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Stability evaluation of lyophilized probiotic bacteria

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Abstract

The unregulated use of antibiotics has emerged as a major driving force of antibiotic resistance and public health hazard, particularly in developing countries. This necessitates to find out a solution that benefits humans and animals. Probiotics are one such alternative; they are simply known as beneficial bacteria that exert health benefits to living beings. Probiotic microorganisms are crucial for the biotechnology and food industries. They are frequently employed as starters for the production of probiotic goods, food (such as yogurt, cheese and fermented meats), and food as well as for green chemistry uses. Bacteria can be conveniently preserved using lyophilization. It permits long-term storage and inexpensive distribution at suprazero temperatures while limiting viability and functionality losses by lowering water activity to values below 0.2. This study aimed to determine the efficacy of lyophilized probiotic after lyophilization. All the lyophilized isolates were found to be Gram positive and showed no catalase activity. The isolates fermented the sugars viz. D-glucose, Lactose, and Sucrose. Lyophilized bacteria showed tolerance against 2% and 4% NaCl concentrations. The bacteria also showed tolerance against 0.3% bile salt and pH 3. The isolates were able to inhibit the growth of enteric pathogens viz. *Salmonella typhi*, *Escherichia coli*, and *Vibrio cholera* and resistant to commercially available antibiotics viz. gentamicin, and tetracycline. It is possible to conclude that lyophilization does not affects the efficacy of probiotic bacteria. The study's findings could open up new avenues for the application of probiotic-based feeds and foods as an alternative to antibiotic among stakeholders.

Keywords: Lyophilization; Probiotics; Biochemical; Feed; Antibiotic

1. Introduction

Probiotics are characterized as "living organisms when ingestion in specific amounts that exerts certain health benefits" [1]. The gastrointestinal tract (GIT) of chicken contains a diverse and complex microbiota (fungi, bacteria, archaea, protozoa, and virus) that plays key role in the development of the immune system and elimination of pathogens in digestion and absorption of nutrients. Interactions between host and chicken GIT bacterial microbiome have been extensively studied and reviewed by many researchers that considered to play an important role in intestinal development. Chicken GI tract components includes the crop, gizzard, duodenum, jejunum, ileum, caeca, large intestine and each section has different metabolic roles that shape the microbial community. Therefore, a well understanding of chicken intestinal function and microbes will provide new opportunities to improve poultry health through probiotic feed consumption [2].

From the very beginning of 21st century poultry industry has become popular and profitable business to meet the huge protein demand of our country. Though the probiotic poultry feed has a large market on the developed world, but it is

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still in mint condition in Bangladesh. Nowadays, antibiotics-based growth promoter for poultry production and management are a random practice. As a result, antibiotic resistance is transmitted from the food supply chain of poultry to human being. Due to the fatalistic impacts of health and environment [3], antibiotic as a growth promoter is banned or regulated in various jurisdiction. In this changed context, probiotic feed can be alternative ways to the antibiotics feed with more beneficial roles. Currently, a huge variety of probiotic feed are available on the market and generally, they are consumed to target gastrointestinal discomfort and pain as well as to improve the properties of the poultry immune system [4]. As a result, in recent years, various alternatives to growth-promoting antimicrobials have been investigated [5]. These techniques have centered on preventing harmful bacteria from multiplying and manipulating beneficial gut microflora to promote health, immunity, and performance [6]. AGP has been replaced with a variety of feed additives in chicken, with variable degrees of success. Probiotics have become a promising antibiotic alternative additive in chickens due to their impact on performance and security when compared to antibiotics. It also has a direct bearing on achieving the highest performance criteria [7]**.** However, the commercial use of probiotics in poultry industry is relatively new. The most common method of administration of probiotics in chickens varies depending on the manufacturer, however it is commonly done via injection in eggs, litter, suspension, and orally. In traditional water-based oral applications, the probiotic may lose viability when it comes into contact with water disinfectants (chlorine) or interacts with other compounds in the water [8]. Even more, they have lower stability and shelf-life issues. The most efficient way of adding probiotics to feed is using dry cell which can be produced by lyophilization process. Lyophilization, a low temperature dehydration process, is used to get freeze dried form of probiotic bacteria that will eventually increase stability, viability and the most importantly shelf-life. The variable rates of dead, alive and injured cells present in the lyophilized powder, depend on the sensitivity of the cells to the environmental stresses induced by the stabilization process. The low cooling rates commonly applied in lyophilized formulations cause compaction of cells in the solidified material and dehydration of the cells [9]. Bacterial cells may experience permanent alterations as a result of dehydration, including modifications to the structure and physical state of sensitive proteins and membrane lipids, which frequently cause a significant decrease in bacterial viability [10,11]. There are still some uncertainties regarding its efficacy as a result. The purpose of the current investigation was to ascertain whether probiotic microorganisms that have been lyophilized are still effective.

2. Material and methods

2.1. Preparation of Lyophilized Probiotics

The isolation of probiotic strains from chicken gut was the first step in the production of lyophilized probiotics. To identify the isolated native probiotic bacteria, the 16S rRNA gene was sequenced.

Table 1 Probiotic isolates used in the experiment

Isolate No.	BLAST Match Sequence			Identified Strain
	NCBI Ref. Sequence	Query length (bp)	Similarity	
01	NR_104919.1	1416	99.44%	*Bacillus tequilensis* strain 10b
02	NR_157736.1	1428	97.19%	*Bacillus tropicus* strain MCCC 1A01406
03	NR_028725.2	1502	98.10%	*Lactobacillus salivarius* strain HO 66
04	NR_036903.1	1425	99.65%	*Staphylococcus gallinarum* strain VIII1

Each bacterial solution was combined with the protective medium in a 2:1 ratio before lyophilization. The protective medium was made up of an equal proportion of 70% glycerol and 5 g/L ascorbic acid as antioxidant solution. 60ml bacterial solution and 30ml protective medium were mixed in a 300ml lyophilization tube and pre-frozen at -20^{o}C in a deep freezer in such a way that there was enough space in the tube. The cooling temperature was set to -40^{o}C after the lyophilizer was started. When the vacuum pressure dropped to 20 mmtor or less, the vacuum valves were closed and the vacuum was turned off. The flasks were then disconnected, and lyophilized samples were taken out. Then, subculture was applied to each probiotic culture that had been lyophilized.

2.2. Gram Staining

On a fresh, dry slide, a single colony was spread out and heated. After being submerged in a crystal violet solution for 30 seconds, the heat-fixed smear was washed with water for 5 seconds. The slide was immersed in the gram's iodine solution for a minute before being washed with tap water for five seconds. Safranin was then applied for 60 to 80

seconds as a counterstaining agent before being washed with water. Then, using a light microscope, the bacteria were evaluated.

2.3. Catalase Test

The catalase test was started with a single isolate on a sterile transparent-looking glass slide. At each of the two opposing ends of the glass slide, a drop of water and a drop of bacterial culture broth were added. On both ends, another drop of H_2O_2 was dropped. The bacterial culture containing H_2O_2 which generate bubbles are catalase-positive.

2.4. Coagulase Test

A test tube containing 9.8 ml of fresh cow milk was inoculated with 200 µl of a pure single isolate at a time. Then the clotting of the solid from liquid was observed every two-hour interval.

2.5. Antimicrobial Activity Test

The zone of inhibition was tested to determine whether or not the putative probiotic isolates have antibacterial activity by co-culturing each isolate with a specific pathogen at a time. Each Probiotic isolate was tested individually for its antibacterial properties using a total of five distinct pathogens. For this same reason, stick plate co-culture was preferred.

2.6. Resistance to Low pH

The pH of the chicken stomach is thought to range between 1.5 to 3.5 [12]. Therefore, resistance to pH 3 is frequently utilized in in vitro tests to assess stomach pH resistance. The MRS broth medium was created for this purpose at a pH of 3 using 5N HCl. The 15 ml broth medium was then inoculated with 20 µl of overnight-grown bacterial cultures. After that, the test tubes were incubated at 37^0C, and absorbance measurements at 620 nm were made every four hours to assess the bacteria's capacity to survive at low pH levels.

2.7. Bile Salt Tolerance

It is believed that the intestinal bile concentration of chicken varies from 0.01% to 0.7% [13]. This experiment was carried out at 0.3% concentration. Using 20 µl overnight-grown cultures, 15 ml of sterilized MRS broth medium containing 0.3% bile was injected. Following this, the tubes were incubated at 37^0C for 24 hours while the optical density was monitored at 620 nm every four hours.

2.8. NaCl Tolerance Test

Test tubes containing MRS broth were adjusted with different concentrations (1-10%) of NaCl. After sterilization, each test tube was inoculated with 1% fresh overnight isolated culture and incubated at 37º C for 24 hr. After 24 hr. of incubation, their growth was determined by observing their turbidity.

2.9. Sugar Fermentation

The screw-capped test tubes were filled with MRS broths (pH 6.5), and phenol red (0.01 gm/l) was added as a pH indicator. The medium underwent a 15-minute autoclave at 121° C. Following autoclaving, 1 ml of various sugar solutions (5%) were injected into various tubes. After that, 200 µl of liquid overnight cultures were added to the broth. To measure the gas generation, a Durham tube was positioned inverted to each test tube.

3. Results

3.1. Biochemical Test

The bacteria were examined using a compound microscope after being lyophilized. The bacteria were clearly gram-positive because they retained a violet-blue color following staining. In addition, the presence of the enzyme catalase, which converts hydrogen peroxide into oxygen and water, is indicated by the appearance of gas bubbles. When hydrogen peroxide was introduced, the isolated bacteria did not produce any bubbles. It means that all isolates tested negative for catalase. Besides, excellent coagulase activity was demonstrated by all of the isolated bacteria, demonstrating their resemblance to common lactic acid bacteria such *Lactobacillus acidophilus*. The result of biochemical test has been shown in Table 2.

Table 2 Biochemical tests of the lyophilized bacteria from chicken GIT samples

Isolate No.	Gram Staining	Catalase Test	Coagulase Test
01	+	-	+
02	+	-	+
03	+	-	+
04	+	-	+

Legends: (+) means reaction positive; (-) means reaction negative

3.2. Antimicrobial Activity Test

The chosen strains were tested for their ability to combat microbes. The indicator microorganisms were used to identify strains for this purpose, and the diameter of the inhibition zones revealed that the majority of isolates had an antibacterial impact on the indicator microorganisms. The result of antibiotic sensitivity test has been shown in Table 3.

Table 3 Antibiotic sensitivity tests of the lyophilized bacteria from chicken GIT samples

Isolate No.	Name of pathogens				
	V. cholera	***Micrococcus***	***S. paratyphi***	***S.typhi***	***E. coli***
01	+	+	+	+	+
02	+	+	+	+	+
03	+	-	+	-	+
04	+	+	+	+	+

Legends: (+) means pathogens are sensitive to the probiotic's antimicrobial activity, (-) means pathogens are resistant to the probiotic's antimicrobial activity

3.3. pH Tolerance Test

One distinguishing trait of probiotic bacteria is their capacity to flourish in environments with lower pH levels. In the current study, the ability of each isolate to survive at various pH levels was assessed by testing it under various pH conditions. All the isolates survived well in pH 4 and 8 and showed tolerance against pH 3 and 9. The result of pH tolerance test has been shown in Table 4.

Table 4 PH tolerance tests of lyophilized bacteria from chicken GIT samples

Isolate No.	0 hr.				4 hr.				12 hr.			
	pH 3	pH 4	pH 8	pH 9	pH 3	pH 4	pH 8	pH 9	pH 3	pH 4	pH 8	pH 9
01	+	++	++	+	+	++	++	+	+	++	++	+
02	+	++	++	+	+	++	++	+	+	++	++	+
03	+	++	++	+	+	++	++	+	+	++	++	+
04	+	++	++	+	+	++	++	+	+	++	++	+

Legends: (++) means highly tolerance; (+) means tolerance.

3.4. Bile Salt Tolerance

The capacity of bacteria to survive in the colon was tested using a bile salt tolerance test. In four different bile salt concentrations, isolates were tested. The percentages were 0.1%, 0.2%, 0.3%, and 0.4%. By measuring the turbidity of the culture broth at different times at a wavelength of 620 nm, the growth of the isolates was detected. Table 5 displays the average value from four replications. The isolates in 0.3% bile salt were found to grow at their fastest rate after 24 hours of incubation at 37°C.

Table 5 Bile salt tolerance tests of lyophilized bacteria from chicken GIT samples

Isolate No.	0 hr.				4 hr.				12 hr.				24 hr.			
	0.1 %	0.2 %	0.3 %	0.4 %	0.1 %	0.2 %	0.3 %	0.4 %	0.1 %	0.2 %	0.3 %	0.4 %	0.1 %	0.2 %	0.3 %	0.4 %
01	+	+	++	-	+	+	++	-	+	+	++	-	+	+	++	-
02	+	+	++	-	+	+	++	-	+	+	++	-	+	+	++	-
03	+	+	++	-	+	+	++	-	+	+	++	-	+	+	++	-
04	+	+	++	-	+	+	++	-	+	+	++	-	+	+	++	-

Legends: (++) means highly tolerance; (+) means tolerance; (-) means sensitive

3.5. NaCl Tolerance Test

Isolates were tested for their tolerance against different concentrations of NaCl. Four concentrations of NaCl and one control were used to determine the NaCl tolerance of isolated bacteria. All the isolates showed tolerance against 2% and 4% NaCl concentration after 24 hours of time. Their growth in different NaCl concentrations was determined by measuring their optical density at 620 nm wavelength. The growth of isolates reduced drastically in 6% NaCl concentration. Almost no growth was observed in 8% NaCl concentration while the growth was highest for all the isolates in control group with no NaCl. The result of NaCl tolerance test has been shown in Table 6.

Table 6 NaCl tolerance tests of lyophilized bacteria from chicken GIT samples

Isolate No.	0 hr.				4 hr.				12 hr.				24 hr.			
	2%	4%	6%	8%	2%	4%	6%	8%	2%	4%	6%	8%	2%	4%	6%	8%
01	++	++	+	-	++	++	+	-	++	++	+	-	++	++	+	-
02	++	++	+	-	++	++	+	-	++	++	+	-	++	++	+	-
03	++	++	+	-	++	++	+	-	++	++	+	-	++	++	+	-
04	++	++	+	-	++	++	+	-	++	++	+	-	++	++	+	-

Legends: (++) means highly tolerance; (+) means tolerance; (-) means sensitive

3.6. Sugar Fermentation

Lactic acid bacteria work to produce lactic acid as a byproduct of the fermentation of carbohydrates. The transformation of broth from purple to yellow indicates the presence of sugar fermentation. Five types of sugar were employed in this investigation. The isolates among them fermented 3 of the sugars. The result of test has been shown in Table 7.

Table 7 Sugar fermentation tests of lyophilized bacteria from chicken GIT samples

Isolate No.	Name of Sugar				
	Glucose	Sucrose	Mannitol	Lactose	D-sorbitol
01	+	+	-	+	-
02	+	+	-	+	-
03	+	-	-	-	-
04	+	+	-	+	-

Legends: (+) means reaction positive; (-) means reaction negative

3.7. Antibiotic Sensitivity Test

The disc diffusion method was used to test each isolate's susceptibility to five commercially available antibiotics viz. Ampicillin, Erythromycin, Gentamicin, Streptomycin and Tetracycline. Table 8 displays the results of the antibiotic sensitivity test.

Table 8 Antibiotic sensitivity tests of lyophilized bacteria from chicken GIT samples

Isolate No.	Ampicillin	Erythromycin	Gentamicin	Streptomycin	Tetracycline
01	S	MS	R	MS	R
02	S	MS	R	MS	R
03	S	MS	R	MS	R
04	S	MS	R	MS	R

Legends: S means susceptibility; MS means moderate susceptibility; R means resistance

4. Discussion

The samples were lyophilized and then pure cultures were prepared following several subculture techniques. Several biochemical tests were used to identify and characterize the isolates, including tests for bile and stomach acid resistance, potent antibacterial activity, and antibiotic susceptibility. All of the isolates were identified as rod-shaped, coagulase-positive, and lacking in catalase activity. The lyophilized isolates fermented the sugars viz. D-glucose, Lactose, Sucrose. However, the isolates did not ferment D-sorbitol or D-mannitol. After 24 hours of incubation, lyophilized bacteria displayed tolerance against 2% and 4% NaCl concentrations and significantly decreased at 6% NaCl concentrations. Additionally, the lyophilized probiotics demonstrated tolerance to 0.3% bile salt, pH 3, and a temperature of 37°C. They were able to stop the growth of intestinal pathogens such *Salmonella paratyphi*, *Vibrio cholera*, and *Escherichia coli*. However, *Salmonella typhi* and *Micrococcus* did not be inhibited by *Lactobacillus salivarius* strain HO 66. Mandal et al. [14] did in vitro characterization of chicken gut bacterial isolates for probiotic potentials on the basis some biochemical tests such as gram-positive, catalase-negative, nonmotile, sugar fermentation pattern, and resistance to inhibitory substances such as pH 2.2, 0.3% bile acid, 0.1∼0.4% phenol, and 1∼10% NaCl. The findings of the current investigation are comparable to those of Mandal et al. [14]. The results of Musikasang et al. [15], who isolated probiotic bacteria from the chicken gastrointestinal system and discovered the same results, were likewise identical to the lyophilized probiotic bacteria. The outcomes were the same as those found by, who found that lyophilized probiotic cells improve viability without degrading any potential feature. The results were consistent with findings those of Rodrigues et al. [16], which indicated that lyophilized probiotic cells increase survivability without compromising any potential feature.

5. Conclusion

The present investigation showed that probiotics that had been lyophilized maintained all of their functional features without suffering any appreciable losses. This study will pave the way for the successful manufacturing of probiotic-assisted foods and feeds since lyophilization extends storage time and preserves viability without causing any biophysical or physiochemical harm to the bacterial cells.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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