	+
GD12	++	+	+	++	S	+
GD18	+++	+	+	++	S	++
GD19	++	+	+++	+++	S	++

Antibiotic resistance abilities: The antibiotic abilities are concerned a dangerous property to clinical microbiology, it can reduce medicine effect. However, microbiology in food and chemistry, this characteristic in allowance range is acceptable, it help them can survive longer and compete to other pathogenic bacteria that we want to destroy. With vancomycine (Va): almost isolates strains sensitive to this type. Exception some strains can resistance to vancomycine such as TD11, GD4, GD5, GD6, GD7; and some strains have intermediate resistance such as TD2, TD12, GD1, GD14, GD17. These strains may have vancomycine resistant gene. They are very dangerous because they may converse to other bacteria.

Bacteriocin productivity: Almost survey strains show bacteriocin active on *E. coli* ATCC 25922 and *St. aureus* ATCC 25923 rather well. However, there are 4 strains inhibited *Lactobacillus acidophillus* NRRL B-2092: TD1, TD5, GD1, GD10 strains.

Resistance to low pH in synthesize gastric juice test: we realize that almost strains could resist acid condition pH 3 up to 3 hours. These abilities may help these strains undergo gastric to small intestine.
With adherence abilities, we detected 10 strains showing the adherence abilities to intestine wall very well, such as TD10, TD12, TD13, GD1, GD5, GD6, GD10, GD7, GD19 strains. With the resistance to pathology bacteria, we realize that there are 6 strains can resist control bacteria such as TD3, TD10, TD11, GD5, GD6, GD7. However, after antibiotic resistance test, we rejected 10 strains that they show the resistance and intermediate to vancomycine (Resistance strains TD11, GD4, GD5, GD6, GD7; Intermediate resistance strains TD2, TD12, GD1, GD14, GD17). Table 2 will give us general information to select probiotic potential strains.

In general, from proiotic properties, we selected 6 *Enterococcus* strains typically: TD3, TD8, TD10, TD13, GD3, GD19. These strains may use in preparation and utilization as probiotics.

CONCLUSION

We isolated 34 bacteria strains from infant faeces. After surveying phisiology, chemistry, and probiotic properties, we obtained six *Enterococcus* strains as TD3, TD8, TD10, TD13, GD3, GD19 that they have equitable good characteristics such as resist to low pH in intestine, resist to intestine enzyme at alkali pH, adherence to epithelium intestine cells. They can resist to some pathogenic bacteria and some antibiotic at given dosage simultaneously. In addition, these trains may produce a small amount of bacteriocin which resists to some pathogenic bacteria.

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PHÂN LẬP, ĐỊNH DANH VÀ CHỌN LỌC CÁC CHỦNG *ENTEROCCOCUS* CÓ TIỀM NĂNG PROBIOTIC TỪ PHÂN TRẺ SƠ SINH

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TÓM TẮT

Vi khuẩn chi *Enteroccocus* hiện diện rộng rãi trong tự nhiên như đất, nước, thực vật, thực phẩm lên men và trong hệ tiêu hóa của người. Về trao đổi chất, các *Enteroccocus* sản xuất acid lactic trong quá trình lên men carbohydrate. Với mục tiêu nghiên cứu khả năng ứng dụng nhóm vi khuẩn trong lĩnh vực probiotic, nghiên cứu này thực hiện việc phân lập các vi khuẩn *Enteroccocus* từ phân trẻ sơ sinh khỏe mạnh trong vòng 1 tháng tuổi không có tiền sử bệnh và không bị tiêu chảy. Việc phân lập, định danh dựa vào các đặc điểm sinh lí, sinh hóa, kết hợp với dữ liệu từ trình tự khuếch đại ITS-PCR. Kết quả đã thu nhận 31 chủng thuộc chi *Enteroccocus*. Các đặc tính probiotic như khả năng sống sót ở pH thấp, có sự hiện của trypsin, khả năng bám dính tế bào ruột đã được khảo sát. Các đặc tính như đối kháng vi khuẩn gây bệnh, tính mẫn cảm khánh sinh và sản xuất bacteriocin đã được tiến hành. Chúng tôi đã thành công trong việc phân lập các chủng *Enterococus* có tiềm năng probiotic. Thông qua nghiên cứu này 6 chủng *Enteroccoccus* được lựa chọn cho nghiện cứu tiếp theo.

Từ khóa: Bacteriocin, *Enteroccocus*, định danh, lactic acid bacteria, phân lập, probiotic.

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