Quantities and antibiotic resistance of microorganisms in some microbial products for animals in Vietnam

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ABSTRACT

The aims of the study were to look into the quantities of live beneficial microorganisms and antibiotic resistance of bacterial strains in several probiotic products used for food animals in the market. Ten probiotic products that claim to contain beneficial bacteria and fungi were examined. Eight products are said on the label to contain *Lactobacillus* spp., nine contain *Bacillus* spp., five contain yeasts and two have molds. The results showed that eight products did not have the microbial quantities or/and composition of microorganisms as saying on their labels. Of eight products which claim to contain *Lactobacillus* spp., the bacteria were isolated from only four, of which three had *Lactobacillus* counts at least ten-fold as low as the numbers on the labels. Spore-forming bacilli were isolated from all nine *Bacillus*-containing products. However, two products had the bacterial counts at least 10-fold as low as the numbers printed on the labels. Among five products stated to contain yeasts, the organisms were recovered from samples of only one. Seven *Lactobacillus* and fifteen *Bacillus* isolates from all samples that had bacterial growth were tested for their susceptibility against seven common antibiotics using Kirby-Bauer disk diffusion method. The results revealed that all the *Lactobacillus* isolates showed susceptibility to the tested antibiotics except kanamycin. All 15 *Bacillus* isolates were susceptible to ampicillin, kanamycin, and ciprofloxacin; five isolates were intermediately resistant to tetracycline; one isolates resisted erythromycin, and one isolates was resistant to vancomycin. The results of this study would provide information for farm practice in choosing antibiotics used together with antibiotics to maintain or/and restore the gut microflora after antibiotic treatment.

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1. Introduction

In the last two decades, the use of probiotics has become more and more popular in food-animal production. They are used for promoting animal health status and disease prevention, and for improvement of productivity. Probiotics have become one of the most potent alternatives to replace antibiotics in animal production. FAO/WHO has provided a definition of probiotics, which has been used as selection criteria for probiotic strains to be used in foods and dietary supplements. Probiotic strains for human use must be (i) sufficiently characterized; (ii) safe for the intended use; (iii) supported by at least one positive human clinical trial conducted ac-

cording to generally accepted scientific standards; and (iv) alive in the product at an efficacious dose throughout shelf life (Binda et al., 2020). Although the selection of beneficial bacteria for use in animals is not as strict as in humans, those criteria are still applied when a strain is studied for probiotic potential. Although most selected probiotic strains are safe, probiotics may possess undesirable properties such as virulence factors and transferable antimicrobial resistance (Alayande et al., 2020).

The objectives of the present study were to look into the quantities of live beneficial microorganisms and the resistance to some antibiotics of bacterial strains in several probiotic products used for food animals in the market.

2. Materials and Methods

Ten different products (named from A to J) containing beneficial microbes – *Lactobacillus* spp., Bacillus spp. and fungi were purchased from veterinary stores. They were all made in Vietnam and recommended to be used as supplements for food animals. Eight products are said on the label to contain *Lactobacillus* spp., nine contain *Bacillus* spp., five contain yeasts and two have molds.

2.1. Enumeration and isolation of the beneficial microbes from probiotic products

Ten-fold serial dilution of each sample was made by suspending one gram of each product into nine ml of sterilized MRS broth (de man, Rogosa, Sharpe; Oxoid, CM0359) or saline. Microbial enumeration was performed using conventional plate count method. One hundred µL of each dilution (10^{-1} to 10^{-6}) was spread on an appropriate agar plate to each group of examined microorganisms as described later. The process (weighing sample, making dilutions and inoculation) was repeated twice. If no growth were detected from the lowest dilution (10^{-1}) from one repeat, the process was done for the third time.

2.1.1. Lactic acid bacteria

MRS agar (de man, Rogosa, Sharpe; Oxoid; CM0361) was used for enumeration of lactobacilli (De Man et al., 1960). One hundred µL of each sample dilution in MRS broth was spread onto an MRS agar and incubated at 37°C/48 h under anaerobic condition (ThermoFisher, AN0025). For each sample, colonies on a plate that had a growth of 20 - 200 colonies were accounted. Three colonies from the counted plate were re-streaked onto new MRS agar plates and anaerobically incubated at 37°C/48 h. Each isolate was microscopically examined for Gram reaction and cell morphology. Test for catalase production was conducted by dripping two drops of hydrogen peroxide 3% onto bacteria on a glass slide. Isolates that were Gram-positive rods, non-spore forming and did not produce catalase was confirmed as Lactobacillus bacteria. Two isolates from each sample were kept in 15%-glycerin MRS broth at -20°C for further studies.

2.1.2. *Bacillus* spp.

TSA agar (Tryptone soya agar; Oxoid, CM0131) were used for enumeration of Bacillus spp. (Gorsuch et al., 2019). One hundred µL of each sample dilution in saline was spread onto a TSA plate and aerobically incubated at 37°C/48 h. Typical colonies of *Bacillus* bacteria were large, wrinkled, and saw-edged. For each sample, colonies on a plate that had a growth of 20 - 200 colonies were accounted. Three colonies from the counted plate were re-streaked onto new TSA agar plates and aerobically incubated at 37°C/24 h. Each isolate was microscopically examined for Gram reaction and cell morphology. *Bacillus* cells were large positive rods of which some were seen with endospores.

2.1.3. Yeasts and molds

Sabouraud dextrose agar (Oxoid, CM0041) containing chloramphenicol 5 mg/100 mL was used for growing yeasts and molds (Ladiges et al., 1974). One hundred µL of each sample dilution in saline was spread onto a Sabouraud plate and aerobically incubated at 30°C, and checked for fungal growth after one day, two days and a week. For each sample, colonies on a plate of 20 - 200 colonies were accounted, from which three colonies were re-streaked onto new agar sabouraud dextrose agar plates. Yeast colonies were large, smooth, raised, with even edges. Mold colonies was large, fuzzy, and pigmented. Two or three colonies from each sample was Gram-stained (yeasts) or Giemsa-stained (mold) and examined under a microscope at magnification 400x (Figure 1).

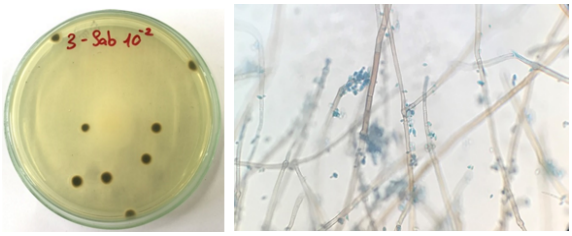

Figure 1. Mold colonies after one-day incubation (left) and Giemsa staining photo showing mold hyphae and spores (right; x400 magnification).

2.2. Antibiotic susceptibility testing

Two isolates of each microbe (*Lactobacillus* or *Bacillus*) from each sample (product) were tested for theirs susceptibility to seven antibiotics (ampicillin, amoxicillin/clavulanic, ciprofloxacin, erythromycin, kanamycin, tetracycline, vancomycin). The test was conducted using Kirby-Bauer disk diffusion method. The antibiotics belong to major groups which can be used for Gram-positive bacteria. Briefly, suspension of each isolate was made with saline to obtain turbidity equivalent to McFarland scale 0.5. The suspensions of *Lactobacillus* isolates each was spread evenly onto an MRS agar plate with a sterile cotton swab. *Bacillus* isolates were spread on Mueller-Hinton agar (MHA, Oxoid, CM0337). Antibiotic discs (Nam Khoa Biotek, Vietnam) were then placed on the surface of inoculated plates. MRS plates were anaerobically incubated at 37°C for 48 h (Anisimova & Yarullina, 2019). Plates of *Bacillus* isolates were aerobically incubated at 37°C/24 h (Jang et al., 2018).

After the incubation, the diameter (mm) of zone of inhibition (ZOI) was measured. Results were interpreted according to the recommendation by CLSI guidelines (Le & Nguyen, 2016; Sharma et al., 2017). Isolates with ZOI $\geq$ 20 mm diameter were considered as susceptible (S); ZOI between 15 and 19 mm were as intermediate (I); and ZOI $\leq$ 14 mm were resistant (R).

3. Results and Discussion

3.1. Enumeration and isolation of microorganisms

3.1.1. *Lactobacillus* spp.

The results are presented in Table 1. Of those four products, the number of lactobacilli recovered from one product (E) was ten-fold as high as that said on the label; from two products (F & G) it was more than ten-fold as low as the numbers on the labels; and from one product (H) the bacterial count was at the lower range of that announced by the manufacturer.

3.1.2. *Bacillus* spp.

Of the nine samples that contain *Bacillus* spp., the numbers of bacteria recovered from four samples were similar as it said on the product labels (product B, D, H, I). The other five were not. From four samples (products E, F, G, J), bacterial counts were higher (approximately 1 to 3 $\log_{10}$ CFU/g), whereas the count from sample C was 1 $\log_{10}$ CFU lower.

3.1.3. Yeast and mold

On the product labels, it was written that samples A and B contained both yeasts (*Saccharomyces cerevisiae* in A and *Torulopsis bovina* in B), and mold *Aspergillus oryzae*. However, the culture showed the growth of the molds but not the yeasts at all (after one week incubation). Furthermore, while the label of product A said that there were 2 x 10^6 *Aspergillus oryzae* CFU/g of the product, the actual count was 4 x 10^3 CFU/g. Similarly, it was 2 x 10^9 *Aspergillus oryzae* CFU/g on the label of product B, but the actual count was 8 x 10^6 CFU/g. It meant the actual viable counts of the molds from two samples were about 1,000-fold as low as it was written on the labels. Three samples (D, H and J) said to have yeast species *S. cerevisiae*. However, no growth was recovered from two samples D and H.

The enumeration and identification of probiotic microorganisms was carried out to evaluate the quality of the products which are available in the market. Four of the products claim to contained *Lactobacillus* spp., but none were recovered. There were five samples with revealed bacterial numbers were much lower than the numbers printed on product labels. In 2002, FAO/WHO gave a definition of probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host". In this document, it is recommended that minimum viable numbers of each probiotic strain at the end of the shelf-life should be described on the label. Of eight products saying to have *Lactobacillus* spp., only four did, of which the counts

Table 1. Microbial counts from probiotic products (CFU/g)

Sample	*Lactobacillus* spp.		*Bacillus* spp.	
	Label*	Counts	Label*	Counts
A	4.8 x 10^6	nd	No	-
B	2.0 x 10^7	nd	2.0 x 10^7	4.4 x 10^7
C	5.0 x 10^6	nd	5.0 x 10^6	5.0 x 10^5
D	10^6	nd	10^6	8.2 x 10^5
E	10^6	1.7 x 10^7	10^6	8.0 x 10^8
F	10^5	2.3 x 104	10^5	5.1 x 10^7
G	105	8.6 x 10^3	10^5	4.2 x 10^6
H	10^4 - 10^7	5.8 x 10^4	10^4 - 10^7	8.3 x 10^6
I	No	-	10^8	9.3 x 10^7
J	No	-	5.6 x 10^5	5.0 x 10^6

*The numbers of microbes stated on product labels; nd: No growth from 100 µL of 10-fold sample dilution; CFU: Colony forming unit.

of three were at least ten-fold as low as those on the labels. On the other hands, *Bacillus* spp. were isolated from all nine samples, of which two had their counts at least ten-fold as low as they were supposed.

So, samples of four products A, B, C and D did not have the counts of all strains as it printed on the labels, either not detected or much less. Samples of products E, F, G had less numbers of *Lactobacillus* than the label stated, and sample of product H did not have yeast as it is mentioned in the label. In summary, eight out ten products did not have the target microbes or/and the numbers of at least one strain were lower than it described on the label.

The facts that *Lactobacillus* spp. were not recovered from samples of four products, while *Bacillus* spp. were isolated from all would indicate difficulties producing and maintaining the survival of the lactic-acid-bacteria species. This is probably due to the anaerobic property of *Lactobacillus* spp.. Although a majority are aerotolerant, optimal growth and survival require anaerobic conditions. In contract, *Bacillus* spp. are aerobes and spore-formers. Therefore, they can grow best in the air and survive harsh environments. Aerobic metabolism and spore forming are among main advantageous traits of *Bacillus* strains for their use as probiotics for farm animals and aquatic animals, when cost of production (that determines the price of the product) and the storage conditions, the way of application are important factors. There were four products that did not meet the statement (on the labels) about the presence and quantities of all beneficial strains. This is a worldwide problem about probiotic market. As reviewed by De Simone (2019), current regulation of probiotics is inadequate to protect consumers. So, the source of probiotics (manufactories and/or stores) should be considered if one want to buy a probiotic product.

3.2. Antibiotic susceptibility of bacterial isolates

3.2.1. Antibiotic susceptibility of *Lactobacillus* isolates

All seven isolates showed susceptibility to the tested antibiotics except kanamycin (Table 2). Among seven isolates of *Lactobacillus*, two were susceptible to kanamycin, one resistant and the other showed intermediate sensitivity to the antibiotic. This finding was consistent with some previous studies, which reported that lactobacilli were highly resistant to aminoglycosides (gentamycin, kanamycin, streptomycin) that act by inhibiting synthesis of protein (Gueimonde et al., 2013).

3.3. Antibiotic susceptibility of *Bacillus* isolates

The test was performed on 15 *Bacillus* isolates. The results are presented in Table 3. All were susceptible to ampicillin, kanamycin, and ciprofloxacin. Five isolates showed intermediate susceptibility to tetracycline, one to amoxicillin/clavulanic and one to vancomycin. This results agreed with previous reports in the literature. For examples, 55.4% *Bacillus* cereus isolates from food samples by Tansuphasiri et al. (2006) and 33% of *B. subtilis* isolates from a study by

Table 2. Antibiotic sensitivity of *Lactobacillus* isolates (n = 7)

Antibiotics	Disc concentration (µg)	Isolates						
		1L	6L-1	6L-2	9L-1	9L-2	10L-1	10L-2
Ampicillin	10	S	S	S	S	S	S	S
Amoxicillin + Clavulanic	30	S	S	S	S	S	S	S
Vancomycin	30	S	S	S	S	S	S	S
Erythromycin	15	S	S	S	S	S	S	S
Kanamycin	30	S	I	S	I	I	I	R
Tetracycline	30	S	S	S	S	S	S	S
Ciprofloxacin	5	S	S	S	S	S	S	S

S: Susceptible; I: Intermediate; R: Resistant.

Table 3. Antibiotic sensitivity of *Bacillus* isolates (n = 15)

Antibiotics	Disc concentration (µg)	Number of isolates					
		S		I		R	
Ampicillin	10	15	100%	0	0%	0	0%
Amoxicillin + Clavulanic	30	14	93.3%	1	6.7%	0	0%
Vancomycin	30	13	86.6%	1	6.7%	1	6.7%
Erythromycin	15	14	93.3%	0	0%	1	6.7%
Kanamycin	30	15	100%	0	0%	0	0%
Tetracycline	30	10	66.7%	5	33.3%	0	0%
Ciprofloxacin	5	15	100%	0	0%	0	0%

S: Susceptible; I: Intermediate; R: Resistant.

Le & Nguyen (2016) showed resistance to oxytetracycline.

Antibiotic resistance of probiotic bacteria can be either or both intrinsic or/and acquired. One of the main functions of probiotics in prevention or/and treatment gastrointestinal disorders in humans and animals is to maintain or/and restore the gut microflora after antibiotic treatment. Therefore, intrinsic antibiotic resistance could be useful. However, when the resistance determinants are located on mobile genetic elements or plasmids, it raises great concerns on public health. Those resistant probiotics can spread resistance genes to others in the gut microflora via horizontal gen transfer, hence creating a reservoir of resistance for potential food or gut pathogens (Sharma et al., 2014). Therefore, in recognition of the importance of assuring safety, determination of antibiotic resistance patterns of probiotic strains should be carried out (FAO/WHO, 2002). The results in this study did not find resistance to the seven common antibiotics among *Lactobacillus* isolates, and most *Bacillus* isolates were susceptible to the drugs except a number of isolates showing intermediate resistance to tetracycline. This would provide information for farm practice in choosing products containing beneficial bacteria used together with antibiotics to maintain or/and restore the gut microflora after antibiotic treatment.

4. Conclusions

This was a small-scale study, which just looked at the quantities of microbial groups announced on the label, but not yet their benefits to animal health and productivity. Nevertheless, the results would provide some evidence for concerns about quality control of products containing beneficial microorganisms used for animals.

Conflict of interest

The authors declare no conflict of interest.

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