

**MOLECULAR IDENTIFICATION OF PROBIOTIC BACTERIA
ISOLATED FROM POULTRY GASTROINTESTINAL TRACT**

**A Thesis
Submitted to
Khulna University, Khulna**



**In Partial Fulfillment of the Requirements for the Degree of
Master of Science
in
Biotechnology & Genetic Engineering
By**

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**Biotechnology and Genetic Engineering Discipline
Khulna University
Khulna-9208**

March, 2020

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Approved as to the style and content by

KM Hossain 24/09/2020

Dr. Khondoker Moazzem Hossain
Supervisor
Professor
Biotechnology and Genetic Engineering
Discipline
Khulna University
Khulna-9208

S. M. Mahbubur Rahman 21.09.2020

Dr. S. M. Mahbubur Rahman
Co- Supervisor
Professor
Biotechnology and Genetic Engineering
Discipline
Khulna University
Khulna-9208

Aslam 21.09.2020

Chairman, Examination Committee
and
Head, Biotechnology and Genetic Engineering Discipline
Khulna University
Khulna-9208



March, 2020

Declaration

This thesis is the original research work of the author, except as otherwise stated. The materials presented have not submitted either in whole or in part of a degree for this or any other university. The findings of the research have not been/will not be published anywhere without the concurrence of the concerned supervisor.

Farhana Hossain

The Author

21.09.2020

Date

DEDICATION

Dedicated
to
My Beloved Parents

Acknowledgment

I feel blessed by Allah for permitting me to complete the thesis successfully. I would love to articulate my earnest gratefulness to my supervisor, Professor Dr. Khondoker Moazzem Hossain, Biotechnology and Genetic Engineering Discipline, Life Science School, Khulna University for his right direction, valuable recommendations and his constant support throughout the process of this research and thesis work.

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ABSTRACT

Probiotics play important role in preventing or mitigating the effects of dysbacteriosis and, specifically, necrotic enteritis in poultry. Recently, the potential usage of probiotics in poultry industry has been increased globally. In Bangladesh, there is no record so far regarding the use of native probiotics in the poultry industry. Therefore, the characterization of beneficial probiotic strains is imperative. The gastrointestinal tract (GI) of poultry can be an excellent source of probiotic as it has a diverse and complex probiotic microbiota that plays a vital role in digestion and absorption of nutrients. The objectives of this study was to identify native probiotic bacteria isolated from the gastrointestinal tract of poultry samples of different regions of the country using 16S rRNA gene sequencing. Thirty poultry isolates were subjected to DNA extraction followed by pure colony isolation and later, PCR of the DNA samples and subsequently 16s rRNA gene sequencing were performed. The 16S rRNA gene sequences were aligned against other bacterial species by BLAST search Using MEGA-X software for the construction of phylogenetic tree. Phylogenetic relationship among the samples was analyzed on the basis of sequenced data and dendrogram was constructed using UPGMA. Four *Bacillus cereus* strains, two *Bacillus subtilis* strains, and three closely related to *Bacillus* spp. strains were identified. Further investigation regarding the development of probiotic based feed product for poultry using native isolates from GI tract of poultry would be worthy to investigate.

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Introduction

The gut is a principal as well as potential source of probiotic bacteria in birds and animals. The microflora in the gastrointestinal tract of broiler chickens influences digestion, health, and well-being. There is a balance of beneficial and pathogenic bacteria in the gut of healthy poultry species. If the balance is at its point then the poultry can gain its maximum developmental efficacy, and if it is not then the poultry can even results in illness. A study shows that in broiler industry, a very common practice is the feedstuff supplement along with the antibiotic to improve the growth performance, but this practice has been retained under inquiry due to the occurrence of small levels of antibiotics in broiler meat and the spread of antibiotic-resistant bacteria in meat. Therefore, if we can provide something in substitute of antibiotic that can keep the balance of gut flora of poultry and it would be a far better solution for current situation (Riaz et al., 2018).

The poultry industry has become an important economic activity in many countries. In large-scale rearing facilities, where poultry are exposed to stressful conditions, problems related to diseases and deterioration of environmental conditions often occur and result in serious economic losses. As most of the farmers use antimicrobial agents for prevention and control of poultry diseases, they are causing the evolution of antimicrobial resistance among pathogenic bacteria which is itself a great threat to both for animal and human (Kabir, 2009).

The gastrointestinal tract is the major digestive and absorbing organ and it plays a crucial role in poultry growth. A diverse microbiota is found throughout the tract and in case of chicken, it is most extensive in the cecum. This microflora has a role in nutrition, detoxification of certain compounds, growth performance, and protection against pathogenic bacteria. The gut microflora influences health and well-being of host animals. In poultry, the absence of normal microflora in the cecum has been considered a major factor in the susceptibility of chicks to bacterial infection. Many studies have shown that the alimentary tract of newly hatched chick is usually sterile but during the posthatch period, microflora has changed (Romach et al., 2004).

The accretive demand of a healthy lifestyle has drawn our attention to the use of probiotics in a variety of dairy and other fermented products (Fujimoto et al., 2008). This is because of the increasing importance of using probiotics in treating several diseases over the past century.

Ongoing research finds numerous health benefits which include development of the immune system, gastrointestinal tract, lactose intolerance, etc. Recent study has made it possible better understanding of probiotic. Therefore, the wide varieties of using probiotics in both industrial sector and human health for beneficial purposes are rising at the peak point (González et al., 2011). The significant role of the intestinal microflora for resistance to disease has been shown before by some researchers. In one study, oral administration of antibiotics to mice rendered the animals more susceptible to infection with *Salmonella typhimurium*, *Shigella flexneri*, and *Vibrio cholerae*. It was observed that, $\leq 1 \times 10^1$ *Salmonellae enteritidis* were sufficient to kill germ-free guinea pigs, whereas 1×10^9 bacteria were required to kill animals with complete intestinal microflora. This study clearly indicates the importance of the presence of probiotic bacteria in the animal gut (Schrezenmeir and Vrese, 2001).

Probiotics are defined as the living microbial food supplement that positively affects the host by improving the gut microbial balance. It is believed by most investigators that there is an unsteady balance of beneficial and non-beneficial bacteria in the tract of normal, healthy, non-stressed poultry. The use of probiotics as an alternative to antibiotic has been rapidly increasing in recent years. The term probiotic, meaning “for life,” is derived from the Greek language first used by Lilly and Stillwell in 1965 to describe “substances secreted by one microorganism which stimulates the growth of another” (Fuller, 1989).

Microbes that are commonly used as probiotics include various species of the genera *Lactobacillus*, *Bifidobacterium*, and *Enterococcus*. Although the microbial communities are distributed throughout the GIT, their composition was found heterogeneous along the different regions of GIT in chicken. These variations in microbial composition can be different in both along different segments and at different locations in the same region (Moreira et al., 2005; Yeoman et al., 2012).

Diverse microbial groups can be located in different regions of the GIT and this might indicate their different role to maintain host health. In the present study, the bacterial communities were characterized across the different regions and locations of the GIT of chickens and duck with a focus on the genus *Lactobacillus*, *Bifidobacterium*, and *Bacillus spp.* which have been frequently considered for probiotics in many studies. However, the species isolated from intestinal tract currently being used in probiotic preparations are different. These are mostly *Lactobacillus*

acidophilus, *Lactobacillus casei*, *Lactobacillus helveticus*, *Lactobacillus lactis*, *Lactobacillus salivarius*, *Lactobacillus plantarum*, *Enterococcus faecium*, *Enterococcus faecalis*, *Bacillus spp.*, *Bifidobacterium spp.* and *Escherichia coli* (Kabir, 2009; Adhikari and Kwon, 2017).

The microorganisms included as probiotics are usually assumed to be non-pathogenic components of the normal microflora, such as the lactic acid bacteria. However, there is good evidence that non-pathogenic variants of pathogenic species can operate in much the same way as traditional probiotics. For example, avirulent mutants of *Escherichia coli*, *Clostridium difficile*, and *Salmonella Typhimurium* can also protect against infection by the respective virulent parent strain. In poultry, the early use of probiotics was instituted by Nurmi & Rantala (1973). In their experiments, the authors observed that the intestinal contents of normal adult birds, orally administered to chicks with one day of age, altered their sensitivity to infection by *Salmonella spp.* From there, several studies have been made and continue being developed with the use of probiotics. Inconsistent results from the use of probiotics in animal production have been a constraint for the promotion of their use. Variations in the efficacy of probiotics can be due to the difference in microbial species or micro-organism strains used, or with the additive preparation methods (Otutumi et al., 2012).

In Bangladesh, the use of probiotics in poultry is gradually being increased. Surprisingly, there is no local probiotic for the huge poultry industry in Bangladesh. The probiotic market in Bangladesh is completely dependent on the importation of probiotic materials and thus, every year the country counts a handsome amount of money for importation of such materials. The reasons of the dependency to the imported probiotics may be that there are no probiotic bacteria isolated yet in Bangladesh even though the existence of potential sources (Kabir et al., 2016).

Researchers claimed that probiotic bacteria (*Lactobacillus*) are diverse in nature, that they can survive in extreme acidic or moderate alkaline condition and also higher salt concentration and lower phenol concentration (Mannan et al., 2017). This diverse nature is the fundamental of the evolution of life. However, to find out the genetic diversity lots of methods is now available like Randomly Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Restriction Analysis of Chromosomal DNA (REAC), Pulsed Field Gel Electrophoresis (PAGE), Variable Number of

Tandem Repeats (VNTR). Among these methods, RAPD is the most prominent (Sowmya et al., 2016).

The purpose of present study was to investigate the genetic variability among the probiotic bacterial species, isolated from selective regional (Dhaka, Comilla, Khulna, Barishal, Sylhet, Chittagong and Rajshahi Divisions) poultry samples of Bangladesh. In this study, suitable strategies were used for the isolation, identification and characterization of potential probiotic strains from poultry sources, in order to evaluate their suitability for commercial use in poultry industry.

Literature reveals so far no such study has been conducted yet. Therefore the present research work was undertaken with the following objectives:

1. To determine the native probiotic isolates from poultry gastrointestinal tract.
2. Molecular identification of the probiotic isolates using 16s rRNA sequencing.

Literature Review



2.1 What Is a Probiotic?

In these years "Probiotic" word has been utilized in many ways. At first it was used to describe substances secreted by one protozoan which is instead stimulated by another, by Lilly and Stillwell in 1965 (Lilly and Stillwell, 1965) but later it was used to describe animal feed supplements which had a beneficial effect on the host animal by affecting its gut flora (Kabir, 2009).

The term "probiotic" means "for life" (Pro-for and biotic-life), derived from Greek language. In 1971 Sperti applied the term to tissue extracts that stimulate microbial growth (Sperti, 1971). Parker was the first man to use this term in the sense that it is used today by defining it as "organisms and substances which contribute to intestinal microbial balance" (Parker, 1974). Later Fuller also gave a definition in opt of improving Parker's definition as- "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller, 1989). In 1992 Havenaar *et al.*, broadened the definition of probiotics with respect to host and habitat of the microflora as follows: "A viable mono or mixed culture of microorganisms which applied to animal or man, beneficially affects the host by improving the properties of the indigenous microflora" (Havenaar and Huis, 1992).

Salminen and Schaafsma broadened the definition of probiotics even further by no longer limiting the proposed health effects to influences on the indigenous microflora. According to Salminen, a probiotic is "a live microbial culture or cultured dairy product which beneficially influences the health and nutrition of the host." According to Schaafsma, Oral probiotics are living microorganisms which upon ingestion in certain numbers, exert health effects beyond inherent basic nutrition (Salminen, 1996; Schaafsma, 1996).

There is a long history of health claims concerning living microorganisms in food, particularly lactic acid bacteria. In a Persian version of the Old Testament (Genesis 18:8) it states that "Abraham owed his longevity to the consumption of sour milk." In 76 BC the Roman historian Plinius recommended the administration of fermented milk products for treating gastroenteritis.

Since the advent of the microbiology era, some investigators (e.g. Carre, Tissier, and Metchnikoff) attributed such health effects to shifts of the intestinal microbial balance. Metchnikoff claimed that the intake of yogurt containing lactobacilli results in a reduction of toxin-producing bacteria in the gut and that this increases the longevity of the host. Tissier recommended the administration of *bifidobacteria* to infants suffering from diarrhea, claiming that *bifidobacteria* supersede the putrefactive bacteria that cause the disease. He showed that *bifidobacteria* were predominant in the gut flora of breast-fed infants. The significant role of the intestinal microflora for resistance to disease was shown by Bohnhoff *et al.*, Freter, and Collins and Carter (Schrezenmeir and Vrese, 2001).

2.2 Health Effects of Probiotics

The health effects attributed to the use of probiotics are numerous. The following outcomes are well documented:

- 1) Lower frequency and duration of diarrhea associated with antibiotics (*Clostridium difficile*), rotavirus infection, chemotherapy, and, to a lesser extent, traveler's diarrhea;
- 2) Stimulation of humoral and cellular immunity; and
- 3) Decrease in unfavorable metabolites, e.g. amonium and procancerogenic enzymes in the colon.

There is some evidence of health effects through the use of probiotics for the following:

- Reduction of *Helicobacter pylori* infection
- Reduction of allergic symptoms
- Relief from constipation
- Relief from irritable bowel syndrome
- Beneficial effects on mineral metabolism, particularly bone density and stability
- Cancer prevention and
- Reduction of cholesterol and triacylglycerol plasma concentrations (weak evidence).

These numerous effects can hardly be explained by a unifying hypothesis that is based on a single quality or mechanism and remains valid for all microorganisms exerting one or the other effect mentioned above (Schrezenmeir and Vrese, 2001).

2.3 Microorganisms in probiotics

Some selective microbial species and their strains are required for getting probiotic mediated food or products. Commonly used microbes for probiotic products are *Lactobacillus*, *Bifidobacterium*, *Saccharomyces* etc. (Simon, 2005). A FAO/WHO joint group report on “Guidelines for the Evaluation of Probiotics in Food”, through some requirements which must be assessed to be considered as probiotics. To identify and evaluate probiotics as health concerned, those requirements have to be fulfilled. According to the findings of the report, Probiotics should be identified as the genus, species and strain level, using accurate molecular and physiological techniques (FAO/WHO, 2002).

2.3.1 *Lactobacilli* species

Lactobacilli have long been the most prominent probiotic microorganisms because of their association with popular fermented dairy products. *Lactobacilli* are Gram- positive rods and part of the large group of lactic acid producing bacteria. Fermentation of carbohydrates by *Lactobacilli* produces lactic acid, so it survives well in acidic environments like stomach. They are rarely pathogenic (Ammor *et al.*, 2007). *L. rhamnosus* is the most studied *lactobacilli* probiotic. It was first isolated in 1987 by Gorbach and Goldin from the feces of a healthy human. This strain is stable in bile and acid and adheres to intestinal cells *in vitro*. It survives passage through the stomach and intestinal tract.

Another useful property of this strain is the modulation of specific enzymes, for example feeding *L. rhamnosus* to healthy volunteers for four weeks decreased faecal β - glucuronidase specific activity (Goldin *et al.*, 1992). The strains of *L. reuteri* are widespread in nature and can be isolated from a variety of food products, animals and the human gastrointestinal tract (Lionetti *et al.*, 2006). It appears to survive passage through the human digestive tract and persists for at least a week after stopping ingestion.

Table 2.1: Important probiotic microorganisms including their genus, species

Microorganisms	Genus	Species
Bacteria	Lactobacillus	<i>L. acidophilus</i>
		<i>L. delbrueckii</i>
		<i>L. casei</i>
		<i>L. casei</i>
		<i>L. plantarum</i>
		<i>L. rhamnosus</i>
		<i>L. reuteri</i>
		<i>L. paracasei</i>
		<i>L. fermentum</i>
		<i>L. helveticus</i>
Bacteria	Bifidobacterium	<i>B. lactis</i>
		<i>B. animalis</i>
		<i>B. bifidum</i>
		<i>B. longum</i>
		<i>B. infantis</i>
		<i>B. adolescentis</i>
	Streptococcus	<i>S. lactis</i>
		<i>S. thermophilus</i>
		<i>S. cremoris</i>
		<i>S. salivarius</i>
	Bacillus	<i>B. coagulans</i>
		<i>B. subtilis</i>
		<i>B. clausii</i>
		<i>B. licheniformis</i>
	<i>Pediococcus</i>	<i>p. acidilactisi</i>
	<i>Leuconostoc</i>	<i>L. mesenteroides</i>
	Enterobacter	<i>E. faecium</i>
		<i>E. faecalis</i>
Fungi	Aspergillus	<i>A. niger</i>
		<i>A. oryzae</i>
Yeast	Saccharomyces	<i>S. boulardii</i>
		<i>S. cerevisiae</i>
		<i>S. carlsbergensis</i>

(Source: Muller *et al.*, 2009, Krishnakumar and Gordon, 2001, Playne *et al.*, 2003, Holm, 2003)

L. acidophilus is widespread in commercially available probiotic products. It is found in fermented dairy products and is part of normal intestinal and vaginal microflora. However, properties of adherence and stability in the gastrointestinal tract are strain specific. Several *L. acidophilus* strains have been shown to produce antimicrobial substances *in vitro*, but production *in vivo* at levels high enough for a direct inhibition of pathogen growth has not been demonstrated (Sanders and Klaenhammer, 2001). *L. casei* is a gram- positive, rod shaped, non-sporing, non-motile, anaerobic bacteria which lacks cytochromes. The lactic acid produced by it is used to make cheese and yoghurt, reduce cholesterol level, enhance immune response, control diarrhea, alleviate lactose intolerance and inhibit intestinal pathogens (Mishra and Prasad, 2005). Similar to *L. acidophilus*, the probiotic properties of *L. casei* are strain specific. *L. casei* strain Shirota has received much commercial attention. Its effectiveness against *Escherichia coli* was shown in mouse models for treatment of urinary tract infections (Asahara *et al.*, 2001), reduction of influenza virus titers in aged mice (Hori *et al.* 2002), against *Listeria monocytogenes* infections in rats (Waard *et al.*, 2002) and reduction of ulcer causing *Helicobacter pylori* in humans (Sgouras *et al.*, 2004).

2.3.2 *Bifidobacteria* species

Bifidobacteria are gram-positive, strict anaerobic, lactic and acetic acid producing bacteria, present in normal flora and are the major component in the intestinal flora of breast-fed infants. They are used in different conditions such as diarrhoea, immune stimulation, as anti-mutagens and anti-cholesterol agents. It also plays an active role in the deconjugation of bile acid, catabolism of dietary carbohydrates and vitamin synthesis. *In vivo*, they are used to restore the immune defense in children (Mombelli and Gismondo, 2000).

2.3.3 *Enterococcus faecium*

A well-studied strain of *Enterococcus* is *E. faecium* SF68, which is marketed as Bioflorin. This microbe is found in healthy adults, capable of producing lactic acid, survives in an environment of low pH, resists antibiotics and inhibits pathogens (Surawicz, 2003). Other strains of *E.*

faecium are pathogenic and resistant to most antibiotics. Although strain SF68 is non-toxic, there has been concern that the probiotic might pick up and share antibiotic resistance genes in the human gut (Lund and Edlund, 2001). Cats and dogs which were fed with *E. faecium* SF68, showed fewer episodes of diarrhoea when compared with controls. This indicates that the probiotic has beneficial effects on the gastro-intestinal tract (Bybee *et al.*, 2011).

2.3.4 *Saccharomyces boulardii*

Saccharomyces boulardii is a well-studied, commercially available yeast probiotic. Unlike the *Lactobacilli*, *S. boulardii* is not usually found in the gastro-intestinal or vaginal tracts. It grows best at 37°C and survives passage into the faeces in both humans and animals. It does not strongly adhere to intestinal mucosa and is eliminated within one to three days if not readministered. Being a yeast, it is not directly affected by antibacterial antibiotics, so it can be given simultaneously during antibiotic therapy. However, it can be adversely affected by antifungal therapy if taken at the same time. The clinical activity of *S. boulardii* is especially relevant to antibiotic-associated diarrhoea and recurrent *Clostridium difficile* intestinal infections. Experimental studies clearly demonstrate that *S. boulardii* has specific probiotic properties, and recent data has opened the door for new therapeutic uses of this yeast as an 'immunobiotic' (Czerucka *et al.*, 2007).

Probiotics can be bacteria, moulds or yeast. But most probiotics are bacteria. A probiotic may be made out of a single bacterial strain or it may be a consortium as well. Probiotics can be in powder form, liquid form, gel, paste, granules or even as capsules, sachets, etc. (Suvarna and Bobby, 2005; Timmerman *et al.*, 2004). Examples of foods containing probiotics in market are yoghurt, fermented and unfermented milk, miso, tempeh, kefir, 30 aged cheese, dark chocolate, pickled vegetables, sausages, sauerkraut, some juices and soy beverages.

2.4 Poultry Gut System

The gastrointestinal tract (digestive tract, digestional tract, GI tract, GIT, gut, or alimentary canal) is an organ system within humans and other animals which takes in food, digests it to extract and absorb energy and nutrients, and expels the remaining waste as feces. The mouth, esophagus, stomach and intestines are part of the gastrointestinal tract. "Gastrointestinal" is an adjective meaning of or pertaining to the stomach and intestines. A tract is a collection of related anatomic structures or a series of connected body organs.

An understanding of the avian digestive system is essential for developing an effective and economical feeding program for our poultry flock and for recognizing when something is wrong and taking necessary actions to correct the problem.

The digestive system of any animal is important in converting the food the animal eats into the nutrients its body needs for growth, maintenance, and production (such as egg production). An animal's body breaks down food through both mechanical and chemical means. In many animals, mechanical action involves chewing; however, because birds do not have teeth, their bodies use other mechanical action. Chemical action includes the release of digestive enzymes and fluids from various parts of the digestive system. After being released from food during digestion, nutrients are absorbed and distributed throughout the animal's body.

2.4.1 Part of a Chicken Digestive tract as an Example

The chicken has a typical avian digestive system. In chickens, the digestive tract (also referred to as the gastrointestinal tract or GI tract) begins at the mouth, includes several important organs, and ends at the cloaca. Here is a short description of anatomy of chicken digestive tract, so that we understand the upcoming discussion of this thesis work.

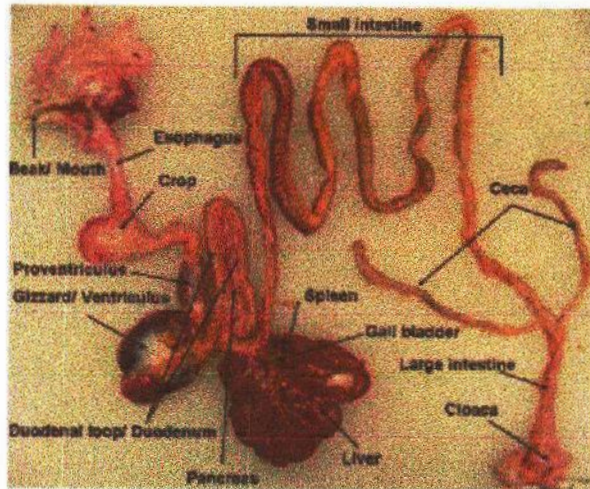


Figure 2.1: Digestive tract of a female chicken (Source: Jacquie Jacob, University of Kentucky)

2.4.1.1 Beak/Mouth

As with most birds, a chicken obtains feed by using its beak. Food picked up by the beak enters the mouth. Chickens do not have teeth, so they cannot chew their food. However, the mouth contains glands that secrete saliva, which wets the feed to make it easier to swallow. Also, the saliva contains enzymes, such as amylase, that start the digestion process. The chicken uses its tongue to push the feed to the back of the mouth to be swallowed.

2.4.1.2 Esophagus

The esophagus is a flexible tube that connects the mouth with the rest of the digestive tract. It carries food from the mouth to the crop and from the crop to the proventriculus.

2.4.1.3 Crop

The crop is an out-pocketing of the esophagus and is located just outside the body cavity in the neck region. Swallowed feed and water are stored in the crop until they are passed to the rest of the digestive tract. When the crop is empty or nearly empty, it sends hunger signals to the brain so that the chicken will eat more.



Figure 2.2: Location of the crop in a female chicken (Source: Jacquie Jacob, University of Kentucky)

Although the digestive enzymes secreted in the mouth began the digestion process, very little digestion takes place in the crop, it is simply a temporary storage pouch. The crop evolved for birds that are typically hunted by other animals but need to move to the open to find feed. These birds can consume relatively large amounts of food quickly and then move to a more secure location to digest that food.

Occasionally, the crop becomes impacted, or backed up. This problem called crop impaction, crop binding, or pendulous crop, can occur when a chicken goes a long time without feed and then eats too much too quickly when feed is available again. Crop impaction also can occur when a chicken free-ranges on a pasture of tough, fibrous vegetation or eats long pieces of string. With crop impaction, even if a chicken continues to eat, the feed cannot pass the impacted crop. The swollen crop also can block the windpipe, causing the chicken to suffocate.

2.4.1.4 Proventriculus

The esophagus continues past the crop, connecting the crop to the proventriculus. The proventriculus (also known as the true stomach) is the glandular stomach where digestion primarily begins. Hydrochloric acid and digestive enzymes, such as pepsin, are added to the feed here and begin to break it down more significantly than the enzymes secreted by the salivary glands. At this point, however, the food has not yet been ground, this organ is called the proventriculus because its location in the digestive tract is before the ventriculus, where food is ground.

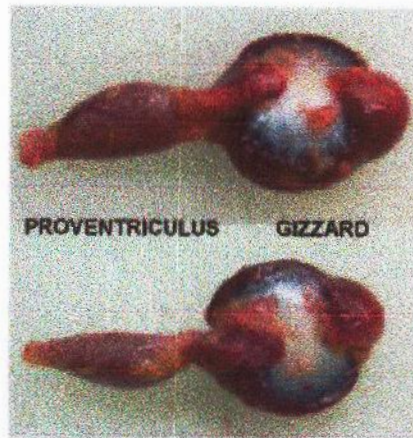


Figure 2.3: Two views of the proventriculus and gizzard from a chicken digestive tract (Source: Jacquie Jacob, University of Kentucky)

2.4.1.5 Ventriculus (Gizzard)

The ventriculus, or gizzard, is a part of the digestive tract of birds, reptiles, earthworms, and fish. Often referred to as the mechanical stomach, the gizzard is made up of two sets of strong muscles that act as the bird's teeth and has a thick lining that protects those muscles. Consumed feed and the digestive juices from the salivary glands and proventriculus pass into the gizzard for grinding, mixing, and mashing.



Figure 2.4: Inside of a chicken gizzard, with the internal lining removed. (Source: Jacquie Jacob, University of Kentucky)

When allowed to free-range, chickens typically eat small stones. The acidic environment in the proventriculus softens the stones, and then the strong muscles of the gizzard grind them into tiny

pieces. The stones remain in the gizzard until they are ground into pieces small enough to pass to the rest of the digestive tract.

2.4.1.6 Small Intestine

The small intestine is made up of the duodenum (also referred to as the duodenal loop) and the lower small intestine. The remainder of the digestion occurs in the duodenum, and the released nutrients are absorbed mainly in the lower small intestine.

The duodenum receives digestive enzymes and bicarbonate (to counter the hydrochloric acid from the proventriculus) from the pancreas and bile from the liver (via the gall bladder). The digestive juices produced by the pancreas are involved primarily in protein digestion. Bile is a detergent that is important in the digestion of lipids and the absorption of fat-soluble vitamins (A, D, E, and K).

The lower small intestine is composed of two parts:

- The jejunum and
- The ileum.

2.4.1.7 Ceca

The ceca (plural form of cecum) are two blind pouches located where the small and large intestines join. Some of the water remaining in the digested material is reabsorbed here. Another important function of the ceca is the fermentation of any remaining coarse materials. During this fermentation, the ceca produce several fatty acids as well as the eight B vitamins (thiamine, riboflavin, niacin, pantothenic acid, pyridoxine, biotin, folic acid, and vitamin B12). Because the ceca are located so close to the end of the digestive tract, however, few of the produced nutrients are absorbed and available to the chicken.

2.4.1.8 Large Intestine (Colon)

Despite the name, the large intestine is actually shorter than the small intestine. The large intestine is where the last of the water reabsorption occurs.

2.4.1.9 Cloaca

In the cloaca, the digestive wastes mix with wastes from the urinary system (urates). Chickens usually void fecal material as digestive waste with uric acid crystals on the outer surface—that is, chickens do not urinate. The color and texture of chicken fecal material can indicate the health status of the chicken's digestive tract: the white, pasty material coating chicken fecal material is uric acid, the avian form of urine, and is normal.

The reproductive tract also exits through this area. When a hen lays an egg, the vagina folds over to allow the egg to leave through the cloaca opening without coming into contact with feces or urine.

2.4.1.10 Intestinal Microflora

Both the small and large intestines normally are populated with beneficial organisms (bacteria, yeast, etc.), referred to as microflora (micro meaning "small" and flora meaning "plants"). These microflora aid in digestion.

When chicks hatch, their digestive tracts are virtually sterile. If raised by a mother hen, a chick obtains the beneficial microflora by consuming some of its mother's fecal material. In artificial incubation and brooding, chicks do not have this option. In such situations, producers can provide the chicks with probiotics, which are preparations containing the beneficial microflora that normally inhabit a chicken's digestive tract. Through the probiotics, the chicks receive the beneficial bacteria they need to fight off infection by pathogenic bacteria, such as salmonella.

Intestinal disease in chickens normally occurs when the balance of normal microflora is upset—that is, the normal microflora are overrun by too many foreign organisms. The result is enteritis, or inflammation of the intestines. Enteritis produces symptoms that include diarrhea, increased thirst, dehydration, loss of appetite, weakness, and weight loss or slow growth. Severe damage to the intestinal tract typically is called necrotic enteritis (necrotic meaning "dead tissue"), which is a problem in many types of production systems (Jacob, J. 2015).

2.5 Poultry GIT and Bacterial Composition in it

Birds use large amounts of energy compared to other animals, and their digestive system is adapted to extract energy from difficult to digest food sources. Intensive selection over the last six or seven decades has resulted in chickens that very efficiently convert food into body mass, making them the most efficient agricultural animal in terms of the input required to produce high-quality meat protein. Energy and nutrient extraction from feed requires interplay between the biochemical functions provided by the chicken and the microbiota present within the gastrointestinal tract (GIT). Highly productive chickens have been developed by selection for elite genetic traits; it is possible that in the future, gains in productivity and health outcomes could be influenced by selection of elite GIT microbiota. Hence, the role of the GIT microbiota in both productivity and health is subject to intensive study. The microbiota within the GIT also has important roles in protection from pathogens, detoxification and modulation of immune system development.

The chicken GIT harbours a very diverse microbiota that aids in the breakdown and digestion of food and comprised over 900 species of bacteria. However, it is estimated that only 10–60 % of caecal bacteria can be cultured and that only around 45 % of chicken intestinal bacteria can be confidently assigned to a known genus. Therefore, much of the GIT microbiota remains largely unexplored making it a major source of untapped biological potential in terms of newly identified bacteria, encoded enzyme activities and potentially probiotic strains of bacteria.

The gastrointestinal (GI) tract of poultry comes into contact with exogenous microorganisms immediately after hatch and thereafter it becomes a warm shelter for a complex microbiome consisting primarily anaerobic bacteria. As host grows, this microbiome becomes very diverse until it reaches a relatively stable yet dynamic state. Compared with other food animals that are mammals, poultry (chicken, turkey, and duck) has a shorter GI tract and faster digesta transit. This anatomic feature selects a very different intestinal microbiome in poultry than in other food animals.

The GI tract of poultry (e.g., chicken, turkey, and duck) consists of esophagus, crop, proventriculus, gizzard, small intestines (duodenum, jejunum, and ileum), cecum, colon, and cloaca. Relative to body length, the poultry GI tract is much shorter than that of

mammalian animals. As such, the digesta passes through the entire GI tract faster in poultry than in mammals. Although diet and feeding can have an effect on passage rate, the average whole tract transit time is less than 3.5 h. Such short retention time selects bacteria that can adhere to the mucosal layer and/or grow fast. However, the ceca, which are two blind pouches that have rather slow passage rate, are ideal habitats for a diverse microbiome that has considerable effect on host nutrition and health. The cecal microbiome is indeed the most studied intestinal microbiome of poultry. The cecum of both chickens and turkeys harbors a complex microbiome, which is almost exclusively composed of bacteria. Early studies revealed low abundances of *lactobacilli* ($>10^4$ /g colony forming units, CFUs) and *clostridia* (10^2 – 10^4 /g CFUs) in the small intestines and high abundance (10^{10} – 10^{11} /g microscope counts) of anaerobic bacteria in the cecum of chickens. Identified bacteria included anaerobic Gram-negative cocci, facultative anaerobic cocci, and streptococci. *Peptostreptococcus*, *Propionibacterium*, *Eubacterium*, *Bacteroides*, and *Clostridium* were the major genera that were recovered from cecum. (Pan et al., 2014)

Large numbers of anaerobic bacteria capable of decomposing uric acid comprise the cecal flora of chicks 3 to 6 h after hatching. During the first 2 to 4 d post hatch, *streptococci* and *enterobacteria* colonize the small intestine and cecum. After the first week, *lactobacillus* predominate in the small intestine, and the cecum is colonized mainly by anaerobes (*Escherichia coli* and *Bacteroides*) with lower numbers of facultative aerobes. A typical microflora of adult birds in the small intestine is established within 2 week, however, it was found that the adult cecal flora, which was mainly obligate anaerobes, took up to 30 days to develop. At that age, *bifidobacteria* and *bacteroides* predominate (Romach et al., 2004).

The bacterial communities originating from different sections of the chicken GIT are so different that it has been suggested that they should be considered as separate ecosystems. Here is a table showing the most abundant bacterial residents of different chicken GIT sections. (Stanley et al., 2014)

Table 2.2: Summary of the most abundant bacterial residents of different chicken GIT sections

Chicken GIT section	Study	Dominant and abundant bacteria
Crop	Sekelja et al. (2012)(S) Rehman et al. (2007)(R) Fuller (1973)(C)	<i>Lactobacillus</i> (dominant) <i>Clostridiaceae</i> , <i>Bifidobacterium</i> <i>Enterobacteriaceae</i> <i>Enterococcus</i>
Gizzard	Sekelja et al. (2012)(S) Rehman et al. (2007)(R) Fuller (1973)(C)	<i>Lactobacillus</i> (dominant), <i>Clostridiaceae</i> <i>Enterococcus</i> , <i>coliforms</i>
Duodenum	Lu et al. (2003)(S) Gong et al. (2002) (T-RFLP) Salanitro et al. (1974)(C) Konsak et al.(2013)(S)	<i>Lactobacillus</i> (very dominant up to 99 %in some birds), <i>Streptococcus</i> , <i>coliforms</i>
Ileum	Lu et al.2003(S) Gong et al. (2002) (T-RFLP) Salanitro et al. (1974)(C) van der Hoeven-Hangoor (2013) (Microarray)	<i>Lactobacillus</i> (very dominant), <i>Streptococcus</i> , <i>coliforms</i> . <i>Enterobacteriaceae</i> , <i>Clostridiaceae</i>
Caecum	Gong et al.2002; (T-RFLP) Zhu et al. (2002) (TTGE) Lu et al. (2003)(S) Stanley et al. (2013a)(S) Torok et al. (2011) (T-RFLP)	Rich in unknown and uncultured, <i>Lactobacillus</i> , <i>Bacterioides</i> , <i>Bacillus</i> <i>Clostridium</i> , <i>Bifidobacterium</i>
Faeces	Sekelja et al. (2012) (S) Zoetendal et al. (1998) (TGGE)	Varies greatly between the time points, <i>Lactobacillus</i> , <i>Clostridium</i> , <i>Fecalibacterium</i> , <i>Ruminococcus</i> , <i>Bacillus</i> , <i>Eubacterium</i> , <i>Fusobacterium</i>

(Source: Stanley et al., 2014)

2.6 Type of probiotic and micro-organisms used in poultry

There are several types of probiotics available in the market to be used in poultry, with a range of micro-organisms present and, therefore, with different metabolic activities and action modes. Also, they present variations as to the capacity of colonizing the intestine or not, which justifies variations on the results of their use. *Bacillus*, *Bifidobacterium*, *Enterococcus*, *E. coli*, *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus* species, and a range of yeast species and non-defined mixed cultures have been used. However, even those belonging to the same

species can have different strains and even these different strains from the same species can have different metabolic activities. These bacteria are used alone or in combination.

Non-defined mixed cultures, known as competitive exclusion cultures, are normally related to the treatment of one-day chicks with an indefinite microbiota derived from adult animals resulting in resistance to colonization against pathogenic micro-organisms.

Among the colonizing species, *Lactobacillus sp.*, *Enterococcus sp.* and *Streptococcus sp.* are worth mentioning, and among the non-colonizing species, *Bacillus spp.* (spores) and *Saccharomyces cerevisiae*.

Another characteristic of probiotics is that some micro-organisms are constituted by microorganisms normal to the intestinal microbiota of poultry, and others by bacteria different from the ones from the digestive tract. According to Kabir (2009) the most commonly used species are: *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus helveticus*, *Lactobacillus lactis*, *Lactobacillus salivarius*, *Lactobacillus plantarum*, *Streptococcus thermophilus*, *Enterococcus faecium*, *Enterococcus faecalis*, *Bifidobacterium spp.* and *Escherichia coli*, and except for *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, all the remaining ones are intestinal strains.

Recently, emphasis has been given to the selection, preparation and application of probiotic strains, especially lactic acid bacteria.

Natural adaptation of lactic acid bacteria to intestinal environment and the lactic acid produced by them have provided advantages for these organisms over other microorganisms used as probiotic (Otutumi et al., 2012).

2.7 Action Mechanisms of Probiotics

The action mechanisms of probiotics on the immune system of broiler mucosa are not completely clear. However, it is admitted that probiotics have immune-modulating effects. According to (Erickson & Hubbard, 2000 and Menten & Loddi, 2003), the bacterium genera present in probiotics that are directly related to the increase in immunity of poultry are

Lactobacillus and *Bifidobacterium*, mainly when related to diseases affecting the gastrointestinal tract.

The immune-modulating effect in poultry happens in two ways:

- (a) From the microbiota, in which the probiotic migrates along the wall of the intestine and is multiplied to a limited extension, or
- (b) The antigen released by the dead organisms is absorbed and thus stimulate the immune system.

The scenario is described through a figure showing the inhibition of enteric bacteria and enhancement of barrier function by probiotic bacteria.

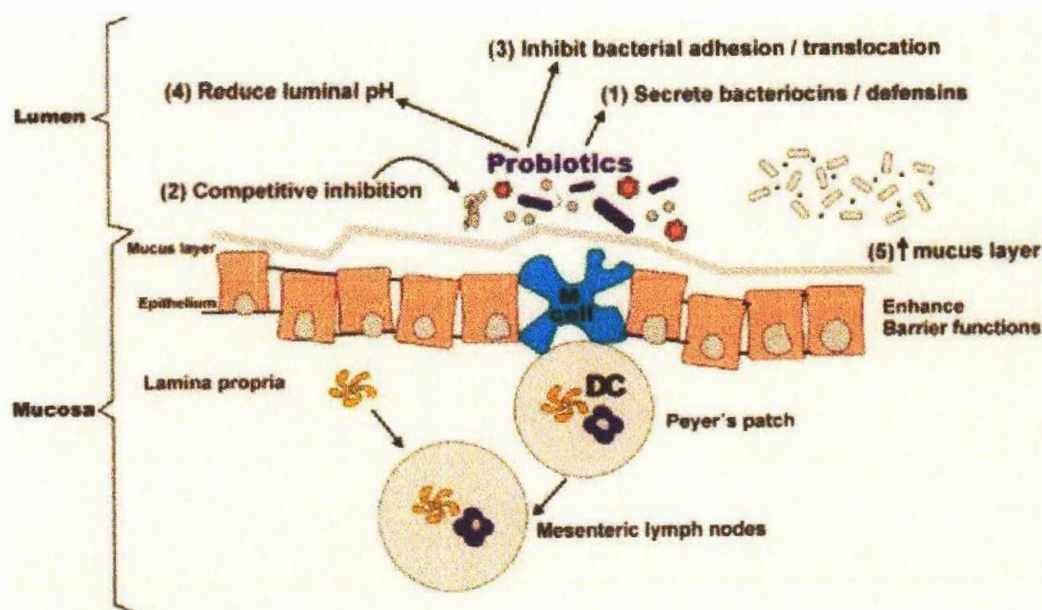


Figure 2.5: Schematic representation of the crosstalk between probiotic bacteria and the intestinal mucosa (Otutumi et al., 2012)

Antimicrobial activities of probiotics include the -

- (1) Production of bacteriocins/defensins,
- (2) Competitive inhibition with pathogenic bacteria,
- (3) Inhibition of bacterial adherence or translocation,

(4) Reduction of luminal pH,

(5) Probiotic bacteria can also enhance intestinal barrier function by increasing mucus production.

According to some studies antigens (lipopolysaccharides and peptidoglycans) are constantly released in intestinal lumen. On the other hand, this release is increased during infectious processes, once these components are fundamental in the development and maintenance of local immune response, since they have chemotactic effect on epithelial cells and cells related to mucosa immunity, and induce changes in the intestinal epithelium of the host (Otutumi et al., 2012).

Probiotic and competitive exclusion approaches have been used as one method to control endemic and zoonotic agents in poultry. In traditional terms, competitive exclusion in poultry has implied the use of naturally occurring intestinal microorganisms in chicks and poults that were ready to be placed in brooder house. Nurmi and Rantala and Rantala and Nurmi first applied the concept when they attempted to control a severe outbreak of *S. infantis* in Finnish broiler flocks. In their studies, it was determined that very low challenge doses of *Salmonella* (1 to 10 cells into the crop) were sufficient to initiate salmonellosis in chickens. Additionally, they determined that it was during the 1st week post-hatch that the chick was most susceptible to *Salmonella* infections. Use of a *Lactobacillus* strain did not produce protection, and this forced them to evaluate an unmanipulated population of intestinal bacteria from adult chickens that were resistant to *S. infantis*. On oral administration of this undefined mixed culture, adult-type resistance to *Salmonella* was achieved. This procedure later became known as the Nurmi or competitive exclusion concept. The competitive exclusion approach of inoculating day-old chicks with an adult microflora successfully demonstrates the impact of the intestinal microbiota on intestinal function and disease resistance. Although competitive exclusion fits the definition of probiotics, the competitive exclusion approach instantaneously provides the chick with an adult intestinal microbiota instead of adding one or a few bacterial species to an established microbial population. Inoculating day-old chicks with competitive exclusion cultures or more classical probiotics serves as a nice model for determining the modes of action and efficacy of these microorganisms. Because of the susceptibility of day-old chicks to infection, this practice is also of commercial importance.

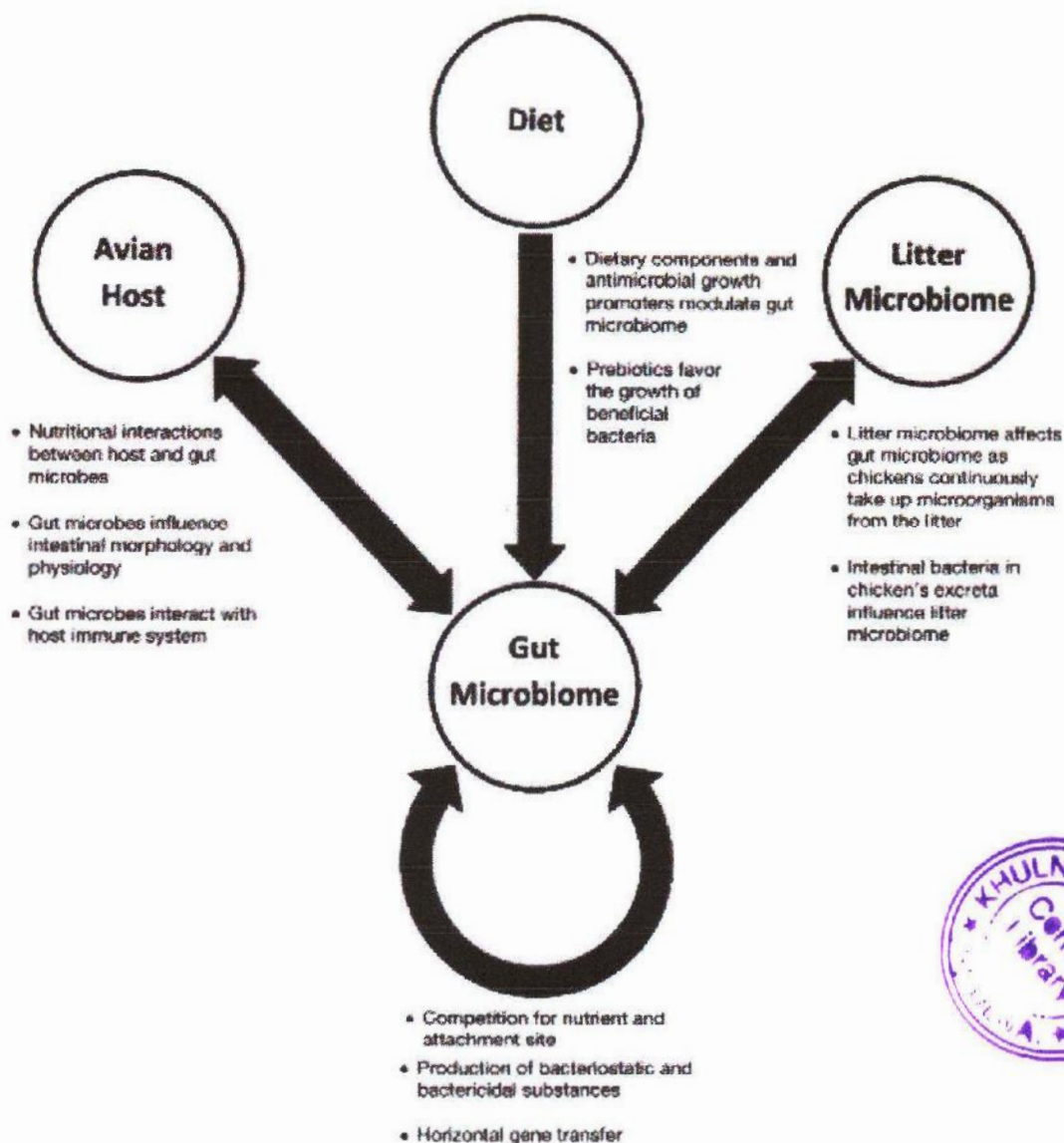


Figure 2.6: Conceptual model of the interactions among gut microbiome, avian host, diet, and litter microbiome (Adapted from Pan & Yu, 2014)

By using this model, a number of probiotics have been shown to reduce colonization and shedding of *Salmonella* and *Campylobacter*. Competitive exclusion is a very effective measure to protect newly hatched chicks, turkey poults, quails and pheasants and possibly other game birds, too, against *Salmonella* and other enteropathogens.

Upon consumption, probiotics deliver many lactic acid bacteria into the gastrointestinal tract. These microorganisms have been reputed to modify the intestinal milieu and to deliver enzymes and other beneficial substances into the intestines. Supplementation of *L. acidophilus* or a mixture of *Lactobacillus* cultures to chickens significantly increased ($P<0.05$) the levels of amylase after 40 d of feeding. The *lactobacilli* colonizing the intestine may secrete the enzyme, thus increasing the intestinal amylase activity. It is well established that probiotics alter gastrointestinal pH and flora to favor an increased activity of intestinal enzymes and digestibility of nutrients. Probiotics may contribute to the improvement of health status of birds by reducing ammonia production in the intestines.

Probiotic is a generic term, and products can contain yeast cells, bacterial cultures, or both that stimulate microorganisms capable of modifying the gastrointestinal environment to favor health status and improve feed efficiency. Mechanisms by which probiotics improve feed conversion efficiency include alteration in intestinal flora, enhancement of growth of nonpathogenic facultative anaerobic and gram positive bacteria forming lactic acid and hydrogen peroxide, suppression of growth of intestinal pathogens, and enhancement of digestion and utilization of nutrients.

Therefore, the major outcomes from using probiotics include:

- Improvement in growth,
- Reduction in mortality, and
- Improvement in feed conversion efficiency.

The manipulation of gut microbiota via the administration of probiotics influences the development of the immune response. The exact mechanisms that mediate the immunomodulatory activities of probiotics are not clear. However, it has been shown that probiotics stimulate different subsets of immune system cells to produce cytokines, which in turn play a role in the induction and regulation of the immune response (Kabir, 2009).

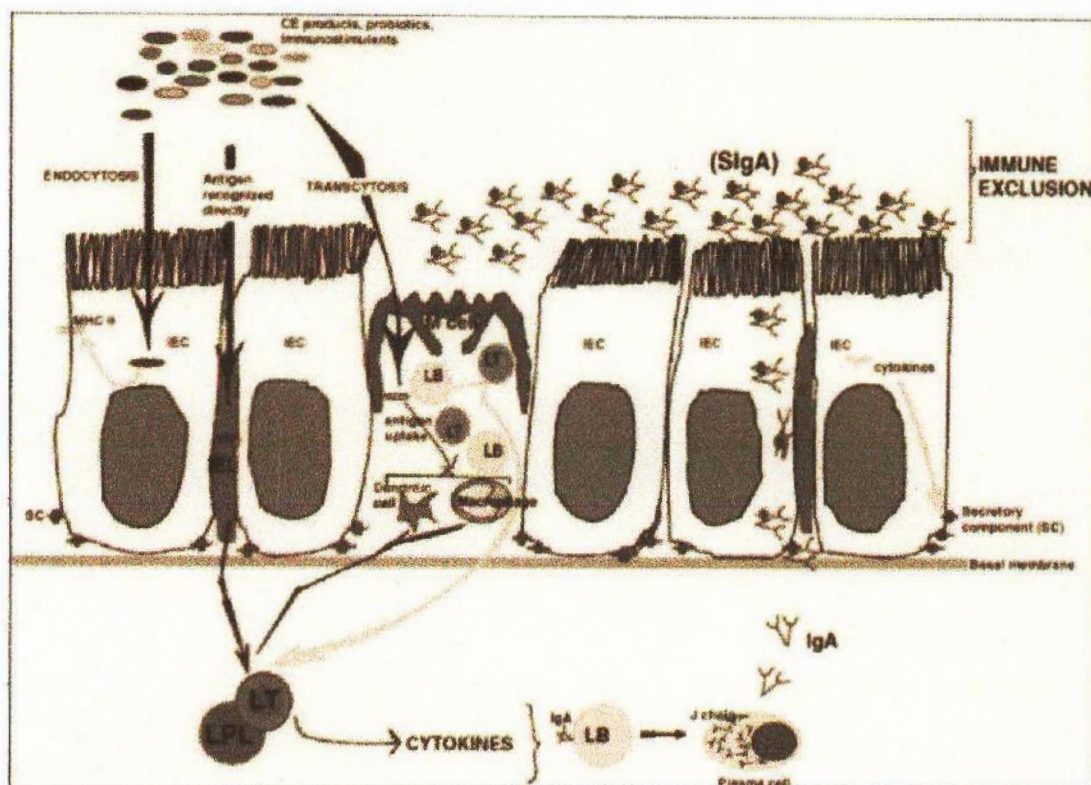


Figure 2.7: Proposed interactions between competitive exclusion products, probiotics/immunostimulants, and avian intestinal immunity (Kabir, 2009)

2.8 Variations on the Efficacy of Probiotics in Poultry:

There is a large range of micro-organisms used as probiotics, with variations in species and strains of the same species, and therefore, they present variations in its metabolic activity and justify variations in the results of their use. However, other factors can justify the variations in the results of using probiotics in poultry, such as the origin species, probiotic preparation method, survival of colonizing micro-organisms in the gastrointestinal tract conditions, the environment where the birds are raised, management (including probiotic application time and application route), the immunologic state of the animals, the lineage of poultry evaluated, as well as age and concomitant use of antibiotics.

Fuller (1986) emphasizes that the specificity of adhesion of *lactobacilli* (one of the most used probiotic genre in poultry) to epithelial cells is specific host and if the colonization is reached, it

is essential to administer bacteria that have been originated from the host species for which they are being given.

On the other hand, it is worth mentioning that there are probiotics presenting efficacy even though they have not been isolated from the original host species. As an example, the works developed by Impey et al. (1984) and Schneitz & Nuotio (1992) showing that the natural microbiota of chicken (Broilact®) and turkeys provide reciprocal protection for chicks and poults. Several beneficial effects of the use of *Lactobacillus* as probiotics are reported in literature in relation to the productive performance of poultry.

Regarding the nutritional status of the animals, studies have shown that improvements in the performance of broilers have been seen when feed does not contain all nutrients in appropriate quantities. In some researches it is shown that the effect of supplementation with *Lactobacillus* spp. on the growth of body mass and feed conversion in broilers is significantly greater when the methionine, cystine and lysine levels in the feed are reduced.

In literature, several treatment methods using probiotics are described, such as through feed, addition to drinking water, spraying on the birds, inoculation via cloaca or in embryonated eggs (in ovo), through the litter used, in gelatin capsules and intra-esophagus. This way, the administration route of probiotics can determine an improvement or worsening in the intestinal colonization capacity by the bacteria present in the product used. Direct inoculation in esophagus/crop (intra-esophageal) is the most efficient, although in practical terms it has little viability.

The duration of treatment can be an important factor in the effect of a probiotic on the intestinal microbiota, once probiotics can be given only once or periodically, in weekly or daily intervals. Despite the little knowledge regarding the minimum required dose to evidence the effects of probiotics, experiments in mice, humans and pigs have indicated that the effect decreases when the probiotic is discontinued.

Regarding age, beneficial effects of probiotics were seen during the initial growth phase, happening before 28 days and not after 49 days of age. Certainly, during the initial stages of life, the intestinal microbiota is in an unstable condition, and the micro-organisms given orally probably find a niche where they can occupy. Therefore, it has been also reported that the

existence of an intestinal microbiota at the time of administration and the health of the host must be considered when a probiotic is supplemented for the suppression of pathogenic bacteria. It should also be noticed that some micro-organisms that can act as probiotics do not resist the action of some antibiotics or anticoccidial used in the feed of birds.

Other factors that might justify the variations in the effects of probiotics in poultry are:

- Variations in the persistence of administered strains (relative intestinal concentration),
- Stability during the manufacturing of feed,
- Absence of statistical analysis of data in previous studies,
- Experimental protocols not clearly defined,
- Micro-organisms not identified,
- Viability of organisms not verified,
- The origin of micro-organisms in probiotics was not reported.

With all these possible variations, it is not surprising that probiotics not always grant the desired result, but the fact that significant results are obtained show that the correct use of probiotics, under appropriate conditions and using the correct administration method, justify the fact that probiotics are an efficient food supplement in animal breeding (Otutumi *et al.*, 2012).

Materials and Methods

3.1 Study Area and Period

This study was conducted in Animal Cell Culture Laboratory of Biotechnology and Genetic Engineering Discipline, Khulna University, Bangladesh since November 2018 to November 2019. Some of its portion was also performed in Plant Tissue Culture Laboratory and in the Microbial Biotechnology Division of National Institute of Biotechnology (NIB).

3.2 Study Design

The whole study has been divided into some parts to enhance the chance of better result. The parts are:

- Isolation of bacteria in pure culture
- Identification of cultural isolates
- Confirmation of isolates by molecular method
- Molecular typing of strains based on randomly amplified polymorphic DNA (RAPD) fingerprint analysis

In this study, pure colony isolation after several subculture, and the genomic DNA extraction has done in Animal cell culture (ACC) laboratory. The PCR, PCR based Gel electrophoresis, Visualization of the band, and the Gene sequencing has been performed in NIB.

For better understanding here is a complete diagram or flow chart of the total methodology of the study:

Flow Chart of the Methodology:

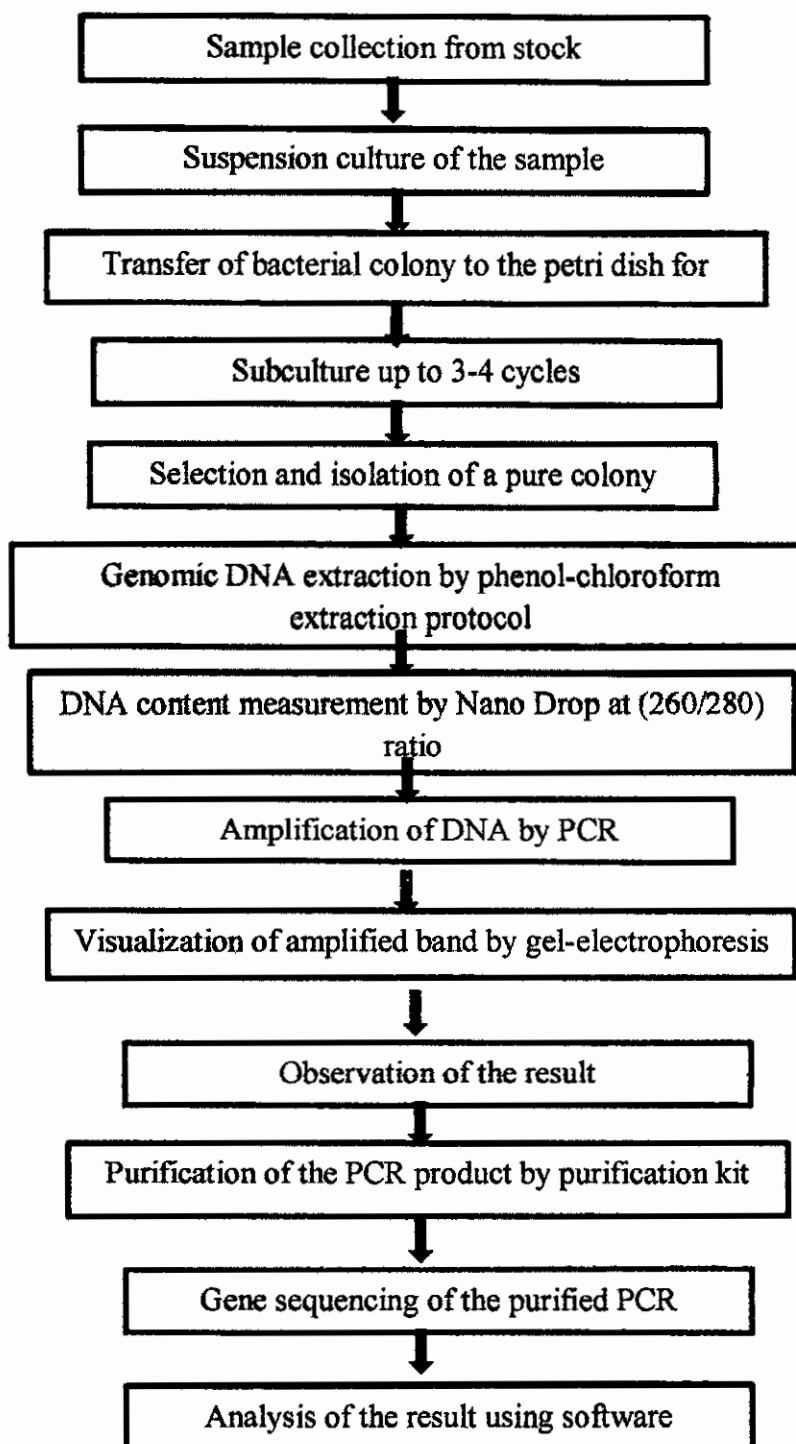


Figure 3.1: Flow chart of the methodology

3.3 Handling of Laboratory Apparatus and Glassware

All glasswares were washed with mild detergents, rinsed at least three times with distilled water and allowed to air dry. Petri dishes, micropipette tips, glass pipettes and micro centrifuge tubes were sterilized by autoclaving at 121°C for 15 minute at 15 psi before every use.

3.4 Sample Source

Sample of this study was collected from the stock, which was preserved from the previous research work. There were total 30 isolates obtained from the 7 different regions all over the country. After collecting the sample, it was serially diluted and made 30 stocks which was then stored in the freezer at -20°C for both immediate utilization and long term preservation.

Here is a table describing the sources of isolates from poultry:

Table 3.1: Sources of poultry samples collected from seven different Divisions of Bangladesh

Sample No.	Sources of isolates	Isolate identity	Sample type (Age)	Name of the Divisions
1	Ceca	C1	Chicken (15d)	Dhaka
2	Crop	CR1	Chicken (15d)	
3	Small intestine	SI1	Chicken (15d)	
4	Crop	CR2	Chicken (30d)	
5	Small Intestine	SI2	Chicken (30d)	
6	Crop	CR3	Chicken (15d)	Cumilla
7	Small intestine	SI3	Chicken (15d)	
8	Ceca	C3	Chicken (15d)	
9	Crop	CR4	Chicken (30d)	
10	Small intestine	SI4	Chicken (30d)	
11	Crop	Cr1	Duck (15d)	Khulna
12	Small intestine	SI1	Duck (15d)	
13	Crop	Cr2	Chicken (15d)	
14	Cecum	C2	Chicken (15d)	
15	Small Intestine	SI2	Chicken (15d)	
16	Cecum	C3	Chicken (15d)	Barishal
17	Crop	Cr4	Chicken (15d)	
18	Cecum	C4	Chicken (15d)	
19	Small intestine	SI5	Chicken (30d)	
20	Crop	Cr5	Chicken (30d)	

Sample No.	Sources of isolates	Isolate identity	Sample type (Age)	Divisions name
21	Crop	CS2	Chicken (15d)	Sylhet
22	Small intestine	SS1	Chicken (30d)	
23	Crop	CS1	Chicken (15d)	
24	Crop	CS3	Chicken (15d)	
25	Cecum	CR1	Chicken (30d)	Rajshahi
26	Small Intestine	SR2	Chicken (30d)	
27	Crop	CR2	Chicken (15d)	
28	Crop	CR3	Chicken (25d)	Rangpur
29	Small intestine	SR1	Chicken (30d)	
30	Crop	CR4	Chicken (25d)	

3.5 Pure Colony Development

Pure culture is usually derived from a mixed culture (one containing many species) by transferring a small sample into new, sterile growth medium in such a manner as to disperse the individual cells across the medium surface or by thinning the sample many fold before inoculating the new medium. Both methods separate the individual cells so that, when they multiply, each will form a discrete colony, which may then be used to inoculate more medium, with the assurance that only one type of organism will be present. Pure colony was obtained after the suspension culture and several times of solid subculture. De Man, Rogosa and Sharpe agar (MRS) media were used for the culture of probiotic bacteria.

3.5.1 Preparation of Suspension Culture

The first step to obtain the pure culture was suspension culture preparation. For this culture the following materials were required:

- Stock solution
- MRS broth
- Test tube
- Micropipette (20-200 μ l) and micropipette tips
- Pipette (10 ml)

MRS broth Preparation

For MRS broth preparation we required:

- Conical flask
- Spatula
- MRS media
- Tween 80
- Distill water

First required amount of distill water in a conical flask was taken. After that required MRS was weighed out in a laboratory weighing scale and was mixed in the conical flask and then tween 80 was also added. At last the pH was checked and maintained to 6.5-6.8. The measurement of MRS and tween was followed by the following protocol:

For 100 ml of broth preparation:

- ✓ 5.5 g MRS
- ✓ 0.1 ml Tween 80

After the broth was prepared it was capped by aluminum foil and autoclaved.

Procedure of Suspension culture preparation

At first the stock sample was thawed to bring it to the normal temperature (avoiding the heat shock of the sample) and then the entire procedure was ready to be started. The entire work was done in the Laminar Air Flow cabinet. All equipment (test tube, pipette, micropipette tips) were autoclaved before use. A flow chart is given to describe the procedure:

All autoclaved equipment along with autoclaved MRS broth media was placed in the Laminar
Air Flow Cabinet



10 ml of broth media was poured into each of the 60 test tube (2 for each sample) by 10 ml of
pipette and cooled



100 μ l of stock solution was transferred by micropipette into the test tube and was properly mixed by pipetting



All test tubes were capped properly by aluminum foil and placed into incubator for 24-36 hours

Figure 3.2: A flow chart describing suspension culture preparation

3.5.2 Preparation of Solid Culture

The next step to obtain the pure colony was solid culture preparation. For this culture, the following materials were required:

- Petri dish
- Pipette (20 ml)
- Micropipette (10-50 μ l) and micropipette tips
- MRS broth media for solid culture (MRS agar media)

MRS Agar Media

For MRS agar media preparation we required:

- Conical flask
- Spatula
- MRS media
- Tween 80
- Agar
- Distilled water

First required amount of distilled water in a conical flask was taken. After that required MRS was weighed out in a laboratory weighing scale and was mixed in the conical flask. And then tween 80 was also added. At last the pH was checked and maintained to 6.5-6.8. In the last step agar was added and mixed properly in oven. The measurement of MRS and tween was followed by the following protocol:

For 100 ml of broth preparation:

- ✓ 5.5 g MRS
- ✓ 0.1 ml Tween 80
- ✓ 1.8 g Agar

After the media was prepared it was capped by aluminum foil and autoclaved.

Procedure of Solid culture preparation

The suspension culture, MRS agar media and required equipment were placed inside the Laminar Air Flow cabinet. Like before all equipment (petri dishes, pipette, micropipette tips) were autoclaved before use. A flow chart is given to describe the procedure:

At first 20 ml of MRS agar media into each of 60 petri dishes were poured and cooled



After the media was coagulated the better growing bacterial culture from each test tube were transferred to each petri dishes by micropipette (each better bacterial growth selected test tube were mixed by vortexing)



The petri dish was then wrapped by paraffin and incubated for 24-36 hours

Figure 3.3: A flow chart describing solid culture preparation



3.5.3 Procedure of Subculture

After incubation period, each petri dish was checked for bacterial growth and the better petri dish was selected for subculture. The bacterial colony was transferred to a freshly prepared MRS agar media with the help of a sterilized inoculation loop. The subculturing was performed for 3-4 times until the pure colony was obtained.

3.6 Stock culture preparation and preservation

A stock culture is a culture of a microorganism maintained solely to keep it viable for subculture into fresh medium.

Requirement

- Glycerol
- MRS broth
- 2 ml screw cap vial
- Test tube
- Micropipette and micropipette tips

Procedure

MRS broth was prepared by dissolving MRS and Tween 80 into distill water



Single colonies from the final subculture was transferred into MRS broth



The mixture was then incubated at 37⁰C for overnight



1000 µl fresh MRS broth was transferred into each 2 ml vial



Then 600 µl culture broth was poured into vial



400 µl glycerol was added to the vial



Screwed the cap of the vial and was labelled



Finally, the vial was stored at -20⁰C temperature

Figure 3.4: Stock culture preparation procedure

3.7 Final suspension culture preparation

Requirement

- Fresh MRS broth
- Test tube
- Inoculation loop

Procedure

MRS broth including with other equipment was autoclaved



Pure colonies containing culture, required equipment was kept in laminar air flow hood



10 ml freshly prepared MRS broth was poured into each of 30 test tubes



A single pure colony was transferred from each culture into the test tube



The test tubes were then incubated for 24 hours at 37°C temperature



After incubation 10 µl from each test tube was transferred into freshly prepared 5 ml MRS broth containing test tube and incubated at 37°C for 10 hours

Figure 3.5: Final suspension culture preparation

3.8 Genomic DNA Extraction

For the extraction of genomic DNA some solution was readily available, those are:

- Lysozyme solution (10 mg/ml)
- RNase A (10 mg/ml)
- Proteinase K (20 mg/ml)
- Isopropanol

But some solution had to be prepared. Those are:

- 1M NaCl solution
- 0.5M EDTA solution
- NaCl-EDTA solution
- 10% SDS
- Phenol-Choloroform solution (1:1)
- Tris solution preparation (1M, 0.5M, 0.1M)

Procedure

Total two extraction protocols were followed. Both are given here:

Protocol no. 1:

2 ml of culture was poured into each of 2 ml vial from the final suspension culture and incubated at 37°C for 1 hour



After incubation, harvested bacteria was then centrifuged at 5000 rpm for 5 min



The media supernatant was decanted and the pellet was washed thrice with 1 ml of NaCl-EDTA



Bacterial pellet was then resuspended in 100 µl freshly prepared Lysozyme solution



4 µl of RNase-A solution was then added to remove RNA



Mixture was incubated at 37°C for 1 h with intermittent shaking



10 µl proteinase k (20 mg/ml) and 50 µl of a 10% SDS solution was then added to the mix



Volume of the mixture was made up to 500 µl additional NaCl-EDTA solution



The mix was then incubated at 55°C for 1 h



Equal volume of Tris-saturated phenol (pH 8.0) was added and mixed gently after incubation



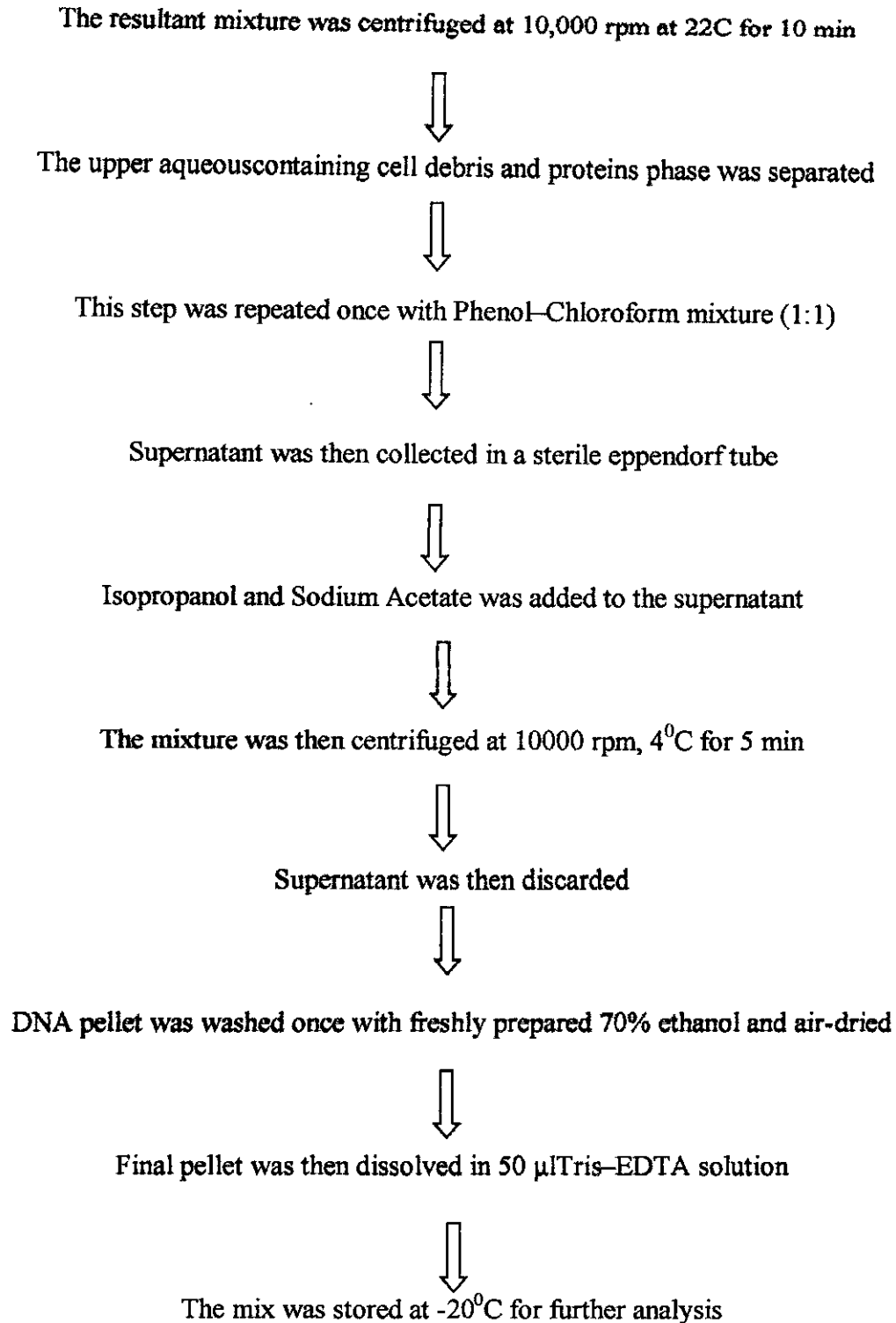


Figure 3.6: A phenol-chloroform DNA extraction protocol (Source: De et al., 2011)

Protocol no. 2:

Bacterial isolates in nutrient broth at 37°C for overnight under shaking



Took about 1.5 ml of culture in a micro centrifuge tube



Centrifuged at 13000 rpm for 5 minutes



Repeated this procedure for 3 times



Discarded the supernatant and collect the pellet



Re-suspended the pellet in 567 TE buffer by repeated pipetting



Added 30.0 µl of 10% SDS and 3.0 µl of 20 mg/ml proteinase K



Incubated at 55°C for 30 min in circulating water bath and then added 100ul NaCl (5 M) and mixed thoroughly and incubated for 30 min at 37°C



Added equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24: 1) to that supernatant.



Mixed thoroughly by inverting tubes several times.



Centrifuged at 13000 rpm for 10 min

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Collected supernatant (aqueous phase) in a fresh tube and discard pellet.



Added equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1)



Centrifuged at 13000 rpm for 10 min and transferred the supernatant to the fresh tube



Added 1/10th volume of 3M sodium acetate



Then added equal volume of chilled iso-propanol and then kept it in ice for 10 min.



Centrifuged at 13000 rpm for 15 min.



Discarded the supernatant.



Washed with 1.0 ml of 70% ethanol, mix by inverting several times. Centrifuged at 13000 rpm for 10 min at 4°C.



Discarded the supernatant.



Spin carefully and again discarded the rest of the supernatant



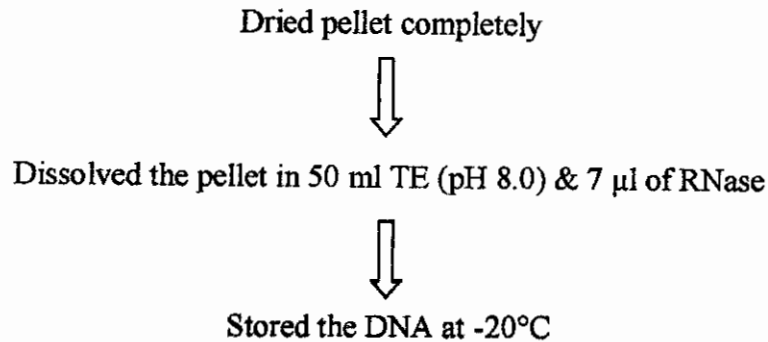


Figure 3.7: Genomic DNA extraction protocol

3.9 Qualitative and Quantitative measurements of DNA

Qualitative assessment was performed to observe DNA presence for ensuring more accuracy. Non PCR gel electrophoresis was performed for the qualitative assessment. And for the quantitative assessment spectrophotometric absorption was performed.

3.9.1 Non PCR Gel electrophoresis

Gel electrophoresis separates DNA fragments by size in a solid support medium (an agarose gel). Application of an electric current at the top (negative) end causes the negatively-charged DNA to migrate towards the bottom (positive) end. The rate of migration is proportional to size: smaller fragments move more quickly, and wind up at the bottom of the gel. Non PCR Gel electrophoresis was performed for detecting the presence of DNA. In this gel electrophoresis process 1% Agarose gel, Ethidium bromide, 50X TAE, 1X TAE were needed to prepare before starting this process.

3.9.1.1 Preparation of 1% Agarose Gel

Requirement: Agarose, 50X TAE buffer, Distilled water

Procedure

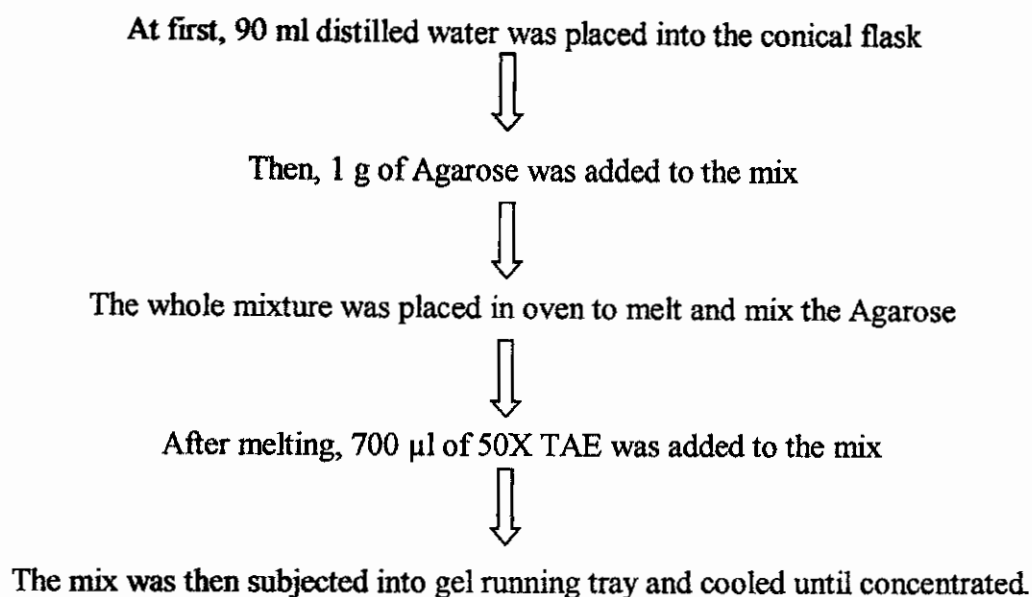


Figure 3.8: Preparation of agarose gel for electrophoresis

3.9.1.2 Gel electrophoresis

Procedure

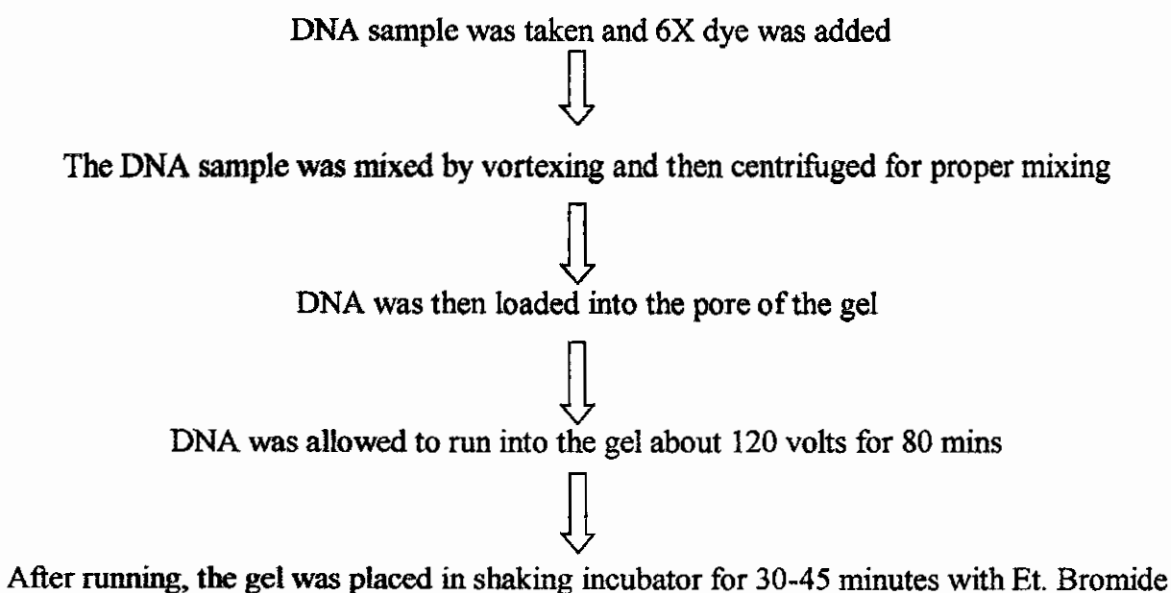


Figure 3.9: Procedure of gel electrophoresis

3.9.1.3 Documentation of the DNA samples

Procedure

After electrophoresis and ethidium bromide staining gel was taken out carefully



The gel was then placed in the dark chamber of UV transilluminator



The image was visualized on the monitor and the photo was taken for further analysis.

(The gel was handled carefully with gloves)

Figure 3.10: Documentation process of DNA samples

3.9.2 Spectrophotometric reading

Requirement

- Frozen DNA sample
- Tris-EDTA solution
- Micropipette and micropipette tips
- Distill water

Procedure:

The wavelength of the spectrophotometer was set at 260 nm



2 ml Tris-EDTA was taken in a 2 ml cuvet as a blank and the absorbance was measured and recorded



The blank value was set zero in the spectrophotometer machine



The cuvet was washed with distilled water



The frozen DNA mixture was thawed



1990 μ l Tris-EDTA buffer and 10 μ l frozen DNA mixture mixed properly by slight vortexing to prepare 2 ml of fresh sample instantly to measure the absorbance.



Each freshly prepared sample was then taken into cuvet to measure the absorbance and each time it was washed with distilled water before next use



Cuvet was placed into the spectrophotometer machine and absorbance was recorded

Figure 3.11: Process of recording spectrophotometric reading

The original concentration of DNA was determined according to the following formula:

$$\begin{aligned}\text{DNA concentration} &= \frac{\text{Abs. at 260nm}}{\text{Pathlength (1cm)}} \times \text{Dilution factor} \times \text{Conversion factor} \\ &= A_{260} \times 200 \times 50 \mu\text{g/ml}\end{aligned}$$

Here,

A_{260} = Spectrophotometric absorbance reading at 260nm.

Dilution factor = $1/0.005=200$

In this experiment, 10 μl of DNA solution was taken to dilute it to a final volume of 2 ml by using the factor of 0.005. To know the concentration of DNA by measuring the absorbance, the determined concentration was multiplied by the reciprocal of this dilution factor ($1/0.005$).

Conversion factor = A conversion factor is a number used to change one set of units to another, by multiplying or dividing. When a conversion is necessary, the appropriate conversion factor to an equal value must be used. The concentration of pure double-stranded DNA with an A260 is 50 $\mu\text{g}/\text{ml}$ which is equal to 1.

3.9.3 Measurement of the DNA Concentration by Nano Drop Spectrophotometer

For getting the exact concentration of DNA we also crosschecked the concentration by Nano drop spectrophotometer where we calculated the OD260/OD280 ratio for an indication of nucleic acid purity. Pure DNA has an OD260/OD280 ratio of ~ 1.8 ; pure RNA has an OD260/OD280 ratio of ~ 2.0 . Low ratios could be caused by protein or phenol contamination.

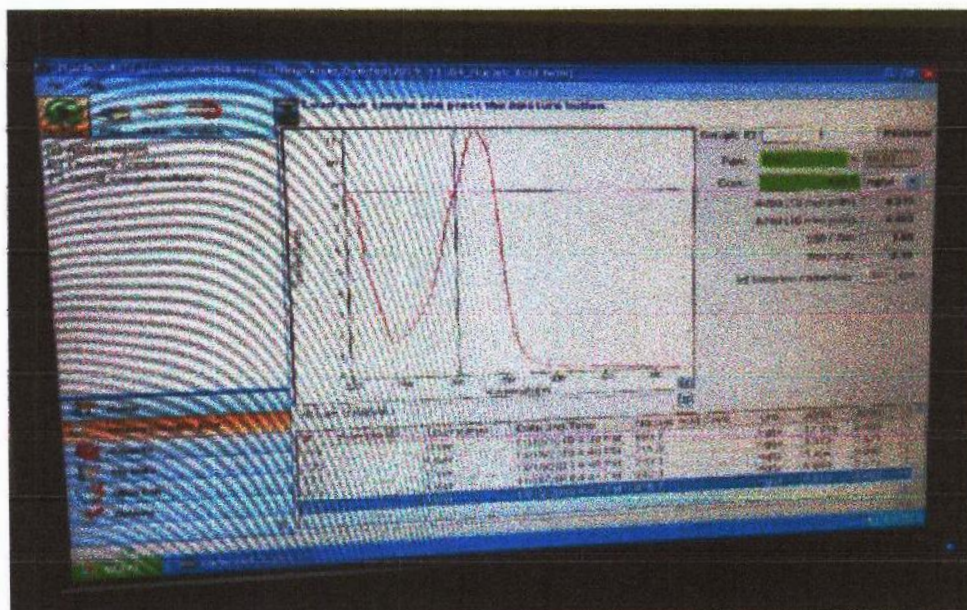


Figure 3.12: DNA concentration measurement by Nano drop

3.9.4 Dilution of the DNA sample for PCR

Before running the samples in PCR machine/program, the DNA concentrations were adjusted to 50 ng/μl by using the following formula:

$$S1 \times V1 = S2 \times V2$$

Where,

S1 = Initial concentration of DNA (ng/μl)

V1 = Required volume of DNA (μl) =?

S2 = Final concentration of DNA (ng/μl) = 50 ng/μl

V2 = Final volume of DNA solution (μl) = 50 μl

After calculating the value of the V1 we added that volume of DNA into a new vial where we have already added the TE buffer of (50-V1) μl. After diluting the DNA we used a slight vortexing and then stored it in -20°C.

3.10 PCR procedure

3.10.1 Master mix preparation

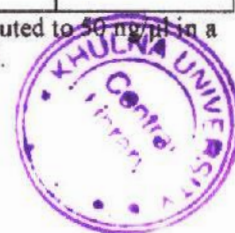
Requirement

- Ready Master mix
- Primer
- Taq DNA
- Nuclease free water

Table 3.2: PCR reaction components

Reagent	Stock Concentration	Working Concentration	Volume(μ l)/ Reaction	No of Samples	Total Quantity
Forward Primer	100 pmol	10 pmol	2.0 μ l	11	22
Reverse Primer	100 pmol	10 pmol	2.0 μ l		22
2X Master Mix			25.0 μ l		275
Taq DNA	5 U/ml	5 U/ml	1.0 μ l		11
Molecular grade water			17.0 μ l		187
DNA sample		50 ng/ μ l	3.0 μ l		
Total volume for one sample			50 μ l		517

Note: 10 samples along 1 control (total 11 samples) were taken for PCR. Each samples were diluted to 50 ng/ μ l in a separate vial for this reaction. Master mix was used as it was, no dilution process was performed.

**Table 3.3: Primer for 16S rRNA Sequencing**

Primer	Sequence
27 F	5' AGAGTTTGATCMTGGCTCAG 3'
1492 R	5' TACGGYTACCTTGTTACGACTT 3'

3.10.2 PCR Optimization

The designing of primer sequences has a pivotal role in obtaining satisfactory results from a PCR reaction. Useful primer parameter values depend on the application and fall within the following ranges: primer length 14 – 40 nt and GC contents 40 – 75%. When amplifying products > 5 kb in size, primers should be ≥ 25 nucleotides in length with a GC content above 55% and matched

T_m values above 65°C; The optimal annealing temperature for PCR is calculated directly as the value for the primer with the lowest T_m :

$$T_a(^{\circ}\text{C}) = T_m^{min} + \ln L$$

where L is length of PCR fragment.

Table 3.4: Thermal Profile of PCR

1 cycle	95°C	2 minutes
35 cycles	95°C	1 minutes
	55°C	1 minutes
	72°C	1 minutes
1 cycle	72°C	10 minutes ((to finish replication on all templates))
1 cycle	4°C	indefinite period (storing the sample prior to further analysis)

3.10.3 Preparation of DNA Samples for Electrophoresis

We needed to prepare a gel first. Once the gel has set, the comb is removed, leaving wells where DNA samples can be loaded. Loading buffer is mixed with the PCR product before the mixture is loaded into the wells. The loading buffer contains a dense compound, which may be glycerol, sucrose, or Ficoll, that raises the density of the sample so that the sample may sink to the bottom of the well. If the DNA sample contains residual ethanol after its preparation, it may float out of the well. The loading buffer also includes colored dyes such as xylene cyanol and bromophenol blue used to monitor the progress of the electrophoresis. The samples were loaded using a pipette.

3.10.4 Sample loading for Electrophoresis

The mixed samples were loaded in holes of gel carefully by using micropipettes. After loading of samples the gel with tray was put in the electrophoresis chamber containing 1X TBE buffer. Switched on the power supply and connected with the electric source. 100V with 180 amp was applied for 40 minutes.

Table 3.5: Preparation of sample for gel reaction

Gene Size	1500 bp
DNA marker	7 μ l
Loading Dye	2 μ l
PCR product	5 μ l

3.10.5 Gel Documentation of the PCR Products

After gel electrophoresis is done the PCR products were observed under UV transilluminator. Generally, a gel doc includes an ultraviolet (UV) light trans-illuminator, a hood or a darkroom to shield external light sources and to protect the user from UV exposure, and a CMOS camera for image capturing. The gel was photographed using gel doc and the image was saved for later analysis.



Figure 3.13: Loading of DNA samples in Agarose Gel



Figure 3.14: Gel electrophoresis of DNA samples

3.10.6 Purification of the PCR Products

Before gene sequencing the PCR products need to be purified. In our experiment we purified our PCR product by using a kit. The following steps we followed are:

1. **Combine:** Add 4 volumes of Binding Buffer with isopropanol to 1 volume of a PCR sample (50-100 L). Mix well.
2. **Load:** Pipet the sample into a Pure Link® Spin Column in a Collection Tube. Centrifuge the column at $>10,000 \text{ xg}$ for 1 minute. Discard the flow-through.
3. **Wash:** Re-insert the column into the Collection Tube and add 650 L Wash
4. **Buffer with ethanol:** Centrifuge the column at $>10,000 \text{ xg}$ for 1 minute. Discard the flow-through and place the column in the same Collection Tube. Centrifuge the column at maximum speed for 2-3 minutes.
5. **Elute:** Place the column into a clean 1.7-mL Elution Tube (supplied with the kit). Add 50 L Elution Buffer to the center of the column. Incubate the column at room temperature for 1 minute. Centrifuge the column at maximum speed for 2 minutes. The elution tube contains the purified PCR product. Store the purified DNA at 4°C for immediate use or at -20°C .

After this procedure the purified PCR product was delivered for Gene sequencing to the molecular division of NIB.

3.10.7 16S rRNA Bioinformatics Analysis

The 16S rRNA gene sequences were aligned against other species by BLAST search Using MEGA-X software for the construction of the phylogenetic tree. Phylogenetic relationships among the samples were analyzed on the basis of sequenced data and dendrogram was constructed using UPGMA.

After sequencing, sequenced data was analyzed by following genomic tools:

1. **DNA Baser:** Bioinformatics software for automatic DNA sequence assembly, DNA sequence analysis, contig editing, file format conversion and mutation detection. DNA Baser was used for assembly of the ABI file format. After assembly it produced contig files.

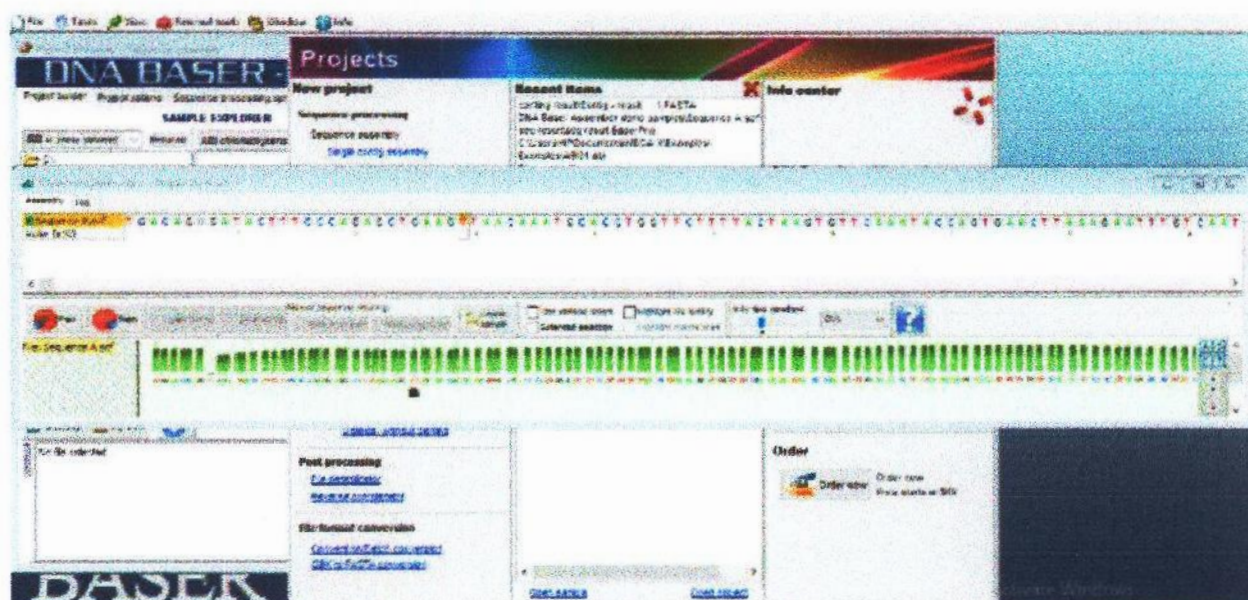


Figure 3.15: Bioinformatics software (DNA Baser) for automatic DNA sequence assembly

2. Basic Local Alignment Search Tool (BLAST)

For comparing the sample nucleotide sequences with a library or database of sequences, and identifying the library sequences that resemble the query sequence above a certain threshold we used BLAST search.

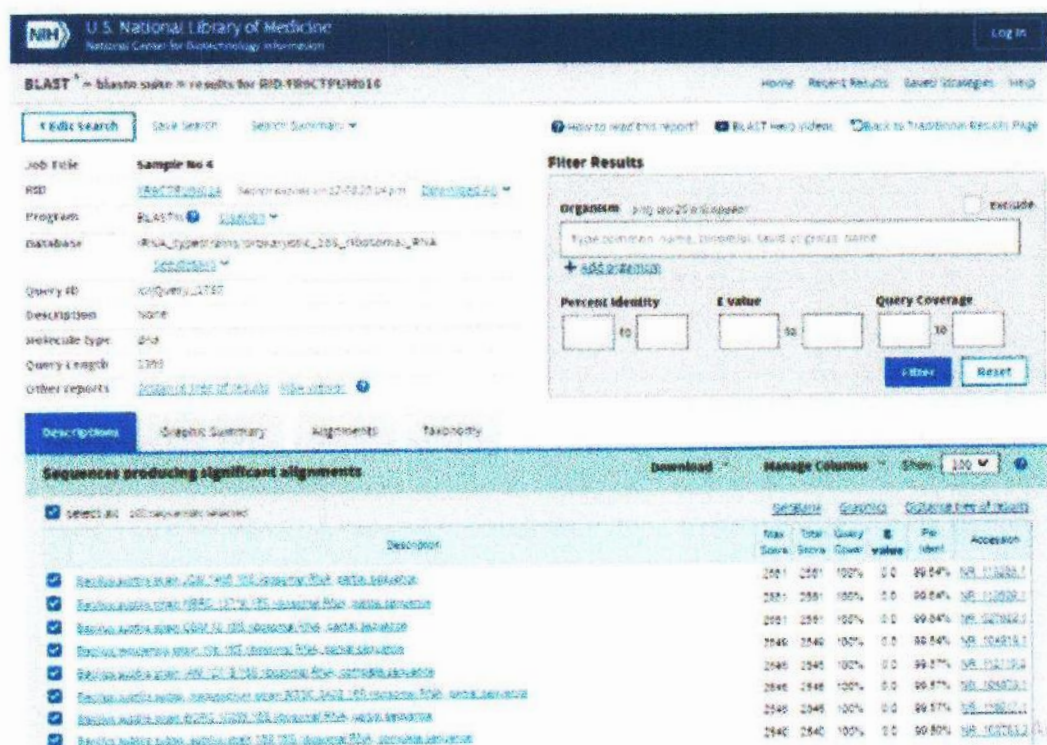


Figure 3.16: The Basic Local Alignment Search Tool (BLAST) search of one sample

3. **MEGA-X: Molecular Evolutionary Genetics Analysis(MEGA)** is computer software for conducting statistical analysis of molecular evolution and for constructing phylogenetic trees using Clustalw Omega and Basic local alignment search tool (BLAST).

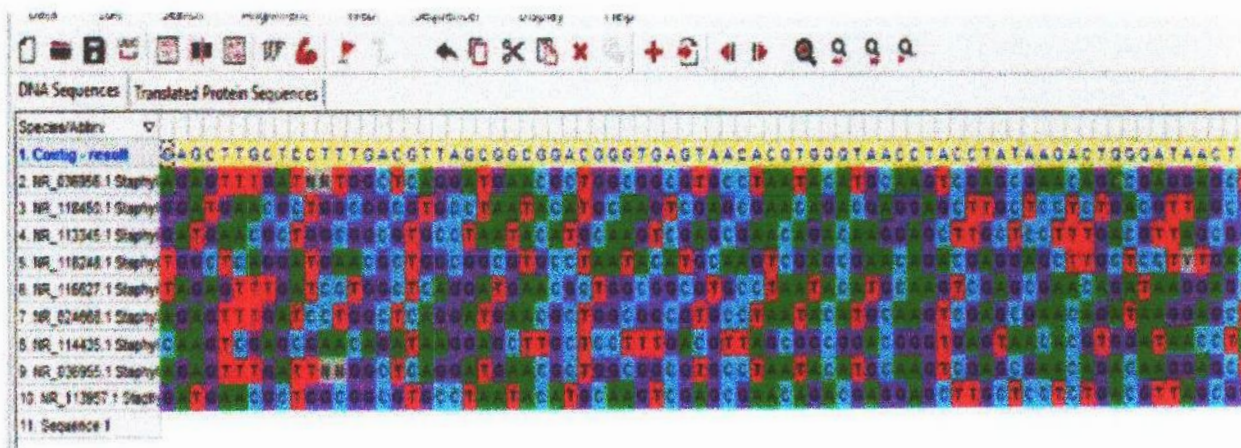


Figure 3.17: Multiple sequence alignment construction by MEGA X and Clustal Omega

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Pure Culture

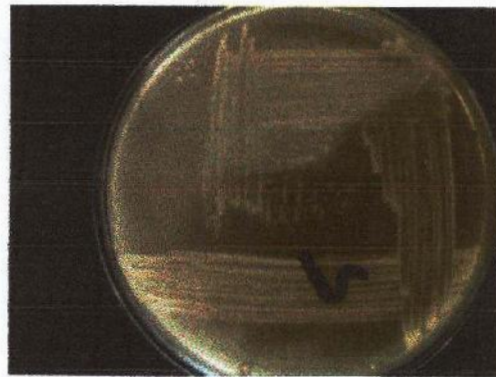
Ten samples (previously isolated from gastrointestinal tract of poultry) were cultured onto MRS media in petri dish. After 3-4 subcultures, pure cultures were obtained. Each plate was observed individually after 24-36 hours of incubation. Initially bacterial identification was performed based on colony characters and macroscopic observation. Among 10 samples, bacterial colony of 7 samples was whitish and rest was watery color. Table 4.1 represents the colony morphology of the bacteria samples.

Table 4.1: Colony morphology of isolates from GI tract of poultry

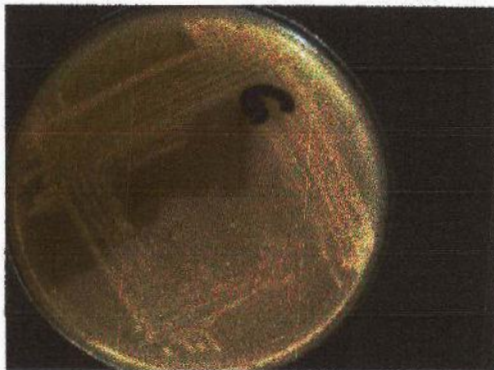
Sample No	Morphology
01	Medium, circular, convex, white colony
10	Medium, circular, convex, white colony
16	Small, circular, water color colony
17	Medium, circular, water color colony
18	Small, circular, white colony
19	Medium, circular, white colony
26	Medium, circular, convex, water color colony
27	Medium, circular, convex, white colony
29	Large, circular, convex, white colony
30	Large, circular, convex, white colony



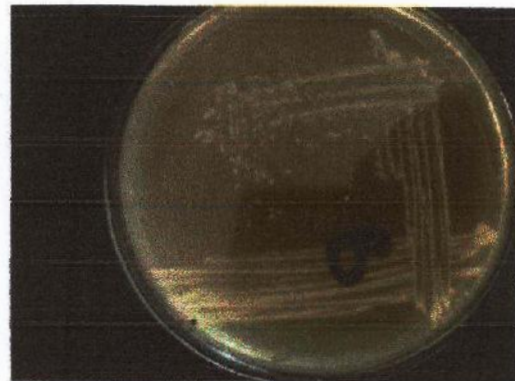
a



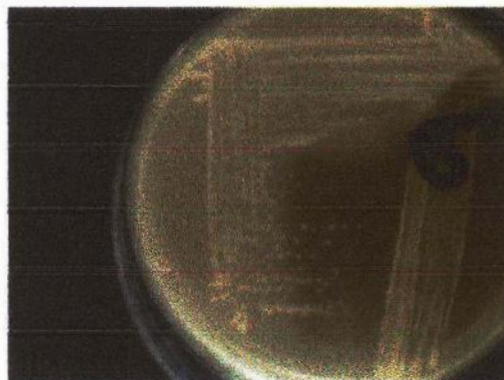
b



c



d



e

Figure 4.1: Pure colony of isolated probiotic bacteria from poultry gastrointestinal tract
(a: sample no. 2, b: sample no. 5, c: sample no. 6, d: sample no. 8, e: sample no. 9)

4.1.2 Genomic DNA Extraction

After obtaining the pure colony of the bacterial isolates, it was transferred in suspension culture (10 ml). The method was phenol-chloroform based that took about 4-5 hours to complete and was laborious. After DNA extraction, the DNA samples were observed by agarose gel run. In Figure 4.2, the gel run image has been showed.

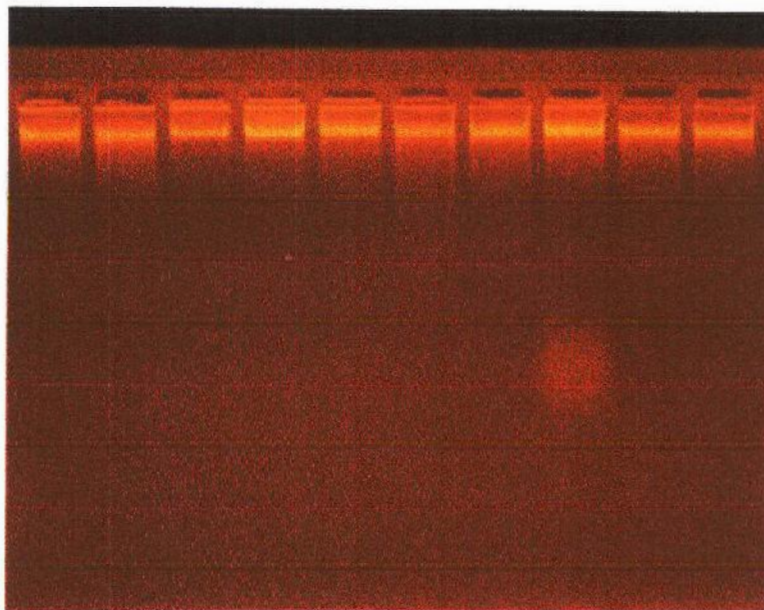


Figure 4.2: Gel documentation image of DNA sample after running on agarose gel; from left to right (1, 10, 16, 17, 18, 19, 26, 27, 29, 30)

4.1.3 Quantitation of DNA Concentration

A pure sample of deoxyribonucleic acid incorporates a quantitative relation of 1.8 at 260/280 and is comparatively free from protein contamination. A deoxyribonucleic acid preparation that's contaminated with protein can have a 260/280 ratio of less than 1.8. A reading of 1.6 does not render the DNA unsuitable for any application, but lower ratios indicate more contaminants are present. If a DNA sample has an A260/A280 ratio of greater than 1.8 this could suggest RNA contamination. Table 4.2 represents the ratio of absorbance of 10 DNA samples.

Table 4.2 Concentration and ratio of the DNA samples

Sample No.	DNA Concentration (ng/μl)	Ratio(260/280UV wavelengths)
1	340.8	1.83
10	332.5	1.82
16	384.0	1.91
17	336.8	1.82
18	368.6	1.86
19	423.4	1.83
26	1019.6	1.85
27	633.2	1.99
29	698.1	1.80
30	681.7	1.74

The following DNA samples were diluted to 50 ng/μl in a separate vial using the $S1V1=S2V2$ formula for proceeding PCR.

4.1.4 Gel Documentation of the PCR product

The extracted DNA samples were run for PCR with 27F and 1492R primers that resulted in the fragments approximately between 1400 to 1500 bp. The uppermost band in marker is of 100 bp, 1500 bp, and 2000 bp, respectively.

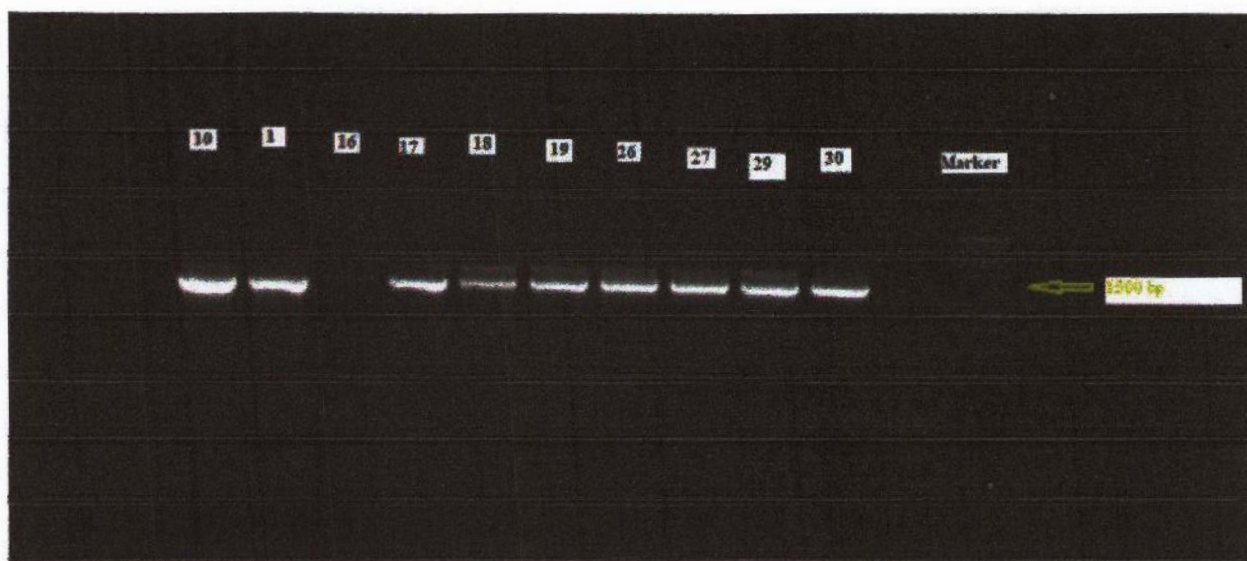


Figure 4.3: DNA bands in Gel Doc images (size of the 16S rRNA is 1500 bp)

4.1.5 Phylogenetic tree construction by MEGA X

The relationship among the aligned sequences was calculated using the UPGMA (unweighted pair group method with arithmetic mean) method. Phylogenetic trees of each of the 10 samples are given below:

A) Phylogenetic tree of Sample 1

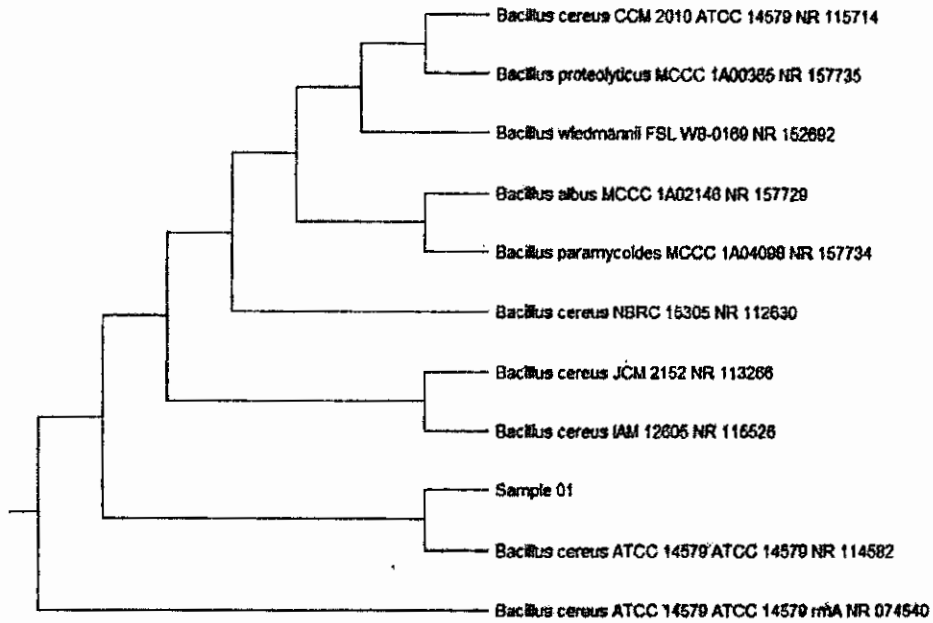


Figure 4.4: Construction of phylogenetic tree for sample 1 showed close relation with *Bacillus cereus* ATCC 14579 ATCC 14579 NR 114582 (sum of branch length 17.80243301; 1540 positions in final dataset)

Phylogenetic tree showing the relationship between *Bacillus cereus* ATCC 14579 and other *Bacillus* species. The tree is rooted at the bottom left. The species listed on the right are:

- Bacillus proteolyticus* MCCC 1A00365 NR 157735
- Bacillus cereus* CCM 2010 ATCC 14579 NR 115714
- Bacillus wiedmannii* FSL W8-0189 NR 152692
- Bacillus paramycoides* MCCC 1A04098 NR 157734
- Bacillus albus* MCCC 1A02146 NR 157729
- Bacillus pacificus* MCCC 1A06182 NR 157733
- Bacillus cereus* ATCC 14579 ATCC 14579 NR 114592
- Bacillus cereus* JCM 2152 NR 113266
- Bacillus cereus* IAM 12805 NR 115526
- Sample 10
- Bacillus cereus* NBRC 15305 NR 112630
- Bacillus cereus* ATCC 14579 ATCC 14579 rna NR 074540

61

C) Phylogenetic tree of Sample 16

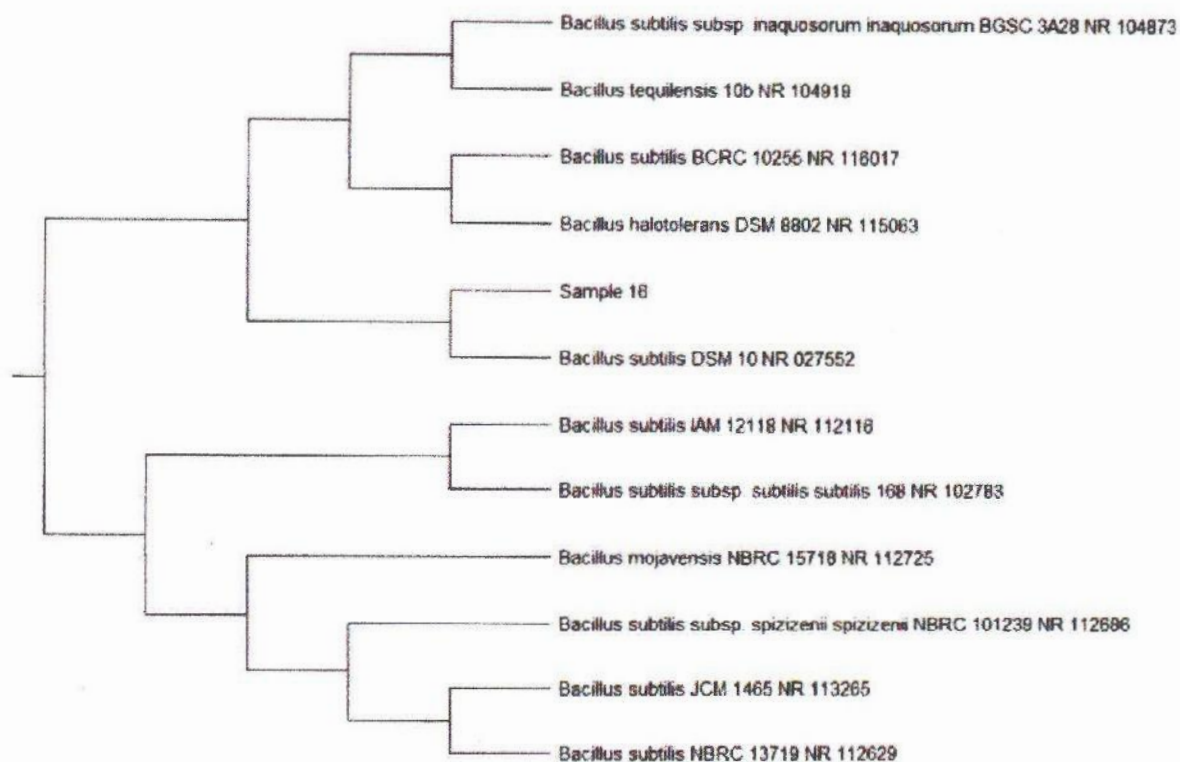


Figure 4.6: Construction of phylogenetic tree for sample 16 formed cluster with *Bacillus subtilis* DSM 10 NR 027552 (sum of branch length 19.94722822; 1550 positions in final dataset)



D) Phylogenetic tree of Sample 17

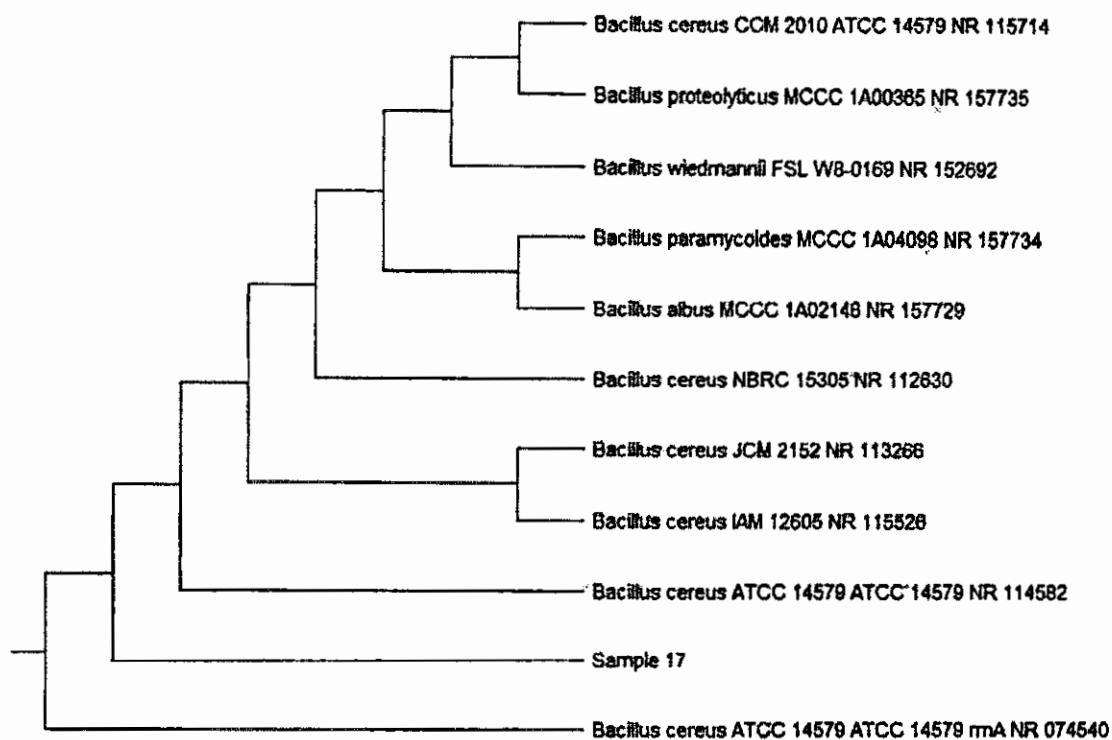


Figure 4.7: Construction of phylogenetic tree for sample 17 formed cluster with *Bacillus cereus*, *Bacillus proteolyticus*, *Bacillus wiedmanni*, *Bacillus paramycoides*, *Bacillus albus* (sum of branch length 18.928332864; 1540 positions in final dataset)

E) Phylogenetic tree of Sample 18

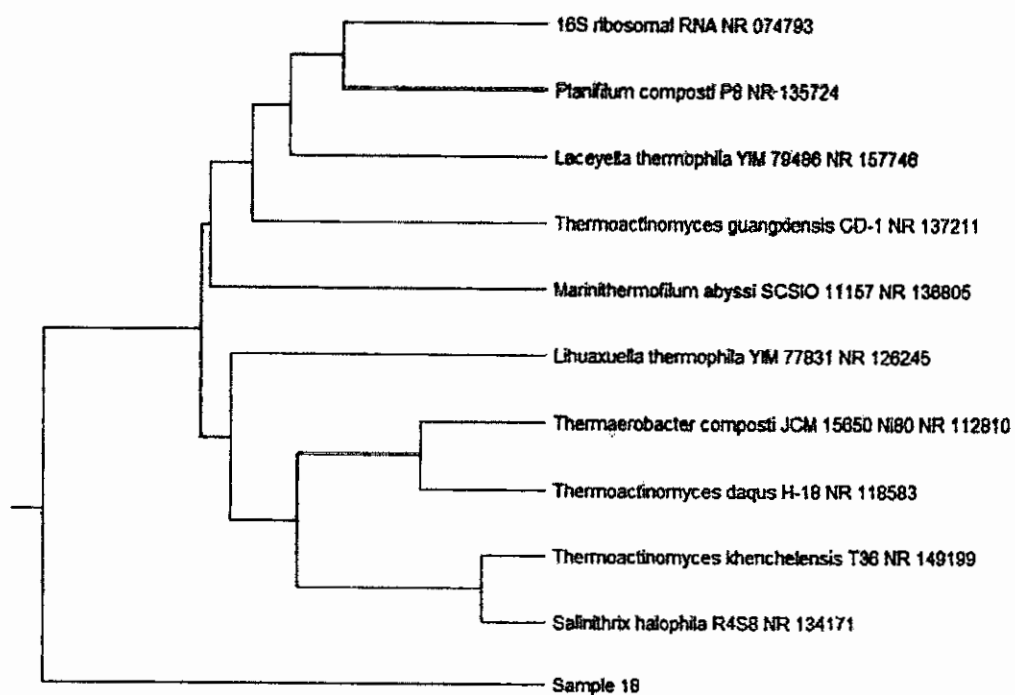


Figure 4.8: Construction of phylogenetic tree for sample 18

F) Phylogenetic tree of Sample 19

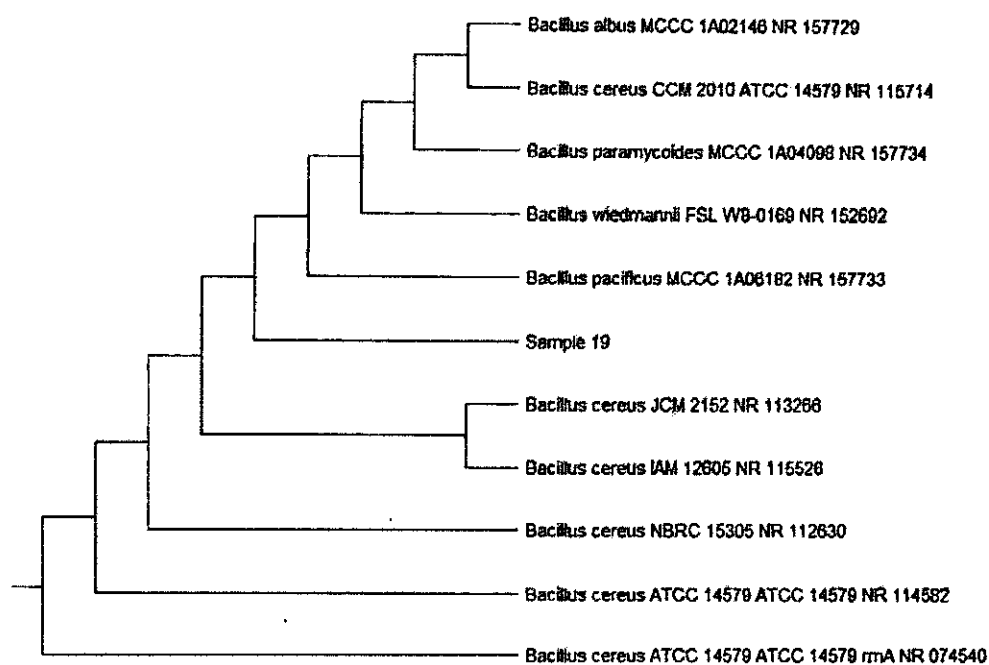


Figure 4.9: Construction of phylogenetic tree for sample 19 formed cluster with *Bacillus cereus*, *Bacillus pacificus*, *Bacillus wiedmanni*, *Bacillus paramycoides*, *Bacillus albus* (sum of branch length 15.04010514; 1540 positions in final dataset)

G) Phylogenetic tree of Sample 26

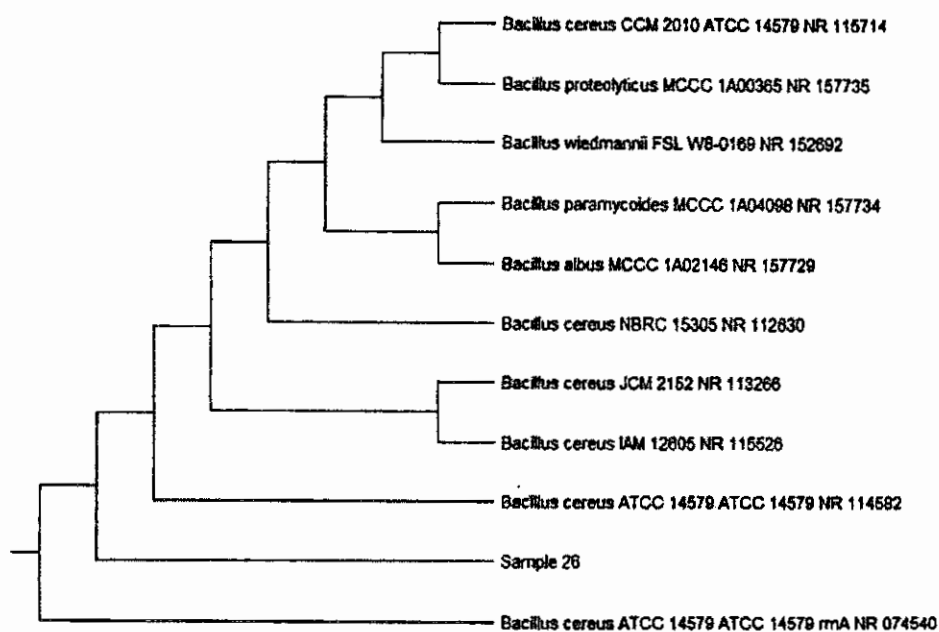


Figure 4.10: Construction of phylogenetic tree for sample 26 formed cluster with *Bacillus cereus*, *Bacillus proteolyticus*, *Bacillus wiedmanni*, *Bacillus paramycoides*, *Bacillus albus* (sum of branch length 18.89201939; 1726 positions in final dataset)

H) Phylogenetic tree of Sample 27

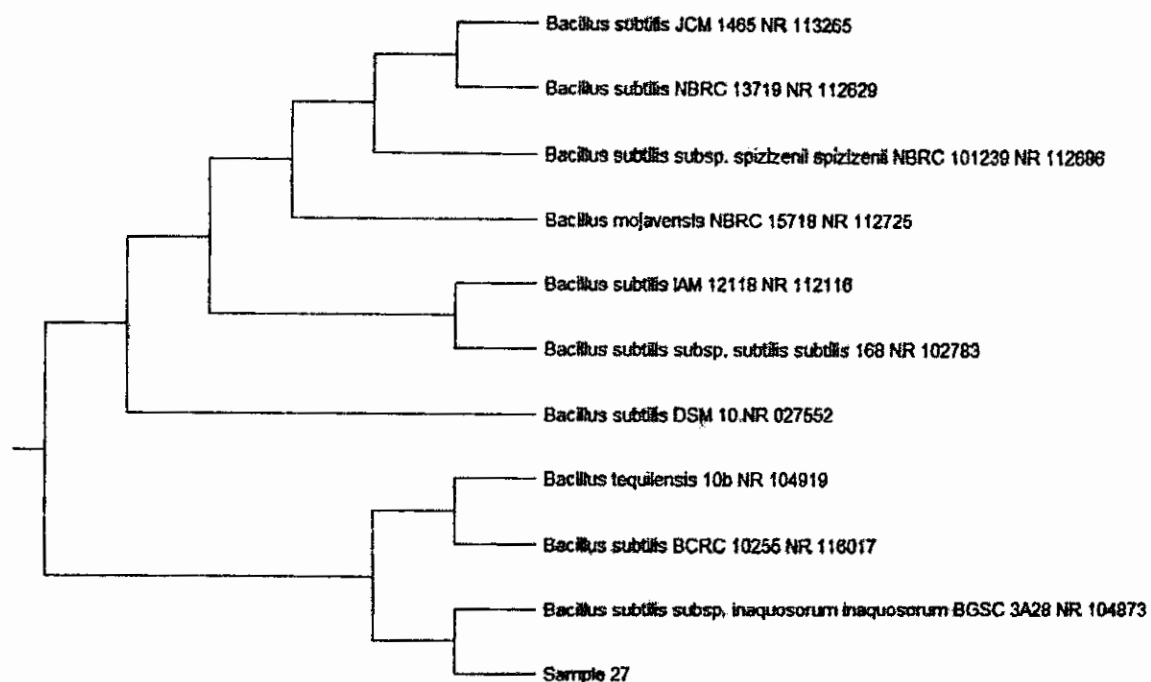


Figure 4.11: Construction of phylogenetic tree for sample 27 showed close relation with *Bacillus subtilis* subsp. *inaquosorum* *inaquosorum* BGSC 3A28 NR 104873 (sum of branch length 16.26644812; 1550 positions in final dataset)

I) Phylogenetic tree of Sample 29

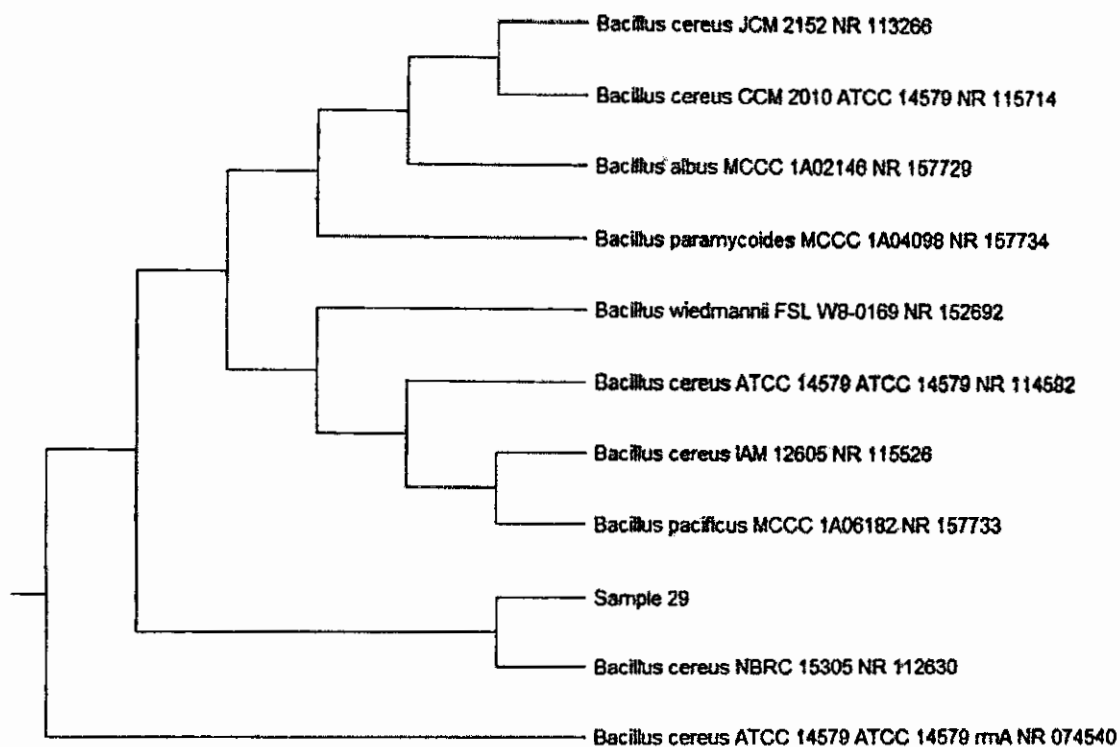


Figure 4.12: Construction of phylogenetic tree for sample 29 showed close relation with *Bacillus cereus* NBRC 15305 NR 112630 (sum of branch length 17.94103500; 1540 positions in final dataset)

J) Phylogenetic tree of Sample 30

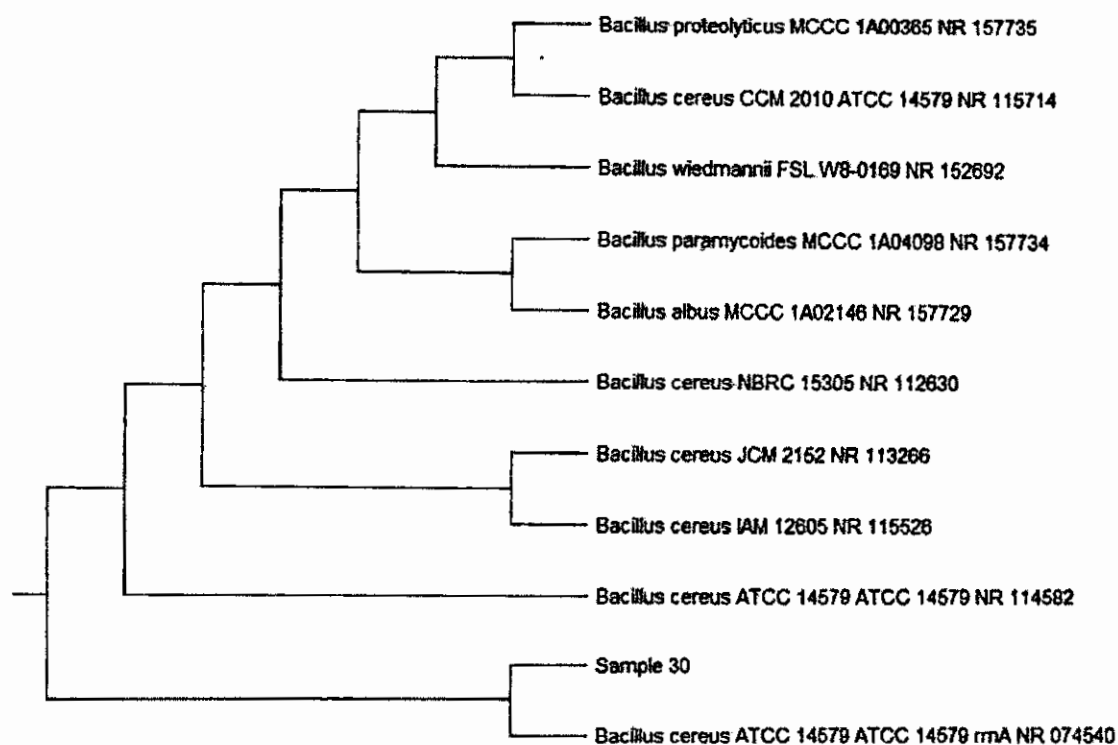


Figure 4.13: Construction of phylogenetic tree for sample 30 showed close relation with *Bacillus cereus* ATCC 14579 ATCC 14579 rna NR 074540 (sum of branch length 17.55702137; 1540 positions in final dataset)

A summary describing the entire results is given here:

Table 4.5: Probiotic isolates with corresponding species

Sample	Location	BLAST Match Sequence			
		Query Length	Similarity	NCBI Reference Sequence	Match with the species
C1	Dhaka	1412 bp	91.98%	NR_114582.1	<i>Bacillus cereus</i>
SI4	Cumilla	1410 bp	96.37%	NR_112630.1	<i>Bacillus cereus</i>
C3	Khulna	1413 bp	94.63%	NR_027552.1	<i>Bacillus subtilis</i>
CR4	Barishal	1421 bp	98.59%	NR_157734.1	<i>Bacillus Spp.</i>
C4	Barishal	-----	-----	-----	No specific matches found
SI5	Barishal	1495 bp	85.99%	NR_157734.1	<i>Bacillus Spp.</i>
SR2	Rajshahi	1726 bp	89.83%	NR_157734.1	<i>Bacillus Spp.</i>
CR2	Rajshahi	1419 bp	97.39%	NR_104873	<i>Bacillus subtilis subsp. inaquosorum</i>
SR1	Rangpur	1493 bp	84.15%	NR_112630.1	<i>Bacillus cereus</i>
CR4	Rangpur	1530 bp	96.66%	NR_074540.1	<i>Bacillus cereus</i>

4.2 Discussions

Bacillus signifies a Gram-positive, rod shaped, spore-forming, aerobic or facultative anaerobic bacterium. Generally, the genus *Bacillus* is known as a group of soil inhabitants. Nevertheless, *Bacillus spp.* can also be isolated from a wide range of sources including air, water, human and animal gut, and also from vegetables and food (Alou et al., 2015; Kotb, 2015). In terms of genotypic and phenotypic characteristics, *Bacillus spp.* has the most heterogeneous group including *Bacillus cereus* and *Bacillus subtilis*, two significant species (Elshaghabe et al., 2017).

The aim of this study was to identify potential probiotic bacteria from poultry gastrointestinal tract. For this reason 30 samples were collected from seven Divisions of Bangladesh and were isolated previously in the laboratory. The DNA samples were carefully extracted from the isolates grown from the stock solution after seven or more continuous subculture on selective MRS media and were evaluated by measuring the concentration with both UV Spectrophotometer and Nano drop. The pure colony was also observed and recorded for latter analysis. Ten DNA samples were selected after considering all the data for 16s rRNA PCR identification of the samples to identify the isolates to the species level. All the 10 samples had the (260/280 UV wavelengths) ratio between 1.74 and 1.99, not more or less than that, for assuring a desirable result. The PCR product had run along marker and a control which produced a uniform band of 1500 bp.

16S ribosomal ribonucleic acid sequences are used extensively within the classification and identification of microorganisms and archaea. The comparison of just about complete 16S rRNA factor sequences has been wide accustomed to establish taxonomical relationships between being strains with 98.65% similarity presently recognized because of the cutoff for delineating species. The comparison of the 16S rRNA factor sequence of associate isolate against sequences of kind strains of all being species provides a correct and convenient thanks to habitually classify and determine prokaryotes. Additionally, a workflow procedure is introduced that permits the analyses necessary for the identification of being strains, ranging from raw Sanger sequencing knowledge to the generation of biological process trees, by MEGA (Molecular Evolutionary Genetics Analysis) package. Application of those procedures can guarantee best apply in the manual redaction of 16S rRNA sequence knowledge and their use for taxonomical functions.

For constructing the phylogenetic trees, the evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. This analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

In the Figure 4.4 a phylogenetic tree of Sample 1 has been constructed from which it is clearly visible that the sample sequence is closely related and forms a monophyletic tree with *Bacillus cereus* ATCC 14579 ATCC 14579 NR 114582. It shows most probably the same characteristics and does the same function as *Bacillus cereus* ATCC 14579 ATCC 14579 NR 114582. Here contig is 16S rRNA gene sequence of DNA extracted from sample C1 which was collected from Dhaka. The Figure 4.5 demonstrates the phylogenetic tree of sample 10 which describes the sample sequence has a close relation with *Bacillus cereus* NBRC 15305 NR 112630. Here contig is 16S rRNA gene sequence of DNA extracted from sample SI4 which was collected from Cumilla. From Figure 4.6, the phylogenetic tree of sample 16, we can see there is a close relation of the sample sequence with the *Bacillus subtilis* DSM 10 NR 027552 strain. Here contig is 16S rRNA gene sequence of DNA extracted from sample C3 which was collected from Khulna.

The tree of sample 17 showed in Figure 4.7 describes the relation of the sample sequence with a cluster of a number of *Bacillus* Spp. including *Bacillus cereus*, *Bacillus paramycoides*, *Bacillus proteolyticus*, *Bacillus albus*, and *Bacillus wiedmanni* species. The contig is 16S rRNA gene sequence of DNA extracted from sample CR4 which was collected from Barishal. From the Figure 4.8 the tree of sample 18 it is visible that it has no close relationships with the species described. It could be a new strain or it can be a negative result. Contig of this sample is 16S rRNA gene sequence of DNA extracted from the sample C4 which was collected from Barishal.

The Figure 4.9 describing the sample 19 phylogenetic tree shows that it is closely related with three strains of *Bacillus cereus* (*Bacillus cereus* MBGIPS 9 KX950679, *Bacillus cereus* MBGIPS 2 KX950673, *Bacillus cereus* BRSC-S-A26MB K C460310) and *Bacillus thuringiensis* BH1 Atriplex halimus L KY910253. Here the contig is 16S rRNA gene sequence of DNA extracted from sample SI5 which was collected from Barishal. In the Figure 4.10, the tree constructed for sample 26 forms a cluster with a number of species of *Bacillus* (*Bacillus cereus*, *Bacillus albus*, *Bacillus paramycoides*, *Bacillus wiedmannii*, and *Bacillus proteolyticus*). Phylogenetic tree of sample 27 shown in Figure 4.11 describes that it is closely related with *Bacillus subtilis* subsp. *inaquosorum inaquosorum* BGSC 3A28 NR 104873 and forms a monophyletic tree with this strain.

The last two samples, sample 29 and 30 both forms a monophyletic tree with two different strains of *Bacillus cereus* (*Bacillus cereus* NBRC 15305 NR 112630, and *Bacillus cereus* ATCC 14579 rna NR 074540) which has been shown in Figure 4.12 and Figure 4.13, respectively.

Overall four samples are closely related with *Bacillus cereus* and two samples with *Bacillus subtilis* species. The other three samples are related with *Bacillus* Spp.

In a recent literature it has been shown that *Bacillus*-based probiotics are being used increasingly to maintain broilers' health condition due to their anti-inflammatory properties. In some other research works, some *Bacillus* species have been identified that are popular as probiotics for

animal feed applications are: *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, and *B. pumilus*. In a guideline of FAO/WHO of 2002 (Canada) it has been noted that these species have the spore-forming capability which is helpful for their management and their ability to replace antibiotics as growth promoters actually made them famous as probiotics. Among the variety of species of *Bacillus* used as probiotics in poultry, the *B. subtilis* strain 29784 (Bs 29784) has shown *in vivo* benefits in broilers, such as improvement of performance parameters under different rearing condition (Rhayat et al., 2019; Huyghebaert et al., 2011; Ukena et al., 2007). A study of 2010 showed the positive effect of a probiotic prepared with *Bacillus cereus* var. *toyoi*, on feed efficiency that was tested in broilers infected with *Salmonella enteritidis* (Gil de los santos et. al., 2005).

Based on FAO/WHO guidelines, analyzing and identifying probiotic microorganisms with 16S rRNA patterns can be considered as an accessible, cost-effective and suitable technique compared with other costly and time-consuming molecular techniques. This technique has also been utilized as an effective method to analyze and isolate probiotic bacteria from the chicken GI tract. However, based on the results and in comparison with the deposited sequences in the NCBI site, the homology levels among some strains are suggested to be more than 99%. This indicates that the 16S rRNA Gene sequencing technique is validated or sufficient enough for discrimination at strain levels.

Health-improving effects of probiotics include resistance to gastrointestinal acid and bile, having high anti-microbial activities and susceptibility against antibiotics. Therefore, probiotic characterization must be performed through standard *in vitro* experiments. The study is extremely promising, which underscores the important role of *Bacillus* strains, having probiotic effects, which can be used to improve the quality and safety of food, feed and beverages.

4.3 Conclusion

- The use of generic primers that did target the 16S rDNA region has the potential to correctly identify DNA template from specific bacterial species.
- The 16S rRNA gene sequencing of DNA extracted from isolated bacteria of 10 different selective samples is closely related to *Bacillus* species.
- Four samples are closely related to *Bacillus cereus* and 2 samples with *Bacillus subtilis*.

4.4 Limitations

- Inadequate funding
- Limited laboratory facilities

4.5 Recommendations

Further investigation of the identified species at strain level is highly recommended. Identification and characterization of the remaining 20 samples followed by an animal model trial is also suggested.

These identified probiotic isolates can be cryo preserved as seed stock for future application in food and new drug discovery. They can also be used for the production of different probiotic feed items for poultry and other mammals.

Findings of the study recommends further animal model trials to observe the beneficial effects of isolated probiotic strain for development of probiotic based poultry feeds which may play crucial role to use as substitutes of antibiotic based food additives.

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APPENDIX A

Gene Sequencing of Sample 1

CTATMATGCAGTCGAGCGATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAAC
ACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTT
GAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAG
CTAGTTGGTGAGGTAAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCAC
ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGA
AAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAACTCTGTTGTTAGGGAA
GAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGT
GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCA
GGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGAC
TTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACA
CCAGTGGCGAAGGCGACTTTCTGGTCTGTAAGTACACTGAGGCGCGAAAGCGTGGGGGAGCAAACA
GGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTT
AGTGCTGAAGTTAACGCATTAAGCACTCCGCTGGGGGAGTACGGCCGCAAGGCTGAAATCAAGGGAA
TTGACGGGGGCCCCGCACAAGCGGTGCAGCATGTGATTACTTCGAGCACGCGAAACCTTTACCAGTCTG
ACATCTTGACACCTAGAGATAGGGATTTTCTTSSGGACCAGAKTGCCAGKTGGKGCATGGTKGTSG
TCAGYTSKGKTCKTGAGATGTTGGGTTAAGTCCCGCAMCGAGCGCAMCCCTTGTTYTTAGTKSCCATC
ATTAAGTTGGCCMYTYTAAGGKGMCKSCCGGTGCCAAMCCGGAGGAAGGTGGGGAKGMSGTCAAAT
CATCATSCCYTTATGCCYTGGSYTCCCCMCGTGYTCCAATGGMCGGTMCAAAGAGYKCCAAGMCCSS
GAGGKGGAGTTAATTTCATAAAMCCGTTYTCAGTSSGATKGTAGGYTGCAATTSGCYTCCATGAAGC
TGGAATCGTTAGTAATCGCGGATCACCAKGCCGCGGGGAATMCGTTCCCGGCCYTKGTCCCMCCSCC
CGCCCCACCACGRGAKTTGWAMCACCSCAAKTSGGKGGGGWAACCTTGAGCCAGCCGCT

APPENDIX B

Gene Sequencing of Sample 10

GCAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTG
GGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAAYATTTTGAAC
YGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAG
TTGGTGAGGTAAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTG
GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGT
CTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAACTCTGTTGTTAGGGAAGAAC
AAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCA
GCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCCAGGT
GGTTTCTTAAGTTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTT
GAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACC
AGTGCGCAAGGCGACTTTCTGGTTCTGTAAGTACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGG
ATTAGATACCTGGTTGTCCACGCCGTAAACGATGAGTGCTAAGTGTAAGGGTTTCCGCCCTTTATGCT
GAAGTTAACGCATTAACCACTCCGCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAAATTGA
CGGGGGCCCCGCMCCAGCGTGAGCATGTGGTTTATTTGGAAGCACCGAAAGAACCTTACCAGTTTGGCC
TTCCTTGACCACCTAGAAATTGGCTTTTCTTCGGAACAGAGTGCCAGTGGTGCAAGGATTGTCTCCAG
TTCGKTTATGAAATGGGKTTAATTCCCCACGGASGCCAMCCYTKRRTYTWAKTKSCCTCCWTTAAG

TKGGCCMTTTTAGGKKGMYKSCCGKKGCCAAMCCGGAGGAAGTKGGGGAGGMSKCCAATTCTCCWK
 SCCCCTAAGGCCTTGGSTTCCCCCKKSTTCCAAGGGCCGTTCAAAGGASYKCAAGGMCCSGGGGKGG
 GASTTATTTCTTAAAMCCKTTTCCATTGTTGGGWTKTTGGSTKCCAMTSCCTTCCAGGAAGCGGGATTC
 GTTGTTATTCSSGGGTTACCAKSCGCGGGGAATMCTTCCCGGCCCTGGTCCCMCCSCCTCAACMC
 CMSGGGAKTTGKWACCMCCSRARKTCGKKGKGGKWAMCYTTTGGAGCCAGCCGCT

APPENDIX C

Gene Sequencing of Sample 16

TGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGSGTGAGTAACACGTGG
 GTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGWTTGAACC
 GCATGGTTCAAWYATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTARCTAGT
 TGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGG
 GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTC
 TGACGGARCAACGCCGCGTGAGTGATGAAGGTTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACA
 AGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAG
 CAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGT
 TTCTTAAGTCTGATGTGAAAGCCCCCGGCTYAACCGGGGAGGGTCATTGGAACTGGGGAACCTTGAGT
 GCAGAAGAGGAGAGTGGAATTCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTG
 GCGAAGGCGACTCTCTGGTCTGTAACCTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG
 ATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTTCCGCCCTTAGTGCTG
 CAGCTAACGCATTAAGCACTCCGCCTGGGGGAGTACGGTCCGACGTAAGTCTAAAGGAATTTGA
 CGGGGGCCCCGACAARCSGTGGARCMGTGGTTAATTYGAARCMACGCGAAGAACCCTTACCMGGTY
 TTGACMTYCTYTGACMATCCTAGAGATWGGRCGYCCCTTYGGGGGCMGARTGACMGGTGGTGAT
 GGTTGTYGTCAGCTCGTGTCGTGAGATGTTGGGTAAAGTYCCGCAACGAGCGCMACCCTTGATYTTAG
 TTGCCAGCMTTCARTTGGGCMCTYTAAGGTGRCTGCCGGTGACMAACCGGAGGAAGGTGGGGAWGA
 CGTCAAATCMTGCCCCCTTATGACCTGGGCTACMCMCSTGCTWCMATGGRCAGAACMAAGGGCA
 GCGAAACCSCGAGGTTAAGCCMATYCCMCAAATYTGTTTCMGTTCCGATYCMGTYTGCAACTYGR
 CTGCGTGAAGCCGGAATCGCTWGTATCGCGGATCAGCMTGCCGCGGTGAATACGTTCCCGGGCCTT
 GTACMCMCCSCCGTCMCMCCMCGRGAGTTTGTAACMCCCGAAGTCGGTGRGGTAACCYTTAGGAGC
 C

APPENDIX D

Gene Sequencing of Sample 17

ATMATGCAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAAC
 ACGTGGGTAACTGCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTT
 GAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAG
 CTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCAC
 ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGA
 AAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAACTCTGTTGTTAGGGAA
 GAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGT
 GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCA

GGTGGTTTCTTAAGTCTGATGTGAAAGCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGAC
 TTGAGTGCAGAAGAGGAAAGTGGAAATCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACA
 CCAGTGGCGAAGGCGACTTTCTGGTCTGTAAGTACACTGAGGCGCGAAAGCGTGGGGAGCAAACAG
 GATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTAGAGGGTTCCGCCCTTTA
 GTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAA
 TTGACGGGGGCGCCGACAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCCTACCAG
 GTCTTGACATCCTYTGACAACCCTAGAGATAGGGCTTYTCCTTYGGGAGCAGAGTGACAGGTGGTGCA
 TGGTTGTGTGTCAGCTCGTGTCTGTGAGATGTTGGGTAAAGTCCCGCAACGAGCGCAACCCTTGATYTTA
 GTTGCCATCATTTAGTTGGGCMCTYTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGA
 CGTCAAATCATCATGCCCTTATGACCTGGGCTACMCMCGTGCTACAATGGRCGGTACAAAGAGCTGC
 AAGRCCGCGAGGTGGAGCTAATYTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTYGCCTA
 CATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGAC
 ACACCGCCCGCTCMCMCCMCGAGAGTTTGTAAACMCCCGAAGTCGGTGGGGTAACCTTTTGAGCCAGC
 CGC

APPENDIX E

Gene Sequencing of Sample 18

AGAGTCCAGGAGTCAGCTCGTAAGGTCTCGMGTGCTCCGATATCCATGTCACGCTGWCGACCCGTCA
 ATCCTTGRGTCARSTTGCGGMCATCTCCAAGGCGARKSTAATGSSTTACGTCGMGTATAAGGCGCG
 AATCCTCTTAMCATWGMGTCWCWCGYGTATMCGCGTGRCWTCCMRGKGATCTAATAYCGTTGCTY
 GCACAGCGYTYGCGCTCTASWGTYAGATGACACAGCAGAGAGTCKCGCTTCGCCACWGTGTGYCTA
 YAYATCTAYRCRCATCACAGCKAYACAYGGAATTTACWCTCTCTCTGCRWCACGTSTCCCACAGTT
 CAATGWGACTCTACGSGTGAGASSYGGGSKTTWCATMTGASWYAWGAGAMCAGYGYGCGCGCKST
 ACACGCAATATTTTCGGATACSGCTSGCTCTACATATGTGTTACTYCRGCGGACTBCGGTGTWACATAK
 ATATMTCGTGGTSTGCTYGGGYRKATGBMGTACAAGGYCCRGGAACGTATWCACSTAGCRGCATGTT
 CTBATCCGCGATTACTACCGATTCMGAGCTTCRCGYAKGCGAGTTGCAACCTGCWWCTCACAMACTG
 AGRRCGGTTCTGTVTSAGATTGCTTNCACMTCGCGAAGAGATCCCMTTGCTGCTCTTTGTCCGGCCCTA
 GGAGTGTGKRGACGTGTGTCACAGCCACAGGTCWTAAGGGGCGATGATGMTCCCCCTCACATGRSGT
 CATSCMCASYKKCTCCGGTTGGTCAGCCGGCWCCTCACCCCWTAAGKGGCCCACTAATGCCCKG
 GGTCACCCCACTATAARTCATAGGRWWAGCGCWCGTTTGCGGGACTTATACCCAWTGAATCTCAYGA
 CACGGAGCTKACRAACAACCATSCGGTATTAGCCACCTGGTTTCMCWAGTTRGCTCCCAGTCAACAGG
 CAGRITACCYACKTRTTCTTCTTASSSATYCCCGTCAVCATCAGGGATGGTCAAGACCTSGTAAGGGTY
 CTYCGCGTYGACTKCGAWGATTAAAACACATGCTCACGCKTGGTGCGGGCCCGTCATTCTTTGAGTTT
 CAGTCTGCACGTAATCCAGGGCGGAAGTGCTATGCGTTTCTGCAGCACTTAAGGGCGAATCTCTAC
 ACTAGCACTCATCGATTACGCCGATGAACTTACCTAG

APPENDIX F

Gene Sequencing of Sample 19

GTCACWTAGCGGCTGGCTCTAAAGGTWACCCCCGAYTTCGGGGGTAAAAYTCTCGGGGGGTGACG
 GSGGGGGGWACAAGGCCCGGAACGWATTCACCGSGGMWTGYTGATCCSCGATTACTASSRATTC
 RGCTTCWGGWAGSCAAGTTGCASCCTACATTCCRAACTGARAACGGTTTATGAAATTGSYTCCACCT

CSGGGCTTTGCRGTTCTTTGWACCGYCCATTGWASMACGKGTGWASCCCAGGYCATAAGGGGGMATGA
TGATTTGACKYCWTCACCTTCCCTCCGGTTTGYCMCCGMRGYCACCTTAAAKGGCCCAAYTAAA
GGAKGGMAAYTAAATCAAGGKTTGCGCTCGTTGCGGAACCTTAACCCACCATCTCACAAACACRASTTG
ACRACAACCATGCMCCACCTGTCACTCTGCTCCCAAAGGARAACCCCYRATCTCTAGGGTTGCMGAAG
GATGTCAAGACCTSKTAASGTTCTTGCGCGTTGCTTCGAATTAAYCACATGTTCCACCGCTTGTGCGG
GCCCCCGTCAATWCCTYTGAGTTTCAGMCTTGCGGACCGTACTCYCCASGGCGGGAGTGCTTAATRGC
GTTWASCKTSCAGCACTAARGGGGCGGAAAACCCCTCTAACACTTTAGMCACTCATTTCGKTACGGG
CGTGAVACTAYCAGGGKTAATCTAATCCTTGTTGGYCTCCCCACGCTTTTCGCGCCTCTCAGTGTCC
AGTTMCMGACTCAGAGAGTTTCGCCTTTGCCCCACTTGAGTRTSCCTCMATCATTCTCTACGCAYTTC
ACCGCTTAACACATGGAAATCHACTTTCTCTTCTGCACTCRAGATCTCCAAGTDTCSAATGACCSTC
CACGRDTGAGCCGTGGGCTTTCACATCAGRCWTAAGAAACCACGTGCGCGCGCTYTACGCGCACTAAT
TCCGGATAACGCTTGCCACMTAYGTATTACCGCGGSTGCTGGCACGTAGTTAKCCGTGGCTTTCTGGTT
AGGTRCCGTCAAGGTGCCAGCTTATTCAASTAGCACWTGTTYTYCCTAACAAACAGAGTTTTACGACC
YGAAAGCCTTCATCACTCACGCGGCGTTGYTCYGTGCACTTTTYGTCCAYTGCGGAAGATTCCCTAYT
GCTGCCTCCCGTAGGAGTSTGGGCGGTGTSTCAGTSCCAGWGTGGCCGATCACCTCTCAGGTGGSTA
CGCATCGTKGCCYYGGTGAGCCGTACCTCWCCAMCTAGMTAATGCGACGCGGGTCCAYCCATAAG
WGACAGCCGAAGCCSCCYTTCAATTTGAACCATGCGGTTCAAAAYRTTATCCGGTATTAGCCCCGGT
TTCCCGGAGTTATCCAGTCTTATGGGCAGGTTACCCACGTGTTACTCACCCGTCCGCCGCTAAMTYYA
YARGAGCAAGCTCTYMATCCRTTCGCTCGAYTGCATKATAGMRAGCAWGCAAAAAGTGWCGATTGG

APPENDIX G

Gene Sequencing of Sample 26

GGGGCAASWSMRAGAATCAATGGCTGKGSACKTATMATGCAGTCGAGCGAAYGGATTARAGCTTGCT
CYTRTRRKTTAGCGGCGGCACGGGTGAGTMACACGTGGGTMACCTGCCATAAGACTGGRATAACTC
CGGGAAACCGGGGCTAATACCGGATAAYATTTGAACCGCATGGTTTCGAAATTGAAAGGCGGCTTCG
GCTGTCACTTATGGATGGACCCGCGTCGCATTAKCTAGKTGGWGAGGTAACGGCTCACCARGGCAAC
GATGCGTASCCGACCTGAGAGGGTGATCGGCCACWSTGGGACTGASACACGGCCASACTCCTACGGG
AGGCAGCAGTAGGGAATCTTCCGCARTGGACRAAAGTCTGACRGAGCAACGCCGCGTGAGTGATGAA
GGCTTTCRGGTCGTAAAACTCTGTTGTTAGGRAAGAACAAGTGCTASTTGAATAAGCTGGCMCCTTGA
CGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCASC CGCGTAATACTAKGTGGCAAGCG
TTATCCGGAATTATTGGGCGTAAAGCGCGCGCASGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCT
CAACCGTGGAGGGTCATTGCGAAACTGGSWCACCTGAGTGCAGAAGMTGGCTCAAAGTGGATTYCCC
CGTGAATGTAGCSGWGAAGTGCGTARARACTMTGGRGTGRRSACCAGTGGCGRRGVGACTTTCWAG
GYCKGGGTAACCTGAATCCACTGAGGCGCGAAWCGTGGACCCGAGMATAACAAGGAATTMCGGWT
WCTGGTAGGYAAGTSGCACGCCTATAAACCRAATGAGTGMWAACGGTKTTDGARRGTTAACTYCCG
CCCTTTYMTGCTGAAGYCTDGAACKCCWTTGAACGGCWCTCCVRTGTGAGAGTACGGGGGAACCG
CAAGGCTGAACTYAAGGGGATGGAGAAKTGAACGGGGGCKCACMMGCSSTWWGCMTCCTGTTGG
TMACTTCGAAGCACGCGAGAACCTTAAAMGAGKCYVACTTMAAGHWGGMCACTAAGAATAAAGGRT
TGCYYYCGTGGCGGAACCTAACCAAMCTTCTCACAAACCAAGTGAASAAMAACAGGMACAMCTGG
YCMTCGGTCCCCAAAGGARAASCCTAACTCCTAGGKTGGTMRAGAAGGYMAAACTGGKWAAGG
TCCTTCSGGTGGTTTSRAATTAAMCMMMTGGYTMMCGSYTGKGSGGSCCCSKYMAATTCTTTKAA
KTTYMRSCYTGGSGSCKWMYCCCMRGGSGAAKTGYTWAWKSCKTTACYTTSCRSMMYWAAGGGS
SGRAAMCCCTYYWAMAMYTWASMMMYMWYSGTTTWSGGSKKGAMYWCMRGGTAWYYYWA
WYCYGKGTTGSYYYCCMMMSSTTTTSCSSGYCTTMRKGGYMRGTTWMMCRACCCARAAAARGT
CSGCCCTTSSCMMYTKGGKKGTTYCCYTCAWAATTTTTYWWAMSCSMWTTTTMACCGSSCTWMA

CACATGGAATTCCCAMCJTITTYCCTYYCTTYYKGCAMYTCAAGGTCTCCCCARGTTTCCAATKGAACC
CTYCCMCGGATTGGAAGCCGGKGGGGCTTTCMACATCARAACTTAGRAAACCACCTGGSCSGMGCG
SCCTTTACGCCTCGAATAAGTTCCCAGATGACGCTTGGCAGCCTAACGTATTATAGTG

APPENDIX H

Gene Sequencing of Sample 27

CTATMATGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAAC
ACGTGGGTAACTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGWT
TGAACCGCATGGTTCAAAYATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATT
GCTAGTTGGTGAGGTAAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCA
CACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACG
AAAGTCTGACGGAGCAACGCCCGGTGAGTGATGAAGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGA
AGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACAGAAAGCCACGGCTAACTAC
GTGCCAGCAGCCGCGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGC
AGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGTCATTGGAAACTGGGGAA
CTTGAGTGCAGAAGAGGAGAGTGAATTCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAAC
ACCCAGTGGCGAAGGCGACTCTCTGGTCTGTAACCTGACGCTGAGGAGCGAAAGCGTGGGGGAGCGAA
CAGGATTAGATACCCTGGTAGTCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCT
TAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGG
AATTGACGGGGGCCCCGACAAAGCGGTGGAGCATGTGGTTTAATTYGAAGCAACGCGAAGAACCTTAC
CAGGTCTTGACATCCTYTGACAATCCTAGAGATAGGRCGTYYCCCTTYGGGGGAGAGTGACAGGTGGT
GCATGGTTGTGTCAGCTCGTGTCTGTGAGATGTTGGGTAAAGTCCCGCAACGAGCGCAACCCTTGATY
TTAGTTGCCAGCMTTCAGTTGGGCMCTYTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGA
TGACGTCAAATCATCATGCCCTTATGACCTGGGCTACMCMCGTGCTACAATGGACAGAACAAAGGG
CAGCGAAACCGCGAGGTTAARCCAATYCCMCAAATYTGTTYTCAGTTCGGATYGCAGTYTGCAACTY
GACTGCGTGAAGCYGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCT
TGACMCMCCSCCGTCMCMCCMCGAGAGTTTGTAAACMCCCGAAGTCGGTGAGGTAACCTTTAGAGC
CAGC

APPENDIX I

Gene Sequencing of Sample 29

CTRMGGSAGCTATAATGCAGTCGAGCGATGGATKRGAGCTTGCTCTTGTGGKTTAGCGGCGGAGGGTG
AGTAACACGTGGGTAATGTGTGCATAAGACTGGGATCTTCCGGGACAGGGGGCTAATACCGGATAAC
ATTTTGAACCGCATGGTTCGAAATTGAAATGCGGYTTCCGCTGAYACTTATSGATGGACCCGCGKSGC
ATTATCTAGKTGGTGAGGTAACGGCTTCTTAYACGRGGVAACKATGCGTACCCBASCTGATTGACGG
STGAYCGGHCAGTGWSCAGGTCYASAYAMGGCCWCRCCTACGAGGMCGGGCATAWGTAGGG
AGTATCKCTCCAAGCAAGTGACGAHGTCTGACGGAGCMACGCCGCGTGAGTGATGAACGCCGTCT
TTCGGAGSWCGWARGAWCTCTGYATGTTAGAAGYGAVGRACAHGYGSTAGWTGAATAAGCTTCGGT
CGTAACTCTTGACGGTACCTAACAKCTDTGAAAGCCGCGRCTRACGTACGTRCCAGMAGCCGCGVTA