IN VITRO ASSESSMENT OF POTENTIAL PROBIOTIC MICROORGANISMS FOR APPLICATION IN ANIMAL FEEDING

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ABSTRACT

This study aimed to evaluate and to select potential probiotic microorganisms. The obtained results would be further studied for application in production of animal feed. A total of 16 strains of microorganisms including 11 strains of *Lactobacillus*, four strains of *Bacillus* and a yeast strain *Saccharomyces boulardii* PLCP were investigated for acid production, digestive enzyme production and antimicrobial activity as well as their survival when exposed to simulated gastrointestinal conditions. The results showed that all 11 strains of *Lactobacillus* bacteria were capable of acid generation (in the range from 18.05 – 19.04 g/l). All four strains of *Bacillus* bacteria were capable of producing protease. Only *Bacillus* sp D7 strain was capable of producing three digestive enzymes (protease, amylase, cellulase) with hydrolyzed hollows ranged from 15.5–18.5 mm. The antibacterial activity of 9/16 test strains was positive against *Salmonella* Typhimurium, *Staphylococcus aureus* and *Escherichia coli*. Survivability of 15 test microorganisms in simulated gastrointestinal conditions was relatively high (c.a. 80 %). Three strains of *L. acidophilus* VAST, *S. boulardii* PLCP and *Bacillus* sp D7 when in mixture demonstrated a great potential as probiotics for animal.

Keywords: animal feed, digestive enzyme, antimicrobial activity, simulated gastrointestinal conditions.

1. INTRODUCTION

In Vietnam, antibiotic use in animal production is not only for therapy, but also for prophylaxis and preventing opportunistic infections to ensure growth. The misuse of antibiotic or abusive use of illegal veterinary drugs without any veterinary prescription and supervision may lead to the presence of residues in animal products, antibiotic resistance in bacteria, and consequently, affect the public health. In recent years, the use of probiotics or beneficial bacteria, which control pathogens through different mechanisms, is increasingly viewed as an alternative to antibiotic treatment. Probiotics are live microbial feed supplements which beneficially affect the host by improving its intestinal microbial ecology balance. The purposes of this study, as a part of a Romanian-Vietnam Joint project funded by Ministry of Science and Technology, was to evaluate and to select potential probiotic microorganisms from the joint microorganism collection that would be applied in production of animal feed. Microbial strains were screened for acid production ability, digestive enzyme production, antimicrobial activity

against intestinal pathogens frequent in animal including *S.* Typhimurium, *S. aureus*, *E. coli*. Then the gastrointestinal survivability was tested. Obtained results could contribute to limit the use of antibiotics in animal production.

2. MATERIALS AND METHODS

2.1. Materials

Microorganisms used in the study were: eleven strains of *Latobacillus* including: *L. rhamnosus* GG, *L. plantarum* NC8, *L. plantarum* 8826 WCH_1, *L. casei* 334, *L. casei* BL23, *L. brevis* 105135, *L. brevis* 103474 (kindly provided by University of Burgundy, Dijon – France); *L. plantarum* NCDN4 (Food Industry Research Institute Collection); *L. acidophilus* VAST (Kindly provided by Institute of Biotechnology, Viet Nam Academy of Science and Technology); *Lactobacillus* IBNA 1, *Lactobacillus* IBNA 2 (Collection of National Research Development Institute for Animal Biology and Nutrition, Romania); four strains of *Bacillus* including: *Bacillus* sp D1, *Bacillus* sp D7 and *B. Subtilis* SBFT (School of Biotechnology and Food Technology, Hanoi University of Science and Technology); *B. subtilis* VAST (Institute of Biotechnology, Vietnam Academy of Science and Technology); one commercial probiotic products *Saccharomyces boulardii* PLCP. Pathogenic strains included: *Escherichia coli* ATCC® 25922TM, *Salmonella enterica* subsp *enterica* serovar Typhimurium ATCC® 14028TM, *Staphylococcus aureus* subsp *aureus* ATCC® 25923TM.

2.2. Methods

2.2.1. Growth conditions

The species of *Lactobacillus* were cultivated in liquid MRS medium then incubated at 37 °C during 24 hours. The species of *Bacillus* and pathogenic strains were cultivated in NB medium then incubated at 37 °C during 24 hours. *S. boulardii* PLCP was cultured in Hansen medium and then incubated at 37 °C during 24 hours. Supplements Carboxymethyl cellulose (CMC), Casein, soluble starch were used for enzymatic tests.

2.2.2. Determination of the lactic acid production

The Thorner method was used to determine the acid forming ability of the lactic acid bacteria [1]. The results obtained were converted into lactic acid content.

2.2.3. Determination of the enzyme production

Enzymes production ability was determined by scoring method and adjusted accordingly [2]. In details, *Bacillus* bacteria strains were reactivated at 37 °C in NB medium, for 24 hours, to concentration of 10^6–10^7 CFU/ml. Then 20 µl of each *Bacillus* strain were dropped on the surface of NA plates supplemented with casein 1 % for protease enzyme, soluble starch 1 % for amylase enzyme, CMC 5 g/l for cellulase enzyme. Plates were then air dried and incubated at 37 °C during 24 hours. Indicator TCA 10 % was added to the casein substrate plate, lugol was added to the CMC and soluble starch plate for colouring. The enzyme production ability was determined measuring the diameter of hydrolyzed areas.

2.2.4. Determination of the antimicrobial activity

Agar well diffusion method was used to evaluate the antimicrobial activity of probiotic microorganisms [3]. Inhibition activity was determined by diameter of inhibition area surrounding each agar well.

2.2.5. Survival of probiotic microorganisms in the simulated gastrointestinal tract conditions

This essay was conducted accordingly [4] with modifications. In details, probiotic strains were reactivated at 37 °C for 24 hours. Cell pellets were harvested by centrifugation and washed twice with sterile saline solution (0.5 % w/v NaCl), then were exposed to simulated gastric juice (0.3 g/l pepsin), adjusted at pH 2.0 with HCl, in sterile saline solution (0.5 % w/v NaCl). Cell pellets were incubated at 37 °C in simulated gastric juice, and samples were taken at initial time 0 min, 90 min and 180 min after gastric exposition to determine the viability by plate counting on MRS agar. Gastric-exposed cells were harvested by centrifugation and supplement simulated small intestinal medium and were incubated at 37 °C. Samples were taken at 0, 90 and 180 min during small intestinal exposition to determine the viability by plate counting on MRS agar.

2.2.6. Interaction between L. acidophilus VAST, *Bacillus sp* D7 *and S. boulardii* PLCP *in liquid culture fluid*

24 hours culture of probiotic strains were adjusted to concentration 10^6 CFU/ml by saline. Then 0.1 ml of *L. acidophilus* VAST, 0.1 ml *S. boulardii* PLCP, 0.1 ml *Bacillus* sp D7 were mixed with 0.7 ml BHI medium. In antimicrobial test, pathogenic bacteria (*S.* Typhimurium, *E. coli*) were inoculated at 10^5 CFU/ml. After 24 hours culturing at 37 ^oC, bacteria were quantified by plate count method on appropriate agars [5].

3. RESULTS AND DISCUSSION

3.1. Acid production ability by *Lactobacillus* strains

Acid production ability of 11 strains of *Lactobacillus* bacteria at 37 ºC were examined. The 11 strains of *Lactobacillus* bacteria were capable of acid generation. The highest acid production was obtained by *L. plantarum* NCDN4 (19.04g/l). Followed by *L. plantarum* NC8, *L. plantarum* 8826, *L. acidophilus* VAST, *Lactobacillus* IBNA 1, *Lactobacillus* IBNA 2, *L. casei* 334, *L. casei* BL23 with the range from 17.64 – 18.87 (g/l). The lowest by *L. brevis* 105135 and *L. brevis* 103474 with the range 10.26–11.88 (g/l).

Organic acids produced by lactic acid bacteria were reported to anhance tract digestibility and growth performance in animals [6]. The main activity of organic acids is associated with a reduction in gastric pH converting the inactive pepsinogen to active pepsin for effective protein hydrolysis. Lactic acids are bacteriostatic and bactericidal, lactic acid has been reported to reduce gastric pH and inhibits the colonization and proliferation of pathogen bacteria by blocking the sites of adhesion or by producing lactic acid and it's metabolites which lower gastric pH. In our study *L. plantarum* NCDN4, *L. acidophilus* VAST, *L. plantarum* NC8 and *L. casei* 334 capable of highest acid generation could be of interest.

3.2. Antimicrobial activity of probiotic microorganisms against pathogenic bacteria

The antibacterial activity of the selected 11 microorganism probiotic was tested against *S.* Typhimurum, *S. aureus* and *E. coli* using the agar well-diffusion method, and the growth inhibition zones of the indicator bacteria were recorded on Table 3.1.

The experimental data shown on Table 3.1 indicated that supernatants obtained from 9/11 strains exhibited varying degrees of inhibitory activity against *S. aureus, S.* Typhimurium and *E.*

coli with inhibition zones ranged from 14.25–33.5 mm. *L. plantarum* NCDN4, *L. acidophilus* VAST, *Lactobacillus* IBNA 1 and *Lactobacillus* IBNA 2 showed strongest antimicrobial activity against *S. aureus*, *S.* Typhimurium and *E. coli*. Inhibition zones ranged from 20 to 22.5 mm against *S.* Typhimurium, from 29.5 to 33.5 mm against *S. aureus* and from 20–25.75 mm against *E. coli*. It appeared that the most sensitive strain was *S. aureus*.

Table 3.1. Antimicrobial activity of probiotic microorganisms against pathogenic bacteria.

Microorganisms Probiotic	**Determined inhibition zone (mm)**		
	***S.* Typhimurium**	***S. aureus***	***E. coli***
L. plantarum NCDN4	20.25 ± 0.03	33.5 ± 0.25	21 ± 0.15
L. acidophilus VAST	21.25 ± 0.03	31.75 ± 0.13	20 ± 0.05
Lactobacillus IBNA 1	20 ± 0.05	31.75 ± 0.15	22 ± 0.15
Lactobacillus IBNA 2	22.25 ± 0.03	29.5 ± 0.10	25.75 ± 0.08
L. casei 334	16.75 ± 0.33	17.5 ± 0.35	16.25 ± 0.33
L. casei BL23	17.5 ± 0.30	17 ± 0.20	16.25 ± 0.28
L. rhamnosus GG	14.5 ± 0.25	15.75 ± 0.18	16 ± 0.1
L. plantarum NC8	18.25 ± 0.28	19.25 ± 0.13	14.75 ± 0.38
L. plantarum 8826	17 ± 0.2	22.25 ± 0.18	14.25 ± 0.53
L. brevis 105135	0	0	0
L. brevis 103474	0	0	0

The study of Hussein et al. [3] reported that *Lactobacillus* sp showed a broad inhibitory spectrum against the indicator organisms tested. Some of the identified antimicrobial compounds produced by *Lactobacillus* strains include organic acid, hydrogen peroxide, diacetyl and bacteriocins. The study of De Keersmaecker et al. [4] showed the high antimicrobial activity of *Lactobacillus* bacteria in MRS medium has been associated with the production of lactic acid. In our study, antimicrobial activity seemed likely in correlation with acid production. Indeed, antimicrobial activity was not observed in *L. brevis* 105135 and *L. brevis* 103474, which previously showed lowest acid production (section 3.1). In contrast, *L. plantarum* NCDN4 *L. acidophilus* VAST, *Lactobacillus* IBNA 1, and *Lactobacillus* IBNA 2 demonstrated the highest antimicrobial activity (corresponded to high acid production ability, section 3.1). Therefore, they were able to be selected for further study.

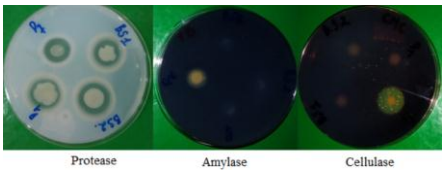
3.3. Enzymes production of *Bacillus* strains

Ability to produce digestive enzymes (protease, amylase, ellulose) of four strains of *Bacillus* bacteria were examined. The experimental data shown on Table 3.2.

Results shown on Table 3.2 and Fig 3.1 indicated that four strains of *Bacillus* bacteria were capable of protease enzyme generation, the highest enzyme production was obtained by *B. subtilis* VAST bacteria with diameter hydrolyzed hollows 22 mm. Followed by *Bacillus* sp D1, *Bacillus* sp D7 and *B. subtilis* SBFT with hydrolyzed hollows ranged from 17–18 mm.

Table 3.2. Enzymes production of *Bacillus* strains

Bacillus bacteria	Determined hydrolyzed areas (mm)		
	Protease	Amylase	Cellulase
Bacillus sp D1	18 ± 0.15	0	0
Bacillus sp D7	17 ± 3.00	15.5 ± 0.50	18.75 ± 0.25
B. subtilis SBFT	17 ± 3.50	0	0
B. subtilis VAST	22 ± 4.50	0	0



Fugure 3.1. Enzymes production of *Bacillus* strains

Three *Bacillus* bacteria strains *Bacillus* sp D1, *B. subtilis* SBFT and *B. subtilis* VAST did not show amylase or ellulose activity in our experiments. Only *Bacillus* sp D7 strain was capable of producing three digestive enzymes (protease, amylase, ellulose) with hydrolyzed hollows ranged from 15.5–18.5 mm. Dietary supplementation with *Bacillus* bacteria, which secretes protease, amylase, and/or ellulose, may aid in nutrient digestion and utilization of feed, therefore, improves growth performance of animal. In our study all four *Bacillus* strains could be used for application in animal feeding based on their enzymatic production pattern.

3.4. Survival of probiotic microorganisms in the simulated gastrointestinal tract conditions

Survival of 11 strains of *Lactobacillus* bacteria and four strains of *Bacillus* in the simulated gastrointestinal tract conditions is expressed through the concentration of bacteria over time in Figure 3.2 and Figure 3.3.

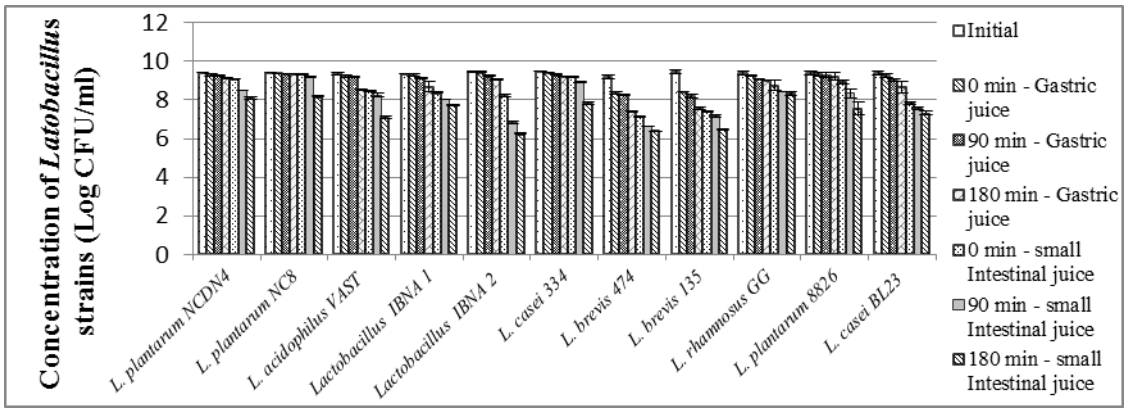


Figure 3.2. Survival of Lactobacillus strains in the simulated gastrointestinal tract conditions.

The experimental data shown on Figure 3.2 indicated that survivability of 11 strains of *Lactobacillus* bacteria in simulated gastrointestinal conditions was relatively high. The biomass of 11 strains of *Lactobacillus* reached 10^9–10^{10} CFU/ml. When cells exposed to simulated gastric condition, survivals of *L. plantarum* NCDN4, *L. rhamnosus* GG, *L. plantarum* NC8, *L. plantarum* 8826, *L. casei* 334, *Lactobacillus* IBNA 2 were reduced by 0.14 - 0.36 log CFU/ml, followed by *L. casei* BL23, *L. acidophilus* VAST, *Lactobacillus* IBNA 1 with survival rate reduced by 0.71–0.83 log CFU/ml. The least was *L. brevis* 103474, *L. brevis* 105135 with survival rate reduced by 2.07 – 2.21 log CFU/ml. However, all of them survived at concentrations ranged from 7.15 log CFU/ml to 9.33 log CFU/ml. Similarly, in simulated small

intestinal condition, slight reduction in bacterial count was observed, however, all of them survived at final count from 6.25 log CFU/ml to 8.34 log CFU/ml.

In previous study [7], strains of *Lactobacillus* when exposed to simulated gastric juice at pH 3.0, showed decreases of less than 1 log cycle with respect to the initial cell concentration and after 180 min of gastric digestion, when exposed to simulated intestinal fluid for a subsequent 180 min at pH 8.0 the survival against simulated intestinal juice was higher than 7 log CFU/ml. When introduced into the digestive system at the count of 7 log CFU/ml, the *Lactobacillus* bacteria likely dominate the digestive and in combination with acids and bile salts increase effective against intestinal pathogenic bacteria include: *S.* Typhimurium, *S. aureus* and *E.coli*. In our case, *L. plantarum* NCDN4, *L. plantarum* NC8, *L. acidophilus* VAST, *L. casei* 334 showed the survival in gastrointestinal tract conditions with a highest rate of 7.1 – 8.18 log CFU/ml and may be of interest for supplementing in animal feeding.

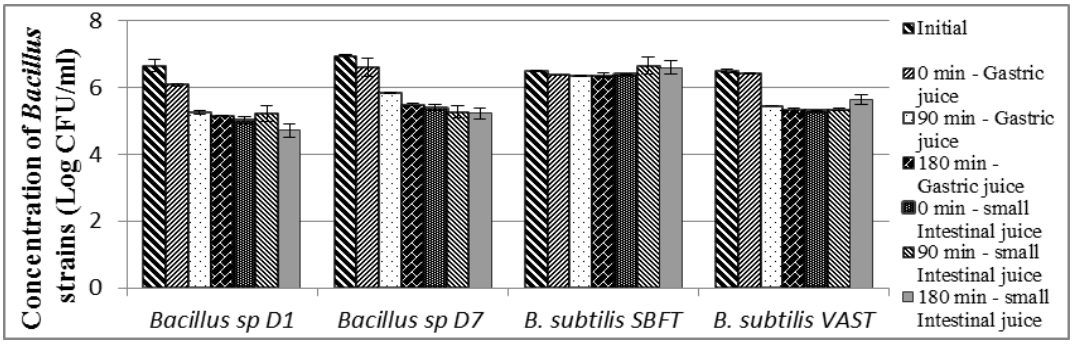


Figure 3.3. Survival of Bacillus strains in the simulated gastrointestinal tract conditions.

Survival of four strains of *Bacillus* bacteria in the simulated gastrointestinal tract conditions (Figure 3.3) and was almost similar to *Lactobacillus* bacteria and followed the same pattern. The count of four strains of *Bacillus* bacteria after exposure to gastrointestinal conditions reduced by maximum of 1.5 log CFU/ml, however, still retained approximately 5-6 log CFU/ml.

Recent research suggested that *Bacillus* spores do germinate in the gastrointestinal tract [8], nevertheless, it remained unclear which form, cell, spores, or both, is actually responsible for the competitive exclusion and probiotic effects. Based on its survivability in gastrointestinal tract and enzymatic production ability (section 3.3), *Bacillus* sp D7 was selected for further studies.

3.5. Interaction between *L. acidophilus* VAST, *Bacillus* sp D7 and *S. boulardii* PLCP in liquid culture fluid

With the aim of combining beneficial effects of probiotic bacteria, *L. acidophilus* VAST, *Bacillus* sp. D7 were selected and tested for survivability and beneficial effect in mixture with another commercial probiotic yeast *S. boulardii* PLCP, which was previously reported with a good bio-therapeutic agent allowing to prevent and/or treat several gastrointestinal diseases [9] and to reduce diarrhea incidence [10]. In our study, *S. boulardii* PLCP demonstrated high survival rate when exposed to gastrointestinal conditions *in vitro* (result not shown). Survivability of *L. acidophilus* VAST, *Bacillus* sp D7 and *S. boulardii* PLCP in mixture was shown in Figure 3.4.

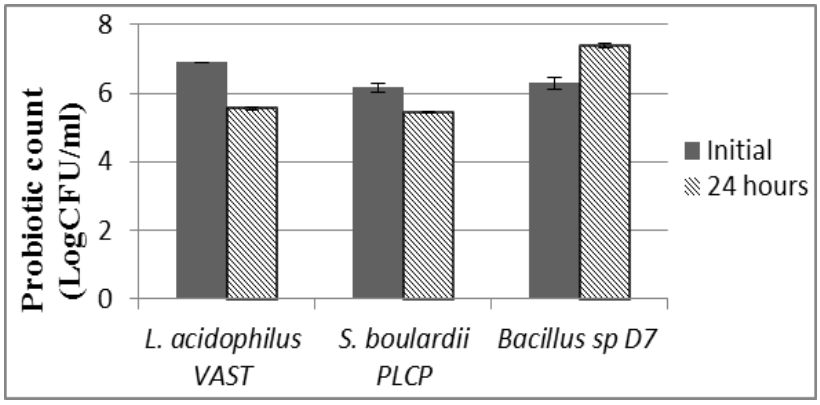


Figure 3.4. Interaction between *L. acidophilus* VAST, *Bacillus* sp D7 and *S. boulardii* PLCP in liquid culture fluid.

After 24 hours of co-culture, the count of three test bacteria were slightly changed, resulting in approximately 1,2 log CFU/ml reduction of *L. acidophilus* VAST; c.a 0,5 log CFU/ml reduction of *S. boulardii* PLCP and c.a. 1,0 log cfu/ml increase of *Bacillus* sp D7. However, when the mixture of test probiotic bacteria was incubated with pathogenic bacteria (*S.* Typhimurium, *E. coli),* the count of pathogenic bacteria were totally depressed (result not shown).

4. CONCLUSIONS

Three potential probiotic strains were selected including of *L. acidophilus* VAST, *Bacillus* sp D7 and *S. boulardii* PLCP. The survival rates of the three strains in combination liquid medium were relatively high. The mixture of three strains together was capable to totally inhibit intestinal pathogenic bacteria. *In vivo* test for their functionality needs to be further studied for potential application in animal feeding.

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TÓM TẮT

ĐÁNH GIÁ *IN VITRO* MỘT SỐ VI SINH VẬT PROBIOTIC TIỀM NĂNG HƯỚNG TỚI ỨNG DỤNG TRONG SẢN XUẤT THỨC ĂN CHĂN NUÔI

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Nghiên cứu này nhằm đánh giá và lựa chọn chủng probiotic tiềm năng hướng tới ứng dụng trong sản xuất thức ăn chăn nuôi. 16 chủng vi sinh vật probiotic bao gồm 11 chủng *Lactobacillus*, bốn chủng thuộc giống *Bacillus* và chủng nấm men *Saccharomyces boulardii* PLCP đã được nghiên cứu khả năng sinh axit, khả năng sinh enzyme tiêu hóa và hoạt tính kháng khuẩn cũng như sự tồn tại của chúng khi tiếp xúc với điều kiện tiêu hóa giả lập. Kết quả là 11 chủng *Lactobacillus* có khả năng sinh axit (trong khoảng từ 18,05–19,04 g/l). Bốn chủng *Bacillus* có khả năng sinh enzyme protease. Chỉ chủng *Bacillus* sp D7 có khả năng sinh 3 loại enzyme tiêu hóa (protease, amylase, cellulose) với đường kính vùng thủy phân từ 15,5–18,5

mm. Chín trong số 16 chủng thử nghiệm đã biểu hiện hoạt tính kháng khuẩn tốt đối với *Salmonella* Typhimurium, *Staphylococcus aureus* và *Escherichia coli*. Khả năng sống sót của các vi sinh vật probiotic thử nghiệm trong điều kiện tiêu hóa giả lập là tương đối cao (khoảng 80 %). Các chủng *L. acidophilus* VAST, *S. boulardii* PLCP và *Bacillus* sp D7 khi được phối trộn trong hỗn hợp vẫn bảo đảm được mật độ sống sót và tiềm năng làm chế phẩm probiotic cho động vật.

Từ khóa: thức ăn chăn nuôi, enzyme tiêu hóa, hoạt tính kháng khuẩn, điều kiện tiêu hóa giả lập.