Evaluation of Control of MDR *Campylobacter jejuni* colonization by indigenous probiotics LAB in Broiler Chickens

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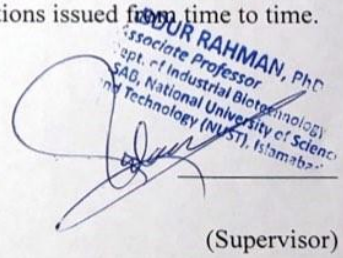

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DEDICATION

This work is humbly dedicated to all my valuable treasures in life:

My beloved parents My adored siblings

For always standing beside me even for better for worst

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GLOSSARY OF ABBREVIATIONS

A number of abbreviations have been used in this study. These abbreviations are either used commonly in the literature or accepted universally. The abbreviations are:

AMR: Antimicrobial resistance

AIDP: Acute inflammatory demyelinating polyadiculoneuropathy

AMAN: Acute motor axonal neuropathy

AMSAN: Acute motor and sensory axonal neuropathy

CDC: Centers for Disease and Control and Prevention

CDT: Cytolethal distending toxin

CFS: Cell free supernatant

CFU: Colony Forming Unit

GIT: Gastro-intestinal tract

GBS: Guillian-Barré syndrome

IBD: Inflammatory Bowel Disease

IBS: Irritable Bowel Syndrome

LAB: Lactic acid bacteria

MDR: Multi-drug resistance

MCCDA: Modified Caracol cefoperazone-deoxycholate agar

REA: Reactive Arthritis

SEM: Scanning Electron Microscope

VBNC: Viable but non-culturable

WHO: World Health Organization

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ABSTRACT

Campylobacteriosis is the most common zoonotic illness affecting people globally and contaminated chicken with *Campylobacter jejuni,* and is one of the major causes of enteric infections in humans. An efficient substitute i.e. the use of probiotics for lowering bacterial contamination in the livestock, which can strengthen the animals' natural defenses against harmful bacteria. In-vitro experiments showed that *Lactobacillus reuteri* and *Enterococcus faecium* isolated from the gut suppressed and control the development of *C. jejuni* count by using a 96-well microtiter plate and the plating technique. We designed an in vivo experiment using a poultry model to test the effectiveness of prospective indigenous probiotic strains against *MDR Campylobacter jejuni* in order to illustrate this impact in vivo. A poultry model was used to assess the effects of an indigenous probiotic combination of *Enterococcus faecium* and *Lactobacillus reuteri* on the growth performance of chicks and cecal microbiota of broiler chicks challenged with MDR *Campylobacter jejuni*. Total forty two, one-day-old birds were given an organic diet with probiotics (10^8 CFU/mL) in their drinking water, and they were given an oral challenge (10^5 CFU/mL) of *Campylobacter jejuni* using a micropipette. Probiotic administration reduced the cecal Campylobacter population ($P<0.001$) and considerably ($P<0.01$) increased feed efficiency and growth performance. Our in vivo study findings indicate that giving broiler chicks probiotics decreased the amount *of C. jejuni* cecal colonization.

CHAPTER-1

1 INTRODUCTION

Globally, there is a serious issue with food safety in both wealthy and developing nations. The World Health Organization (WHO) estimates that diarrheal illnesses, primarily from consuming food that is contaminated, cause about 500 million illnesses and 240 000 deaths each year. (World Health Organization, 2017). Among the pathogenic bacteria, Campylobacter species is highly ranked compared to its rivals, including Salmonella and Escherichia coli (Javed et al., 2013). In Pakistan, the prevalence of Campylobacter spp. was found to be significantly more in the food items that not only includes raw food but also the undercooked food. The overall prevalence of *Campylobacter* that was found to be in Pakistan was found to be 21.5%, and out of which 71.6% were identified as *C. jejuni* and 28.4% as *C. coli*. Campylobacter infections is one of the main causes of bacterial gastroenteritis in people in the whole world. (Hussain et al., 2007). Every year, reports of human *Campylobacte*r infections total 1.3 million in the United States alone. There are around 17 different species of *Campylobacter* known to exist, but only *Campylobacter jejuni* is actually thought to be the one of the main cause of 95–99% of human infections (*Campylobacter (Campylobacteriosis)* | *Campylobacter* | *CDC*, 2021). The majority of cases of Campylobacter enteritis resolve on their own, although certain serious post-infectious consequences, including reactive arthritis and Guillain-Barré syndrome, have been documented. There are several known sources of *Campylobacter jejuni*, but chicken is thought to be the main way that people become infected with the bacteria. It has been revealed that *Campylobacter jejuni*, which poses a major risk to human health, is prevalent in almost 90% chicken flocks. Therefore, lowering or getting rid of

Campylobacter in chicken flocks will greatly lower the prevalence of campylobacteriosis in humans (Kaakoush et al., 2015). In an effort to lower the frequency of *Campylobacter jejuni* in chicken flocks, a number of preharvest intervention techniques have been studied, including biosecurity, vaccines, bacteriophages, vaccines, organic acids that includes medium chain fatty acids, and prebiotics. Regretfully, none of them are able to totally eradicate Campylobacter from chickens. (Al Hakeem et al., 2022)

In response to public pressure, the negative effects of antibiotics on avian health, and the need for alternative medicines to maintain low rates of death and morbidity as well as to improve feed and growth efficiency, Antibiotics can undoubtedly be replaced with a variety of non-therapeutic alternatives. The most widely used of these are probiotics. Nonpathogenic bacteria known as probiotics have beneficial effects on mammals and are defined as "Live microorganisms which, when administered in adequate amounts, can confer beneficial effects on host health". They haven't maintained genetic stability or passed on antibiotic resistance genes to other organisms up to now (Prabhurajeshwar & Chandrakanth, 2019). These microorganisms, which can withstand lysozyme enzyme, intestinal alkalinity, and stomach acid, are mostly found in the crop, gizzard, and ileum regions of chicken. Probiotics possess a potent ability to attached to the walls of to the intestine and combat pathogens that cause gastroenteritis. Several probiotics, including *Lactobacillus reuteri, Enterococcus lactis, and Enterococcus faecium*, have been shown to reduce the pathogenicity of pathogens in poultry. The following are the ways that probiotics work in poultry: mucin adhesion, competitive exclusion, competition for life, competitive adhesion to the intestinal site, and metabolite synthesis. Due to their exceptional ability to survive and attach to the environment of intestine, as well as their

function in reestablishing the gut microbiota, lactic acid bacteria are the preferred. In order for probiotics to successfully combat infections and In order to accommodate GIT, their source is thought to be a crucial component. Therefore, for the best possible poultry production, host-specific probiotic strain selection is essential. In vitro experiments involving competitive inhibition, growth dynamics, and co-aggregation are used to characterize possible probiotics against infections. One useful method for assessing and testing the efficacy of probiotic isolates against multidrug-resistant (MDR) infections is in vitro characterization (Tareb et al., 2013).

Other than probiotics, parabiotics- the heat killed from of probiotics can be used to control the colonization of Campylobacter in poultry. A number of techniques, including heating, use of deadly chemicals that includes formalin, use of radiations like gamma and other UV radiation, and the use of sonication, can be used to inactivate living bacteria; nevertheless, heat treatment is still the most used technique. However, the manner in which various techniques of inactivation operate, their impact on the structure of cell and its elements, and their influence on biological activities continue to differ. To guarantee that all of the bacteria in the solution are killed, heat treatments use a variety of time-temperature combinations. Combining the processes of tyndallization and cell freezing up can also result in inactivation. Pour plating was used in the study to validate the deadly impact of the heat-killed suspension of bacteria, which was made by heating the suspension of cell (10^8 CFU/mL) to 80 °C for 30 minutes. The possible mechanism by which parabiotics can control the *Campylobacter jejuni* number is by competitive adhesion that has proved effectiveness. In terms of their capacity to contend with food-borne and diarrheal pathogens for adhesion sites on the cells of gut, parabiotics show

encouraging promise. Furthermore, in vitro and the other in vivo trails have shown the anti-inflammatory markers like IL-6, TNF-α, and to enhance anti-inflammatory cytokines like IL-10) and anti-oxidative (ability to scavenge the free radicals) effects of parabiotics of Lactobacillus and Enterococcus to control different pathogens in poultry. But probiotics showed more promising effects in controlling the number of *Campylobacter jejuni* in poultry than parabiotics (Nataraj et al., 2020).

Introducing probiotic bacteria into chicken is one tactic that could prevent or lessen the colonization of *Campylobacter jejuni*. Food-borne infections including Salmonella, E. Coli, Listeria, Clostridium, etc. were successfully decreased by probiotics. Probiotic treatment, however, may not always result in a decrease in *Campylobacter* colonization in broiler chicks. Such uneven outcomes in the fight against *Campylobacter jejuni* colonization indicated the need for improved probiotic bacterial screening techniques (Taha-Abdelaziz et al., 2019a).

The research objectives of the study are as follows:

1) The first objective is the in-vitro assessment of indigenous probiotics and parabiotics of *Enterococcus faecium* and *Lactobacillus reuteri* to control Campylobacter *jejuni.*

2) The second objective is in-vivo testing of probiotics to control *Campylobacter jejuni* in poultry.

CHAPTER 2

2 REVIEW OF LITERATURE

One of the biggest issues facing public health today is food-borne disease, which is mainly brought on by consuming food tainted with bacteria, viruses, parasites, and/or chemicals. According to reports, the poor sanitation and socioeconomic circumstances that predominate in emerging and underdeveloped nations increase the risk of food-borne diseases. On the other hand, a growing number of food-borne illnesses in wealthy nations have been reported by recent research. The overall prevalence of *Campylobacter* in the whole Pakistan was found to be 20.5%, out of which about 71.6% were identified as *C. jejuni* and 28.4% was identified as *C. coli* (Hussain et al., 2007). Most of the prevalence was found in the food commodities in Pakistan. Despite having one of the cleanest food sources in the world, 1 in 6 people in developed countries are said to become ill from foodborne illnesses, which leads to 129,000 hospital admissions of the people that were infected with *Campylobacter* infection and 3,000 fatalities annually in the US. Worldwide, one of the main causes of food-borne disease in humans is campylobacter infection (Kaakoush et al., 2015). In 2013, there were around 13.85 instances recorded for every 100,000 persons in the United States alone. and it's predicted to cost the economy $1.7 billion a year. Similarly, it has been estimated that there are around ten million human cases of campylobacteriosis in the European Union, which results in an annual economic loss of € 2.4 billion. It was shown that the total frequency of Campylobacter in poor nations such as Pakistan was 21.5% (Hussain et al., 2007).

2.1 CHARACTERISTICS OF CAMPYLOBACTER

2.1.1 History, Taxonomy and Nomenclature of *Campylobacter*:

Theodor Escherich initially noticed a distinct rod shaped and S-shaped bacteria in stool samples from newborns who had diarrhea in 1886, and this bacteria was subsequently recognized as Campylobacter. Two scientists McFadyean and Stockman recovered the species of *Campylobacter* from the embryonic tissues of lambs that had been aborted in the early 1900s. Since then, three scientists have identified comparable microbes from sheep and calves that had diarrhea and aborted bovine fetuses. The organisms were formerly categorized under the genus Vibrio, but the scientists Véron and Sebald advocated the creation of the new genus Campylobacter in 1963 after separating Campylobacter from the genus Vibrio. Because of their low DNA base makeup, non-fermentative metabolism, and microaerophilic growth, Campylobacter and Vibrio vary significantly. Due to their distinct traits from the genus Vibrio, Véron and Chatelain further categorized Vibriolike organisms in 1973 into the type species, *C. fetus*, along with *C. sputorum, C. coli* and *C. jejuni*. Later, the genera Helicobacter, and Sulfurospirillum, and Acrobacter were added to the Campylobacter genus, which was reorganized as the Campylobacteraceae family. While Campylobacter has been detected in at least 17 species, more than 90% of cases in patients of campylobacteriosis are caused by *Campylobacter jejuni* and the other *Campylobacter coli* (Smialek et al., 2018).

Originating from the Greek terms "kampulos" and "bacter," which mean "curved" and "rod," the name "Campylobacter" was created. Every member of the genus Campylobacter is a gram-negative, microaerophilic, rod that is 0.5–5 µm length and 0.2–0.8 µm broad. Because of its solitary polar flagellum, which is about twice as long as the

cell, campylobacteria are incredibly motile and exhibit a distinctive corkscrew-like movement. Certain species, such *C. gracilis,* do not move means have no flag, whereas *C. showae* possesses numerous flagella to move. These are the exceptions (Santini et al., 2010).

Because they are picky eaters, campylobacter spp. require complicated growth media and microaerophilic ambient conditions in order to flourish. Under microaerophilic circumstances, which means under very low oxygen concentration optimal development is shown at 42°C. Although *C. jejuni* requires a high temperature to flourish, they exhibit physiological activity as low as 4°C. According to reports, *C. jejuni* may withstand environmental challenges by transitioning from spiral-bacilla to coccoid forms, which are indicative of a lack of culturability but are still alive. The shift from spiral to coccoid viable but the condition that is non-culturable and is also caused by adverse environmental growth circumstances, including as variations in temperature, pH, loss and gai n of water, and loss of nutrients and other ingredients in the medium. This Viable but non culturable condition has been discovered to have the capacity to infect hosts. Regarding the capacity of Viable but not culturable condition forms to become active metabolically and cause illness when exposed to favorable settings, conflicting views have been put forward. It is still unknown what molecular mechanism underlies the creation and resuscitation of the VBNC state (Santini et al., 2010).

2.2 Sources of Campylobacter infection in the Environment

Campylobacter species are common and typically present in a variety of warm-blooded animals, including those that produce food, such as chickens, lambs, beef cattle, and turkeys. In addition to animals that produce food, human infections with Campylobacter

can also be acquired through contact with pets. Another way that people might become infected with Campylobacter is by drinking untreated water. According to several study papers, raw or unpasteurized milk can have Campylobacter bacteria, which can cause gastroenteritis in humans. It has been documented that eating fruits, vegetables, and mushrooms can occasionally expose people to low levels of Campylobacter. Poultry is regarded as the main source of illnesses among the several reasons that affect humans. Human campylobacteriosis is mostly caused by handling poultry infected with Campylobacter and eating undercooked chicken. Additionally, it has been shown that a common pathway for human Campylobacter infections during the preparation of food in the kitchen is the cross-contamination of chicken that is raw with other uncooked or undercooked food products. (Elmi et al., 2021)

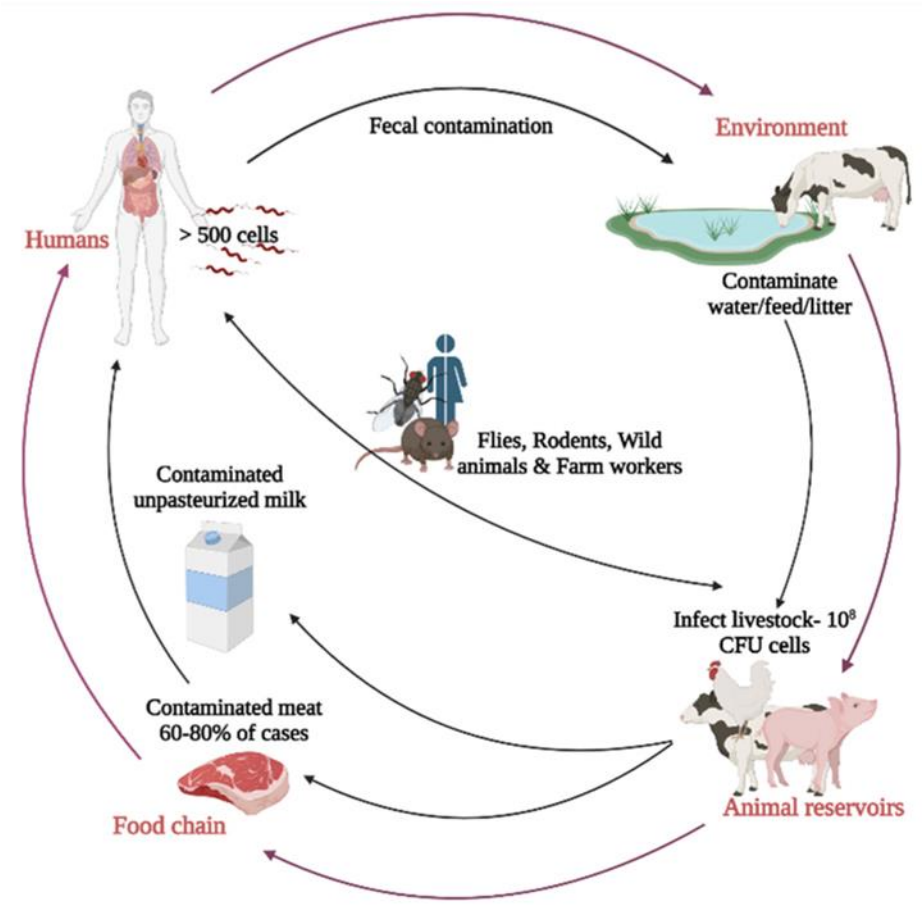


Figure 1*:* Sources of *Campylobacter jejuni*

(Elmi et al., 2021)

2.3 Pathogenesis of *Campylobacter jejuni*

It is unclear whether molecular pathways are involved in this process. Nonetheless, it is thought that the symptoms of campylobacteriosis are primarily caused by connection, colonization, and attack of the epithelial lining of host. (Santini et al., 2010). The fibronectin binding proteins FlpA and other periplasmic or protein that is associated with

membrane (PEB 1) that Campylobacter has are in charge of attaching to and colonizing host cells.

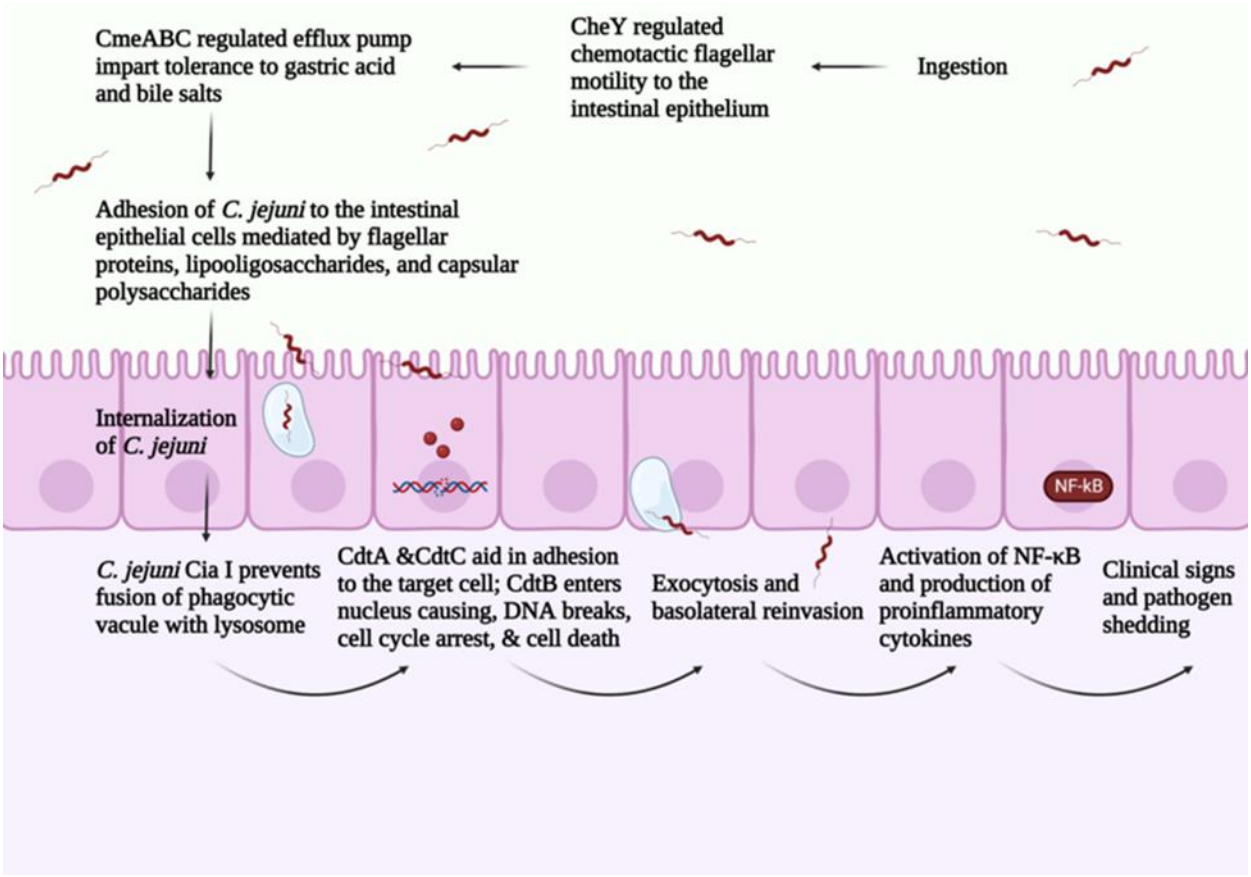


Figure 2: Pathogenesis of *Campylobacter jejuni*
(Santini et al., 2010)

Protein secretion by the flagellar type 3 secretion system mediates gastroenteritis. The Campylobacter invasion antigen and flagellar-driven motility both contribute to the infection. It has been discovered that the interaction of the host cells' microfilaments and microtubules initiates the internalization of *C. jejuni* into the host cells. Cytolethal

distending toxin (Cdt) is a toxin that is produced by *C. jejuni*. In order to stop cells from going into the M phase and trigger host cell death, Cdt induces a host cell cycle arrest. In the late 1990s, the genes encoding Cdt were sequenced for *Campylobacter* and in the 2007 for *C. fetal* and *C. coli* (Dasti et al., 2010).

2.4 Human Infections:

One of the main causes of bacterial intestinal disease in humans is infection with Campylobacter. It has been proposed that individuals with impaired immune systems and youngsters are more vulnerable to infections of *Campylobacter*. Globally, thermotolerant Campylobacter species, particularly *C. coli* and *C. jejuni* , are responsible for around 99% of instances of campylobacteriosis in humans (Santini et al., 2010). It may only take 500–800 live cells to produce sickness in people, depending on the infectious dosage. There have been reports of an incubation period of up to 10 days, however the range is 2–5 days. The majority of individuals may have fever, myalgia, diarrhea, cramping in the abdomen, and malaise (Kaakoush et al., 2015). Because *C. jejuni* is invasive, diarrhea not only loose, watery but also bloody, which may indicate infection. Less commonly observed are extra-intestinal symptoms such as meningitis, osteomyelitis, and newborn sepsis. Patients with campylobacteriosis may heal on their own without the need for therapy because the illness often resolves on its own. Serious post-infectious sequelae such RA, Guillain-Barré syndrome, Inflammatory Bowel Disease and IBS have been linked to some instances of campylobacteriosis (Prabhurajeshwar & Chandrakanth, 2019).

2.4.1 Guillain-Barré Syndrome.

Guillian-Barré syndrome (GBS) represents a potential severe long-term consequence of infection with Campylobacter. This neuromuscular condition is typified by ascending

paralysis, which results in reflex loss, respiratory muscle weakness, and limb weakness. It has been determined that a prior *C. jejuni* infection was linked to 20–40% of GBS infections. GBS can occur in around 2 in 1010 instances of infections. GBS often appears 1-3 weeks following the beginning of Campylobacter enteritis in patients. Roughly 20% of GBS patients need to stay overnight in the critical care unit in order to get breathing support. The formation of antibodies that are auto generated and a role in the pathophysiology of GBS have been suggested as potential outcomes of molecular mimicry between the lipooligosacccharides of *C. jejuni* and GM1 gangliosides of hsot. Acute motor and sensory axonal neuropathy (AMSAN), Acute motor axonal neuropathy (AMAN), acute inflammatory demyelinating polyadiculoneuropathy (AIDP) and Miller Fishers syndrome are the four subtypes of generalized basal ganglion syndrome. The AMSAN subtype of GBS is most commonly linked to Campylobacter infections among these four subtypes (Elmi et al., 2021).

2.4.2 Reactive Arthritis:

A spondyloarthropathy known as reactive arthritis is brought on by microbial gastrointestinal infections, such as Campylobacter. Inflammation of the tendons, joints, tissues, and skin are possible ReA symptoms. Reactive arthritis has been linked to 1-5% of Campylobacter cases, while figures as high as 16% have been seen. ReA is more prevalent in adults even though Campylobacter infections are more common in youngsters. The disease's pathophysiology is yet unknown. Two theories have been proposed on the relationship between the development of illness and the production of antibodies against pathogens that have an affinity for HLA-B27 and poor cellular

immunity, or decreased production of interleukin-2 against the inciting bacterium(Epps et al., 2013).

2.4.3 Irritable Bowel Syndrome:

Often occurring, with three or more episodes per month, irritable bowel syndrome (IBS) is a functional gastric illness marked by bloating in the abdomen and recurrent pain of abdomen or discomfort related with changing one's bowel habits. In North America and Europe, the prevalence of IBS varies between 10-16%. About 10% of IBS patients are thought to have Campylobacter infections as an antecedent illness. Although the precise process by which Campylobacter produces IBS symptoms is not fully understood, it is known that certain Campylobacter species create cytotoxins, which may be linked to the onset of IBS (Guyard-Nicodème et al., 2016).

2.4.4 Inflammatory Bowel Disease:

Ulcerative colitis and Crohn's disease are included under the umbrella name of "inflammatory bowel disease" (IBD). Tenesmus, diarrhea, constipation, stomach cramps, fever, discomfort, and bleeding of rectum with movement in bowel are some of the symptoms of this chronic, relapsing disease. Ten percent of IBD patients have been shown to have *Campylobacter jejuni*. In the pathophysiology of inflammatory bowel disease (IBD), campylobacter facilitates the translocation of bacteria by interfering with transcellular transport that occurs across the epithelial lining of cell. (Haddad et al., 2010).

2.5 Campylobacter Epidemiology in Poultry:

It has been shown that while Campylobacter is common in chicken flocks, the proportion of broiler flocks that are colonized with the bacteria differs by nation. Nearly 90% of flocks in the US and England, 41.1% in Germany, and 47.5% in Japan have

Campylobacter colonization (Mazziotta et al., 2023). Nonetheless, incidence rates range from 19 to 91% throughout Europe, with the northernmost nations having noticeably lower percentages. Variability in *Campylobacter jejuni* adulteration with retail chicken products has been demonstrated by several research findings. The degree of variation in Campylobacter infection in chicken and poultry products can be attributed to several factors, including but not limited to collection of sample, detection methods, season, geographic location, and production procedures. According to an epidemiological research conducted in the Washington area, 71.7% of raw chicken flesh may have *Campylobacter jejuni*. Up to 91%–99% of raw chicken flesh is infected with Campylobacter, according to other research.

2.6 Campylobacter jejuni Colonization in Poultry :

In poultry, Campylobacter jejuni is usually not harmful. When chicks are first deposited, environmental contamination is the main cause of illness. Campylobacter colonizes the digestive tract of chicks at the age of two to three weeks as a commensal bacteria. It has been observed that the infectious dosage for chicken is as low as 50 organisms. Campylobacter is mostly found in the ceca, which is the bottom portion of the gut. Up to 10^8 CFU of campylobacter can be found per gram of cecal contents. There is no periodic fluctuation in the frequency of *Campylobacter jejuni* in broiler flocks, according to a UK research. However, other research indicates that the proportion of positive flocks peaks in the summer. It is unclear exactly how the colonization process works in the gut of the bird. It is postulated that *C. jejuni's* chemoattraction to mucin, which it uses and colonizes in large numbers in the cecal crypts, is a major factor in colonization. Similar to this, an immunological study on the host's immune response to *Campylobacter jejuni* in chickens

revealed that a key factor in the bacteria's continued high degree of colonization is the downregulation of certain host genes. The intestines are where *Campylobacter jejuni* typically localizes. Nonetheless, reports have also indicated systemic invasion of the liver, spleen, heart, and lungs (Willis & Reid, 2008).

2.7 Transmission:

Several investigations have revealed that a variety of animals, including rodents, insects, wild and domestic birds, are hosts for *Campylobacter jejuni*. In poultry, transmission of Campylobacter is the most common mechanism of transmission. The aforementioned sources naturally cause flocks of poultry to get colonized, and birds having *Campylobacter* quickly excrete the germs in their feces, which serve as a source for other birds and quickly spread from one bird to another, contaminating the entire flock. Chick coprophagy and food and water pollution contribute to the flock's quick transition from completely colonized to almost 100% infected with *Campylobacter jejuni* (Elgamoudi & Korolik, 2021).

2.7.1 Vertical transmission

It is debatable if *C. jejuni* is transmitted from parent hens to chicks. Any bacteria can spread vertically by two different methods: primary infection, which involves contaminating the egg inside the hen's reproductive canal, or secondary infection, which involves contaminating the eggshell with feces after the egg is laid. Numerous studies have shown Campylobacter in different regions of the female and male reproductive systems in chickens, suggesting a potential for transmission of *C. jejuni* to chicks. Numerous studies have been carried out to confirm that Campylobacter may spread vertically in chickens. About 11% of the resultant chicks developed Campylobacter in

their digestive tracts after inoculating viable eggs with *C. jejuni*. Nevertheless, naturally, Campylobacter finds it difficult to penetrate the egg shell and, even if it does, it is unlikely to live for longer than 48 hours when kept at room temperature. Several studies have shown, however, that Campylobacter may survive for up to 14 days in the egg yolk but only for around 8 days in the albumen and air sac (Haddad et al., 2010).

2.8 Treatment:

Fluoroquinolones are the recommended medication if antibiotic therapy is required for Campylobacter infections, as these infections typically resolve on their own without it. However, strains of Campylobacter that are resistant to fluoroquinolones have been appearing in recent years. Other antibiotics such as novobiocin, rifampin, vancomycin, ciprofloxacin, bacitracin, and tetracycline may also cause resistance in Campylobacter species. Nowadays, erythromycin is the most commonly used medication to treat Campylobacter infections because of its inexpensive, low toxicity, and restricted spectrum (Royden et al., 2016).

2.9 Handling *C. jejuni* in broiler chickens: (Pre harvesting)

Within twenty-four hours, *C. jejuni* colonizes the chicken's lower gastrointestinal system, especially the ceca. Infected birds can have a concentration of up to 1×10^9 CFU/g of *C. jejuni.* Usually, birds get infected between the ages of two and four weeks, and the infection persists until the bird reaches market age. As a result, management measures are required to lower the prevalence of *C. jejuni* in broiler farms (Ocejo et al., 2023).

2.9.1 Biosecurity

Tight biosecurity protocols are essential for stopping the spread of *Campylobacter* in broiler houses. The first steps towards effective biosecurity measures are determining the

possible sources and techniques for farm-level *C. jejuni* detection. Limiting entry to poultry buildings is essential for preserving a flock free of *C. jejuni*, and adhering to stringent biosecurity protocols can reduce the prevalence of *C. jejuni* in broilers by over 50% when they reach market age. Between cycles, cleaning and disinfecting chicken buildings can help lower the incidence of *C. jejuni*. Strict hygiene measures including hand washing, footbaths, and boot coverings can also reduce the spread of *C. jejuni*. In order to lower the amount of *C. jejuni* in the litter, standard litter management techniques are also essential. Transmission between flocks is also lessened with adequate downtime. Thinning down broiler flocks somewhat might raise the chance of *C. jejuni* spreading to other areas of the farm. To guarantee a low rate of *C. jejuni* transmission throughout the thinning process, stringent biosecurity measures must be taken. Furthermore, the summer and early fall seasons are when the incidence of *C. jejuni* rises. The peak of bug populations coincides with *C. jejuni's* seasonality. Therefore, to guarantee that *C. jejuni* is not widely distributed on the farm, stringent biosecurity precautions are required throughout the summer, early fall, and during thinning (Hansson et al., 2005).

2.9.2 Organic Acids:

Organic substances with acidic qualities are known as organic acids. Three types of fatty acids are classified as organic: (1) medium-chain (C7:C10), which includes capric and caprylic acid; (2) long-chain (≥C11), which includes lauric acid; and (3) short-chain fatty acids (SCFAs), which includes butyric, fumaric, propanoic and acetic and lactic acid. Millions of microorganisms, including organic acid producers, are found in the gastrointestinal tracts of bird species (Ghareeb et al., 2012). The synthesis of organic acids serves as a mediator for the antibacterial action in probiotics. It is thus anticipated that

adding organic acid will improve the bird's health. Supplementing with organic acids lowers the pH of the stomach, which improves the digestion of nutrients and proteolytic enzymes. Furthermore, organic acids are a good substitute for antibiotics since they have the ability to operate as bactericidal, bacteriostatic, or both against gram-negative pathogenic bacteria. *C. jejuni* colonization was stopped by supplementing with 2% formic acid and 0.1% sorbate. But adding 2% formic acid as a supplement wasn't enough to stop *C. jejuni* colonization. Formic acid causes the gut's pH to drop, which affects environmental microorganisms that are acid-sensitive. Conversely, sorbate lowers the pH of *C. jejuni* and targets the bacteria by entering the cell membrane (Tawakol et al., 2023).

2.9.3 Bacteriophages

Bacteriophages are actually the viruses that infect bacteria and archaeal cells and are widely distributed in nature. Félix d'Hérelle discovered bacteriophages in 1917. Since bacteriophages are commonly isolated from human saliva and feces, they are regarded as non-pathogenic to humans. Bacteriophages are often found in food and drinking water, and humans can consume them without experiencing any negative effects. Furthermore, the human gut virome is dominated by bacteriophages. But the characteristics of the chicken virome are still unknown (Liu et al., 2018).

Because they are simple to isolate, have a restricted specificity, and don't change the microbita of the treated host, bacteriophages are recommended as an antibiotic substitute for managing foodborne infections. Despite the fact that over 180 *C. jejuni* phages have been identified, most of these phages only have a limited ability to suppress foodborne bacteria. The two types of *C. jejuni* phages are lytic and lysogenic. The best bacteriophages are lytic ones since they can quickly lyse the intended cell. On the other

hand, since they integrate into the bacterial genome and spread virulence amongst bacteria, lysogenic bacteriophages are not employed. Based on their size, lytic Campylobacter phages are divided into three groups (Efimochkina et al., 2020).

Large phages with a range of 320–425 kbp are included in the first group, whereas phages with a range of 175–183 kbp and a strong affinity for *Campylobacter* are included in the second category. The Campylobacter phages that are the smallest in size and have the highest lytic capacity and affinity for *C. jejuni* are found in the third group. Foodborne diseases can be controlled both before and after harvest using the adaptable instruments known as campylobacter phages (Van Gerwe et al., 2005).

2.9.4 Bacteriocins:

Bacteria release antimicrobial peptides that are produced by ribosomes. When used against similar bacterial species, bacteriocins have both bacteriostatic and bactericidal effects. Bacteriocins are secreted, which destroys the targeted bacterium without harming the host. The mechanism of action of bacteriocin involves membrane permeabilization and subsequent cell lysis. Bacteriocin supplementation effectively lowers the load and contamination in the food chain of *C. jejuni* in broiler infections. Purified encapsulated bacteriocins generated by *L. acidophilus* NRRL B-31514 considerably decreased the load of C. jejuni in seven-day-old broilers (Royden et al., 2016). Similarly, the *C. jejuni* cecal burden was reduced by two log CFU/g upon supplementing two pure forms of L. salivarius and P. polymyxa. Reuterin has recently shown promise as a bacteriocin to manage *Campylobacter* colonization in broiler chickens. Reuterin is an antibacterial substance that is created when Lactobacillus reuteri forms glycerol anaerobically. Reuterin has a broad spectrum of antimicrobial activity against mold, yeast, and bacteria,

both gram-positive and gram-negative. Reuterin works by blocking the target bacteria's redox-base defenses and inducing oxidative stress through the interaction of acrolein with the functional group of glutathione. The lack of glutathione synthase protein in *C. jejuni's* genome suggested that the bacterium is incapable of detoxifying acrolein. The vulnerability of *Campylobacter* reuterin during in vitro investigations may be explained by the lack of glutathione biosynthetic protein. Due of the high metabolic cost of bacteriocin synthesis, probiotic species will not overproduce it. Probiotic species addition to encapsulated bacteriocins may be crucial in competitively keeping *C. jejuni* out of the avian stomach (Dasti et al., 2010).

2.9.5 Vaccines:

Vaccination is still a potentially useful method to reduce the amount of pathogens (Salmonella and *C. jejuni*) in production of chicks. The goal of vaccination is to lower the market-age *C. jejuni* burden and induce a mucosal anti-*Campylobacter* immune response (Haddad et al., 2010).

2.9.5.1 Live Attenuated Vaccine

At 37 days of age, Japanese Jordi chickens were subcutaneously injected with a whole cell vaccination of *C. jejuni* that had been formalin-killed and contained 2.7×10^8 CFU/mL. The vaccine was administered either with an adjuvant. At 58 days of age, the group that got the aluminum hydroxide adjuvant received an additional dose. After 72 days, *C. jejuni* was given to the birds as a challenge. High anti-*Campylobacter* IgG levels were produced by both vaccination groups. Similarly, an adjuvant was added to or removed from a *C. jejuni* whole-cell vaccination that had been formalin-killed. When compared to the non-vaccinated group, the vaccination group's anti-*Campylobacter* levels

were higher and their *Campylobacter* colonization decreased from 17% to 98% (Soto-Beltrá N et al., 2023).

2.9.5.2 Subunit Vaccine

The Type 6 secretion system is essential for the communication of bacteria with the host's cells, interbacterial rivalry, and the ability of bacteria to infect nearby cells. The type 6 secretion system of *C. jejuni* contributes to the bacteria's ability to survive and elude the immune system. A 60 μg pure recombinant hemolysin co-regulated protein encased in chitosan nanoparticles was used to create a vaccine. The broilers received an oral gavage with the subunit vaccination at day 7, followed by booster shots at 14 and 21 days. When the broilers were 28 days old, *C. jejuni* was given to the immunized birds. The *C. jejuni* burden in the ceca decreased by one log CFU/g in the immunisation group (Prajapati et al., 2023).

2.9.5.3 Bacterial Vector Based Vaccine

Potential vaccination possibilities against gastrointestinal infections include live or genetically modified bacterial strains. Appropriate vectors are those bacteria that cause an immune response and are avirulent to hens. The birds' immune systems may be exposed to pathogenic antigens of *C. jejuni* through these vectors. Mutants of *C. jejuni* do not survive long enough to elicit an immunological response, and they exhibit a transitory colonization model in the gut of chicken. The preferred bacterial vectors for developing a vector-based vaccination against *C. jejuni* are actually the non-virulent strains of Lactobacillus and Salmonella. At one and fourteen days of age (booster), broilers were gavaged orally with an non-virulent strain of Salmonella Typhimurium χ3987 that expressed CjaA. *C. jejuni* presented a challenge to the grill when it was 28 days old. The

C. jejuni cecal burden decreased by 6.0 log CFU/g as a result of the vaccination (Gharib-Naseri et al., 2012).

Lastly, the possible vaccine development to reduce campylobacteriosis globally is complicated by strain variations and phase variation in most of the *Campylobacter* strains.

2.9.6 Quorum Sensing Inhibitors:

The cecal burden of *C. jejuni* in broilers might amount to 1×10^9 CFU/g. Using quorum sensing, *C. jejuni* can recognize and react to abrupt anychanges in bacterial populations. Bacteria use a process known as quorum sensing to communicate from cell to cell by producing, detecting, and reacting to signaling molecules called autoinducers. The sequence in which autoinducers accumulate is depending on density. A signal cascade is triggered upon reaching a specific threshold in the autoinducer concentration. The signal cascade modifies gene expression, which causes the bacteria to change morphologically and improve its ability to survive in the environment.

Initially, research in *C. jejuni* quorum sensing revealed a gene encoding an orthologue of the LuxS system, which is responsible for mediating the synthesis of autoinducer-2 (AI-2). A crucial function of luxS in controlling moving capacity in *C. jejuni* was demonstrated by the mutants in the same research, which displayed a reduction in motility in semisolid medium. Additionally, a research comparing the *C. jejuni* mutant strain's capacity for colonization to that of the *C. jejuni* wild-type strain assessed the function of luxS in host colonization. Seven days after inoculation, the luxS mutant exhibited a reduced ability to colonize chickens; yet, certain birds injected with the luxS mutant strains continued to colonize at a comparable rate to those injected with the original strain.

Additionally, an experiment expressing competitive fitness comparing the normal and mutant revealed a reduction in the recovery of mutant relative to the wild-type, suggesting a significant contribution of luxS to *C. jejuni* fitness (Javed et al., 2013).

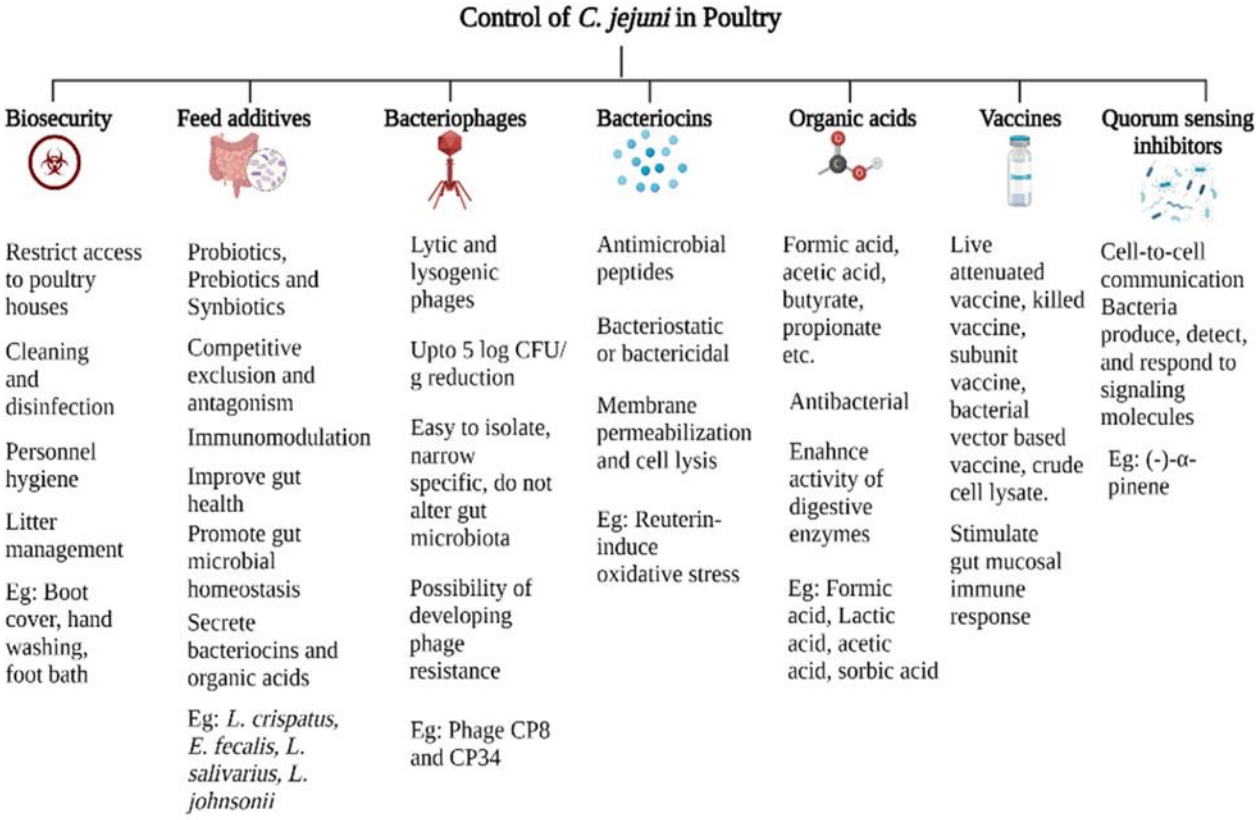


Figure 3: Pre-Harvest Control of *Campylobacter jejuni* in poultry

(Javed et al., 2013)

2.10 Probiotics, Prebiotics, and Symbiotics: An Overview

In poultry, probiotics can help maintain gut health and guard against intestinal disorders. Probiotics work by (1) competitively excluding and antagonistically opposing enteric pathogenic bacteria, (2) lowering pH through the synthesis of organic acids, (3) creating

bacteriocin, (4) stimulating and modulating the host immune response, and (5) changing the virulence features of pathogen that is enteric. Contrary prebiotics are indigestible feed elements that help the host by encouraging the growth of good bacteria un the stomach. Synbiotics are a mix of prebiotics and probiotics. Probiotics, prebiotics, and synbiotics have all been shown to be effective against *C. jejuni* in field research, in vivo, and in vitro experiments. Bacteria that are Gram-negative are susceptible to the antibacterial action of organic acids secreted by probiotics. The supernatant of *L. salivarius, E. faecium, P. acidilactic* and *L. reuteri* suppressed the growth of *C. jejuni* in vitro. After six hours, the *C. jejuni* burden in ceca was reduced by one log CFU/g following the injection of the E. faecalis strain. Lactic acid-producing bacteria's capacity to combat Campylobacter was mediated by their ability to produce organic acids. Enteric diseases' virulence factors can be disrupted by probiotic species. The combination of Lactobacillus species induced the expression of costimulatory molecules in macrophages, specifically CD80, CD86 and CD40. The supplementation of probiotics can start the innate and adaptive immune response against *C. jejuni* because costimulatory molecules are necessary to start an immune response both adaptive humoral (Lopes et al., 2021).

Enteric pathogens are driven out of the gut mucosal surfaces by the competitive action of probiotic bacteria. Examining the probiotics' mechanism of action requires in vitro research. However, in vivo research offers a thorough evaluation of probiotics' capacity to assist the host. Not every encouraging in vitro outcome is confirmed in vivo. In vitro experiments with the strain of *E. faecalis* shown a two log CFU/g reduction in the burden of *C. jejuni*. But in vivo, the *E. faecalis* strain was unable to lessen the burden of *C. jejuni.* Similar to *C. jejuni* connection and invasion of the main chicken cell line*, Y L. lactis, L.*

paracasei JR, *L. rhamnosus* 15b and *L. lactis* FOA all inhibited these processes. Through the upregulation of tight junction gene expression, probiotics improve intestinal barrier's integrity. In one research, Ht-29 cell line added with *E. coli* Nissle 1917 increased the expression of genes that encodes the tight junction, which decreased the intracellular invasion of *C. jejuni*.(Al Hakeem et al., 2022)

2.11 In vitro safety of LAB strains:

Naturally occurring lactic acid bacteria are a component of animals', particularly birds', healthy gut microflora, or GIT. These days, this microbial community serves as a massive gene bank for antibiotic resistance. These microbes have the ability to cause bacteria in the host's body to become resistant to antibiotics. "Intrinsic resistance" refers to a form of resistance that occurs when bacteria possess an innate resistance to an antibiotic, which is specific to a given bacterial species. All the strains within that species then exhibit this kind of resistance to a certain drug (Prajapati et al., 2023). It is believed that acquired resistance, which is mediated by the insertion of genes, has a significant potential for lateral gene transfer, but intrinsic resistance is thought to have relatively limited potential for horizontal spread. On the other hand, acquired resistance happens when a strain resistant to a certain antimicrobial medication comes from a class of species that are normally sensitive to that antibiotic. Genes acquired by the bacterium by exogenous DNA acquisition or gene mutations already present in the bacteria can both result in acquired resistance. The European Food Safety Authority (EFSA) considered published resistance profile data while developing the probiotic safety scheme. All strains intended for use as chicken feed additives must first pass this evaluation (Mazziotta et al., 2023).

2.12 Parabiotics:

Non-viable microbial cells or in-animate form of microbial cells are known as parabiotics, inactivated probiotics. When given in sufficient amounts, these products can help human or animal health. Research has demonstrated that parabiotics can have effects similar to those of postbiotics when used to treat gastrointestinal disorders (Hosseini et al., 2024). In addition, parabiotics have a few advantages over live bacteria and probiotics. These advantages include the absence of a risk of translocation from the lumen to the blood, a zero chance of developing antibiotic resistance, ease of extraction, transport, and storage without losing efficacy, and an enhanced ability to directly interact with epithelial cells when damaged cell components are present. It is possible to hypothesize that using parabiotics derived from the natural gut microbiota of chickens might be beneficial in treating poultry infections, based on documented literature and evidence of the effectiveness of parabiotics (Siciliano et al., 2021).

2.12.1 Mechanism of Action of Parabiotics:

The components of parabiotics are found in the cell envelope. Since this portion of the microbial cell interacts with host cells initially, the probiotics' cell surface components that are extracted by heating the live cells are regarded as a crucial component of effector molecules (Nataraj et al., 2020). These include Membrane Polysaccharides, which have sticky qualities and use the competitive exclusion approach similar to probiotics, Teichoic Acid, which has anti-inflammatory effects, and Peptidoglycan, which has immunomodulatory function in the host; EPS that suppresses inflammation and lowers the generation of cytokines, controls metabolism, and lowers the concentration of Tri glycerol and cholesterol ester in the host liver; proteins on the surface layer that are

beneficial to the host's biological functions while blocking extracellular contact with host cell proteins; Moonlight proteins aid in plasminogen binding and activation and prevent infections like Campylobacteriosis from using plasminogen; LPTXG proteins attach to mucus membranes and improve host-bacterial contact; and Pilli proteins produce reactive oxygen species and shield intestinal epithelial barrier (Halloran & Underwood, 2019).

2.13 Mechanisms of LAB to regulate *Campylobacter jejuni*:

2.13.1 Exclusion of Pathogenic Microorganisms via Competition Exclusion:

A key component of microbial adhesion, attachment, and colonization is competitive exclusion. Probiotics can reduce the colonization of harmful microbes primarily by adhering to particular receptors found in the intestinal epithelium.

2.13.2 Antagonistic Effect on Pathogenic Microorganism:

Lactic acid bacteria produce antimicrobial substances, such as organic acids and $H_2O_2$and less molecular weight constituents, which have positive effects against pathogens that are primarily responsible for bird illness. This prevents pathogenic colonization in the gizzard and cecum region (Prajapati et al., 2023).

2.13.3 Immune Response Stimulation:

Probiotics can either migrate or multiply in the gut to activate. Probiotics have been shown to have a non-immune mechanism that increases the gut defense barrier, stabilizing the ecology of the gut microbiota and intestinal absorptivity he immune system. Certain lactic acid bacteria are skilled at producing cytokines, which aids (Bermudez-Brito et al., 2012).

2.13.4 Growth Stimulators:

Due to their biochemical, physiological, and immunological effects on the host as well as their ability to fend off hazardous diseases, probiotics are also known as growth and health

promoters. They may be found in a variety of foods, pharmaceutical products, seafood, and poultry. A variety of microbial species, including *Bacillus, Lactobacillus, Bifidobacterium Enterococcus*, and *Saccharomyces,* are often employed in cattle and poultry, and their effects on growth stimulation, feed consumption, and metabolism are noteworthy. On the other hand, feeding *Lactobacillus* to chickens has become more and more popular. Combinations of certain *Lactobacilli, Enterococcus, Streptococci,* and *Bacillus* bacteria are recognized to be advantageous probiotics for animals, particularly cattle. Probiotics have long been recognized as a safe, non-toxic feed supplement that helps animals develop. When compared to control broilers, *Lactobacillus* is said to have increased body weights and feed-to-gain ratios. (Saint-Cyr et al., 2017)

2.13.5 Probiotics' impact on intestinal morphology

Research has been done to investigate how probiotic use affects the morphology and histology of the gut. Probiotic Lactobacillus sp. have been shown to affect the depth of the crypt and villi in the small intestine of broilers. Probiotics are advised to lengthen villi by inducing cell mitosis and gut epithelial-cell proliferation. Lactobacillus ingestion contributes to the increased height and crypt intensity of villi in the intestine of broilers compared to hens administered antibiotics. Probiotic-assisted villi enhancement increases

broiler nutrient absorption because of the increased and expanded surface area of the villi (Cerdó et al., 2019).

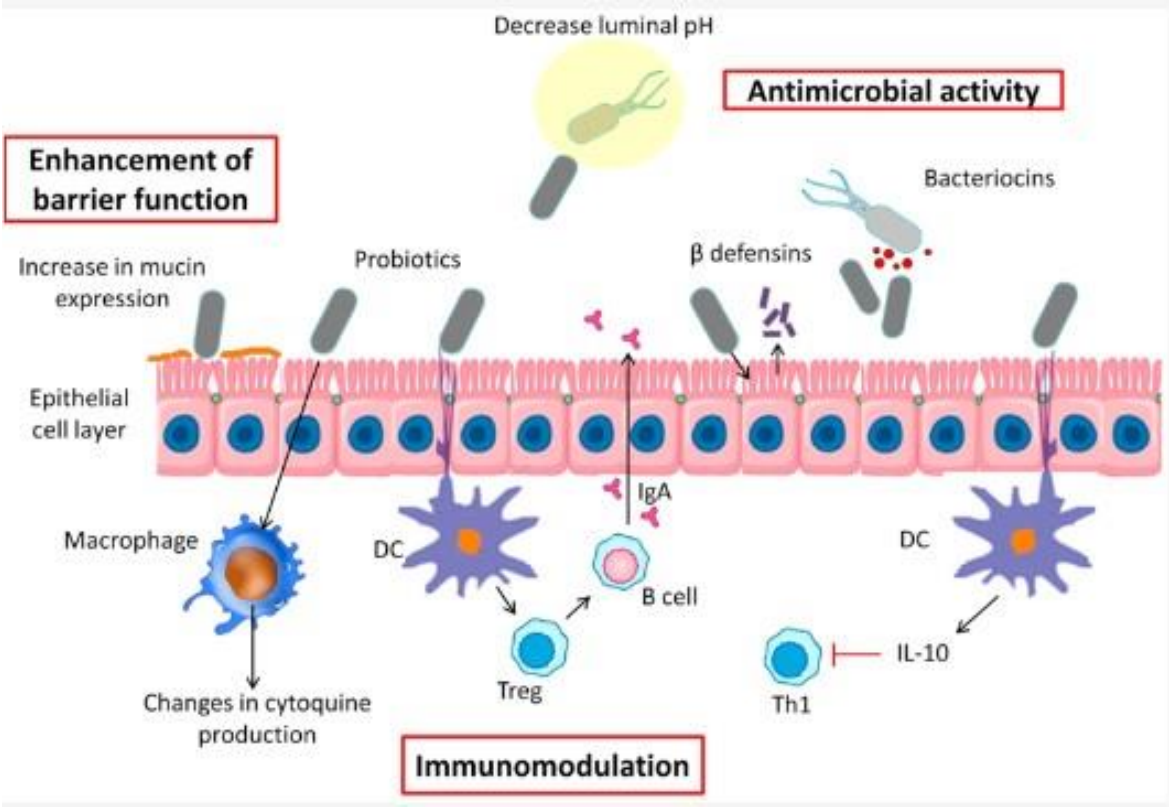


Figure 4: Mechanism of Action of Probiotics

(Cerdó et al., 2019)

CHAPTER 3

3 METHODOLOGY

Ethical Approval for the study

The field trial was actually approved by the Board of Institutional review of the Atta Ur Rahman School of Applied Biosciences, NUST, (Ref: No: IRB-132). The health status of the chicks were routinely monitored.

3.1 Probiotic and Campylobacter isolates:

In this study, two potential probiotics strains *L. reuteri* PFS1 and *E. faecium* PFS15 that were already present in our lab were selected and the *Campylobacter jejuni* AH1 was borrowed from NARC and their efficacy was tested against *Campylobacter jejuni*. The efficacy of the probiotics isolates and the heat-killed form of probiotics i.e parabiotics was tested first by time kill assay using 96-well microtiter plates. After that only probiotics was then tested by time kill assay using plating method. After that the Scanning Electron Microscope was done to identify the changes or any damage in cell morphology and biofilm of *Campylobacter jejuni* due to the effect of cell free supernatent of *Lactobacillus reuteri* and *Enterococcus faecium* strains.

3.2 Determination of the Antimicrobial Activity of the Probiotics:

3.2.1 Time kill Assay By using Parabiotics:

Time kill assay was actually performed by using 96 well microtiter plate using parabiotics i.e. heat killed cells of probiotics. For this the *Campylobacter jejuni* was grown in Muller Hinton (MH) broth for 24 hours at 37°C. 15µL of overnight grown *Campylobacter jejuni* and 15ul of heat killed cells of probiotics (80°C for 30 minutes) of *L. reuteri* PFS1 and

E. faecium PFS15 strains were actually mono cultured and co-cultured separately or in combination in 96 well sterile microtiter plates and was given an incubation at 41 °C. The O.D using the microtiter well plate reader was being obtained at 0, 4, 8, 12, 24, and 48h after incubation. Campylobacter without heat killed cells of probiotics strains were taken as the positive control. The experiment was actually performed in the triplicates (Nataraj et al., 2020).

3.2.2 Time Kill Assay by 96-well Microtiter Plate Using Probiotics:

Time kill assay was then performed by using 96 well microtiter plate. For this the Campylobacter was grown in Muller Hinton (MH) broth for 24 hours ℃. 15µL of overnight grown *Campylobacter jejuni* and 15ul Cell free supernatent of *L. reuteri* PFS1 and *E. faecium* PFS15 strains were co-cultured separately or in combination in 96 well sterile microtiter plates and incubated at 37 °C. The O.D using the microtiter well plate reader is being obtained at 0, 4, 8, 12, 24, and 48h after incubation. Campylobacter without cell-free supernatant of LAB strains were taken as the positive control. The experiment was performed in triplicates.(Taha-Abdelaziz et al., 2019b)

3.2.3 Time Kill Assay by Plating Method:

The overnight culture of Campylobacter and probiotics strains *L. reuteri* PFS1 and *E. faecium* PFS15 were grown in MH Broth and MRS Broth respectively at 41°C in separates falcon tubes. After that the CFS of probiotics were made by centrifuging the overnight culture at 6000 rpm for 10 minutes. After that the in the separate falcon tubes, equal volume 4.8ml each of MRS and MH is being added and 1.2 ml of overnight culture of 10^5 CFU of campylobacter and 1.2 ml of CFS of probiotics separately or their combination were cocultured. Positive control without CFS of LAB strains were taken. They were

incubated at 41 °C. After that 100ul is taken from each falcon tube and was being spread on MH agar plates at 0,4,8,24 and 48 hours. The number of viable cells of *Campylobacter jejuni* were counted from MH plate and the number of CFUs were calculated (Taha-Abdelaziz et al., 2019a).

3.2.4 Scanning electron microscopy:

Scanning electron microscopy was the analysis which was done to identify or analyze any changes or any rupture or destruction in cell morphology of *Campylobacter jejuni* due to the effect of Cell free supernatant on probiotics strains as described in (Kaur et al., 2018). A sterile 24-well microtiter plate with the glass slides of 12 mm was taken and the overnight-grown *Campylobacter jejuni* culture was combined with Cell free supernatent of *L. reuteri* PFS1 and *E. faecium* PFS15 alone and in combination. The plate was then place in an incubator for 48 hours at 41°C. The control was an overnight-grown *Campylobacter jejuni* culture in Muller Hinton (MH) media. The microtiter plate was carefully cleaned to get rid of any non-adherent cells after incubation, and then it was fixed with 2.5% glutaraldehyde and washed twice with PBS. Chilled ethanol was used to dehydrate the cover glass in increments of 30, 50, 70, 90, and 100% (v/v). The specimens were critical dried, coated with gold and photographed and ready for SEM examination using scanning electron microscope (JSM-IT500HR, JEOL, Akishima, Japan).

3.3 Statistical Analysis:

All the experiments were carried out in triplicate. A t-test was performed to determine the statistical significance of the data.

3.4 In vivo assessment of indigenous probiotic strains to reduce Campylobacter Jejuni

3.4.1 Chicks, Housing, and Diets

In total 42, 1-day-old Hubbard chicks weighing an average of 40g were purchased from a hatchery based in Rawalpindi (Tarnol Hatchery Pvt Limited, Rawalpindi) and were randomly assigned to 2 groups. One is positive control and other is treatment group. Each group consists of three replicates with 7 birds each. The chicks were grown in an area with a regulated temperature. Corn, wheat, and soybean meal were combined to provide an antibiotic-free baseline diet for the birds, which was prepared in compliance with NRC (1994) guidelines. The broilers were fed the starter diet for 21 days and the grower-finisher diet for the final 4 weeks of the research. Wood shavings were used as litter in each floor enclosure used for the experimental groups. Throughout the experiment, lighting was available for 18 hours every day, with the exception of the brooding period, which lasted from day 1 to day 7. After 28 days of brooding, the room temperature was lowered from 33 °C to 28 °C progressively. Water and food were available ad libitum means all the time and then the performance of chicks was assessed by recording the weight of chicks of each group and the feed that they take in each group the whole week. The broilers were given the vaccine against Newcastle diseases, Ghumbhuru and avian pox on the 7th day.

Table 1: Composition of all the ingredients used in experimental diets for broilers in the study.

Item	Starter (days 1–21)	Finisher (days 22–28)
Ingredient (%)		
Corn	49.30	59.6
Soybean	5.58	5.0
Wheat	26.86	16.05
Corn gluten	10.00	11.48
Oil of Soybean	3.50	3.34
Salt	0.36	0.36
DCP	1.95	1.80
Vitamin	0.25	0.25
Mineral	0.25	0.25
Limestone	1.45	1.27
Calculated composition of chemical, g/kg		
ME, (Kcal/Kg)	3000	3100
Protein	205.1	194.2
Fat	51.7	53.4
Fiber	34.13	44.2
Calcium	9.2	9.56
Phosphorus	6.34	6.53
Sodium	1.67	1.64
Chloride	2.16	2.11

3.4.2 Probiotic Strains used for the study

Two indigenous strains of probiotics, *L. reuteri* PFS1 and *E. faecium* PFS15, were being selected because of their previous proved anti-bacterial activity against *Campylobacter jejuni* in time kill assay using microtiter plate and time kill assay using plating method. Parabiotics (heat-killed form of probiotics) were not being used because they had not inhibited the growth of *Campylobacter jeuni* when performed in 96-well microtiter plate. So, only the indigenous probiotics were being used. The results of SEM also showed the ruptured morphology of *Campylobacter jejuni* after treated with CFS of indigenous probiotics. The combination of probiotics were administered from the day 1st to the treatment group. Before their administration to the chicks, the probiotics were being cultured in MRS broth and was placed in incubation at 37 °C for 24 hours. After that the final concentration of each probiotic strain was adjusted to approx. 8 log CFU/ml concentrations and then they were added in the drinking water by spectrophotometry (610 nm) (Biochrome Libra S22). The dose was administered to the chicks for the 2 hours in a day. But the fresh water was being administered throughout the day.

3.4.3 Challenge of *Campylobacter jejuni:*

Campylobacter jejuni was cultured in MH broth and then placed in incubator for 24 hours at 41°C. The dose was prepared by centrifugation of overnight culture (two times at 7000 rpm for 15 minutes) and then washed in PBS twice. On day 11th of the trial, the 100ul of 10^5 CFU of campylobacter. Birds were examined throughout the experiment as the campylobacter is the commensal organism in poultry, so it does not causes any kind of disease in poultry.

3.4.4 Collection of Body organs for the weight:

Prior to assigning the chickens to their appropriate treatment enclosures, each chicken's unique weight was recorded. Every week, the residual feed from each group and individual birds were weighed, and the results showed the body weight gain (BWG) and feed Conversion ratio (FCR). For every treatment, any applicable mortality was also calculated. On days 7, 14, 21, and 28, there were four planned sampling sessions (with three replicates from each group). One chicken was taken out of each group before to injection in order to look for *Campylobacter jejuni*. Broilers were rendered unconscious with an intraperitoneal injection of 6.8% sodium pentobarbital at a dose of 0.6 mL/kg. The weight of the small intestine, caecum, and gizzard was measured on days 7 and 28 after the contents were emptied into sterile containers. In addition, the weight of the liver was noted, and the organ weight was then reported as a percentage of the total weight of the body.

3.4.5 Enumeration of Campylobacter jejuni , total Lactobacillus , and total Enterococcus from cecal contents:

The chickens were dissected on day 1st, 7th, 14th, 21st and 28th. 2-3 grams of fresh caecum digesta were collected in an hour for the microbial enumeration. Using Phosphate buffer saline solution, about 1 g of the fresh samples of ceca were serially diluted for the microbial enumeration of *Campylobacter jejuni, Lactobacillus* spp., and *Enterococcus* spp. by using the same and conventional microbiological techniques using selective media of campylobacter Modified charcoal-cefoperazone-deoxycholate agar (mCCDA agar , MRS agar and M17 agar plates respectively. The enumeration of pathogen and probiotics were actually conducted in triplicate, and the average was taken. Results were

expressed as log CFU/g of the bacterial counts obtained from the cecal digesta contentsof the chicks.

CHAPTER 4

4 RESULTS

4.1 In vitro assessment of indigenous probiotic strains to control MDR *Campylobacter Jejuni:*

4.1.1 Time Kill Assay by 96-well Microtiter plate using Parabiotics:

The results of the time-kill assay by 96-well microtiter indicated that the dead cells of probiotics (*Lactobacillus reuteri* PFS1 and *Enterococcus faecium PFS15*) were not reducing the growth of *Campylobacter jejuni* culturing with parabiotics in all of the time points of 4.8.24.48 hours as compared to the positive control without incubated with parabiotics.

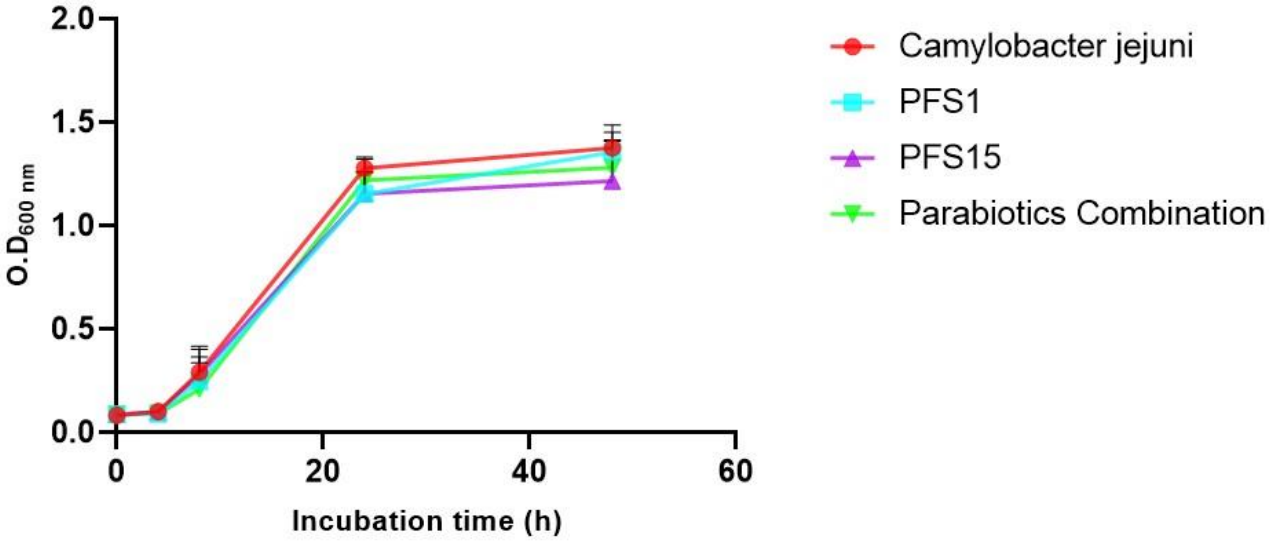


Figure 5: Growth kinetics of *Campylobacter jejuni* monoculture and co-culture with Parabiotics of two LAB strains alone and for the probiotic combination.

4.1.2 Time Kill Assay by 96-well Microtiter Plate Using Probiotics:

The results of the time-kill assay that was obtained by using by 96-well microtiter indicated the ability of Cell free supernatant to reduce the growth of *Campylobacter jejuni* inhibited after culturing with Cell free supernatant in all of the time points 4,8,24,48 hours compared with the control cultures incubated without Cell free supernatant. The inhibitory effect of probiotics was actually more prominent especially after an incubation of 20 hours. The Cell free supernatant of the probiotics combination reduced the count of *Campylobacter jejuni* by a single log as compared with the control *Campylobacter jejuni* that was without any cell free supernatant of probiotics after an incubation of 20 hours.

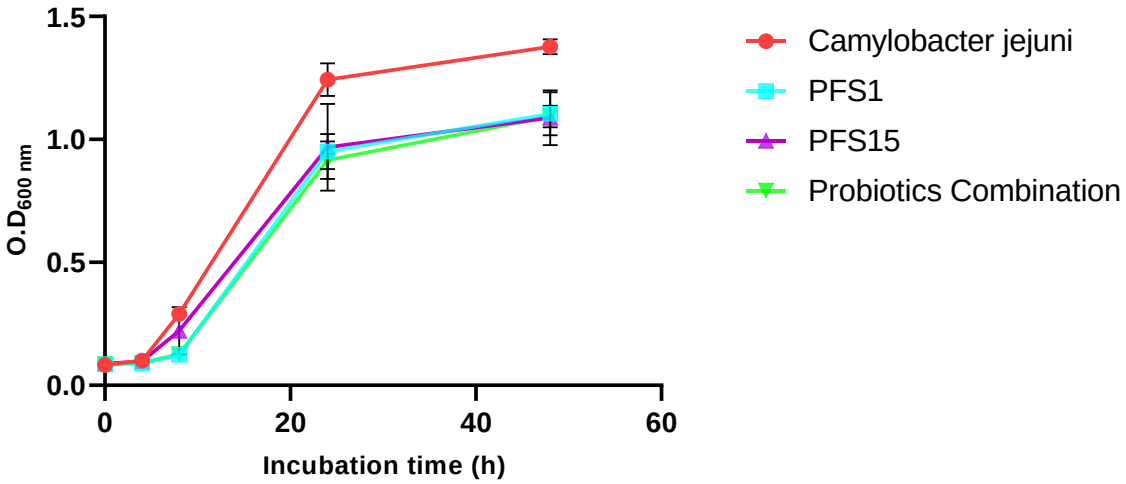
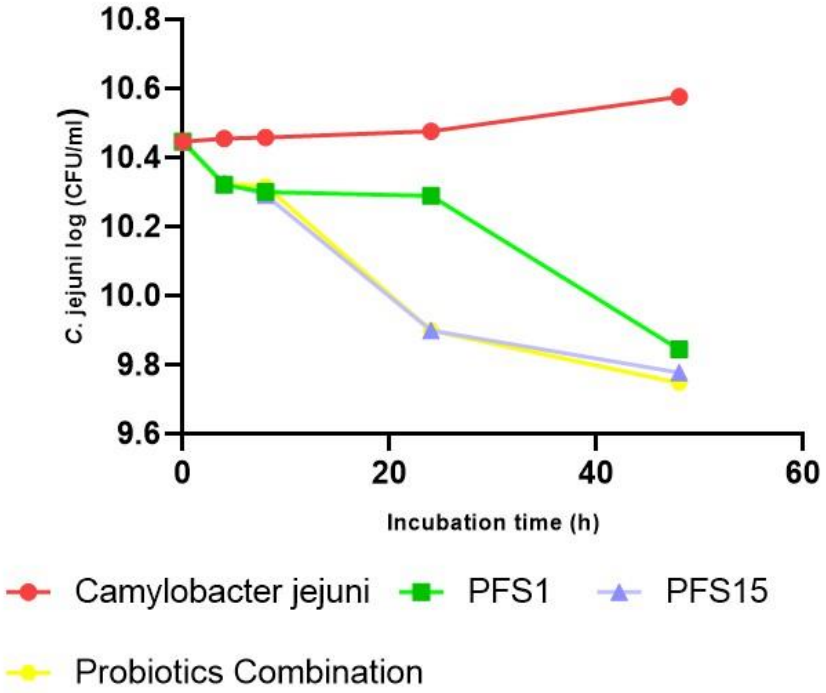


Figure 6: Growth kinetics of *Campylobacter jejuni* monoculture and co-cultured with Cell free supernatant of two probiotics LAB strains alone and for the combination of probiotic.

4.1.3 Time Kill Assay by Plating Method:

The results of the time-kill assay that was performed by plating method indicated the ability of Cell free supernatant to reduce the growth of *Campylobacter jejuni* inhibited after culturing with Cell free supernatant in all of time points of 2,4,8,24 and 48 hours as

compared with the control cultures incubated without Cell free supernatant. The inhibitory effect of probiotics was more prominent particularly after 24 h of incubation. The Cell



free supernatant of the probiotics combination reduced the count of *Campylobacter jejuni* by a single log compared with the control of *Campylobacter jejuni* without any Cell free supernatant after an incubation of 24 hours.

Figure 7: Growth kinetics of *Campylobacter jejuni* monoculture and co-cultured with Cell free supernatant for two probiotics strains alone and for the combination of probiotics in time kill assay by using plating.

4.1.4 Scanning Electron Microscopic:

The biofilm that was produced by *Campylobacter jejuni* was validated by SEM. The control sample, which was *Campylobacter jejuni* grown in sterile MHB, was shown to have a high cell density of the pathogen. After 24 hours of incubation, the Campylobacter's adhesion and aggregation were lessened by the Cell free supernnatant of the probiotics strains in comparison to the control. The SEM result has verified the

maximum inhibition is done by the probiotics combination of *Lactobacillus reuteri* PFS1 and *Enterococcus faecium* PFS15 than the *Enterococcus faecium* PFS15 only.

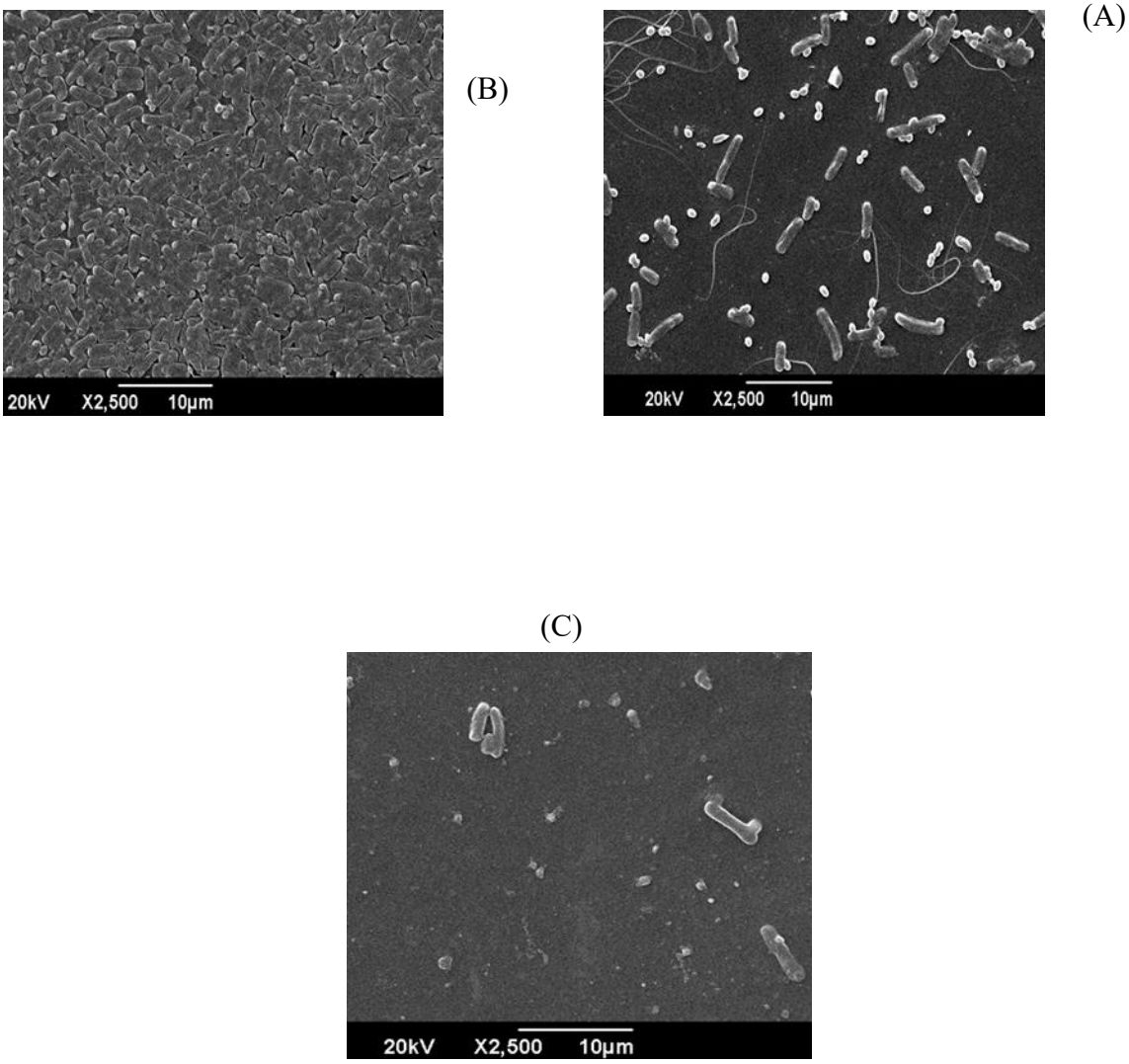


Figure 8: Scanning electron microscope images. (A) Positive biofilm of *Campylobacter jejuni* after 24 hours. (B) *Campylobacter jejuni* treated with Cell free supernatant of *E. faecium* PFS 15 (C) *Campylobacter jejuni* treated with Cell free supernatant of a combination of probiotics *(*PFS 1 *and E. faecium* PFS 15. *L. reuteri.*

4.2 In vivo assessment of indigenous probiotic strains to control MDR *Campylobacter jejuni*:

4.2.1 Effect of indigenous Probiotics on the Growth performance of Chicks

Table 2 shows the variations in body weight, feed consumption, and FCR. During the first week of the study, there was observed a significant changes between the two groups' body body weight gain (BWG), or feed conversion ratio (FCR). On days 7 through 28, there was an interaction between the effects of probiotic treatment and positive control on broiler body weight (BW). Probiotic treatment also significantly raised the broiler body weight when challenged. On the other hand, at 1-28 days of age, birds fed native probiotics acquired considerably and prominently more body weight and had a higher Feed conversion ratio (1.46) ($P < 0.01$) than the positive control.

Figure 9: Birds in Treatment Group more active and more feed consumption Birds in Positive Control group that are not as much active as the birds that had been administered with probiotics.

Table 2: Effect of indigenous probiotics treatments on performance of Broiler

Items	**Groups**		***P* value**
	Treatment Group	**Positive Control**	
day 1 to 7	**1st week**		
ABW[1] (g/bird)	105	100	<0.0001
FCR[2]	0.96	1.14	0.0002
Mortality[3] (%)	0	0	0
day 1 to 14	**2nd week**		
ABW (g/bird)	280	240	0.01
FCR	1.24	1.34	0.01
Mortality (%)	0	0	0
day 1 to 21			
ABWG (g/bird)	513	585	0.01
FCR	1.40	1.46	0.01
Mortality (%)	0	0	0
day 1 to 28	**4th week**		
ABW (g/bird)	100	800	0.0072
FCR	1.46	1.84	0.0020
Mortality (%)	0	0	0

[1]AWB: Average body weight of birds

[2]FCR: Feed conversion ratio

[3]Mortality rate

Birds were fed with the organic diet and probiotic were given in drinking water from the day 1st to 28 days of experiment. The average body weight and the feed conversion rate was obtained and the birds fed with native probiotics acquired considerably and prominently more weight as compared to the positive control and had a higher FCR (1.46) ($P < 0.01$) than the positive control.

4.2.2 Impact of Probiotics on Relative Organ weights

The mean weight of organs percentage in relative to the Body weight are shown in (Table 3). On day 28, the relative weights of the liver, caecum, and small intestine were higher ($P < 0.05$) in the positive control of *C. jejuni* than in treatment group.

Table 3*:* Effects of Probiotics on relative organ weights (% body weight) of broilers in two groups on d 7 and d 28

Item	**Group**		***P-value***
	Positive Control	**Treatment Group**	
Day 7			
Liver	4.0	3.8	0.0273
Gizzard	9.46	9.25	0.087
Small intestine	6.6	5.1	0.0247
Caecum	1.07	0.92	0.0208
Day 28			
Liver	3.10	2.8	0.0315
Gizzard	6.1	4.8	0.0015
Small intestine	4.6	3.8	<0.0001
Caecum	0.63	0.43	0.038

Chicks were fed with the organic diet and probiotic were supplemented in drinking water from the 1st day to 28 days of experiment. The body organs including the small intestines

where the probiotics have more effect, liver, gizzard and caecum where the microbiota number varies between both the groups were taken and the organs were weighed and the relative weights of the liver, caecum, and small intestine were significantly higher in the positive control than in treatment group.

4.3 Slaughtering of birds for Caecum Microbial Analysis

The birds were slaughtered in Figure A on day 1^{st,} 7th, 14th, 21st and 28th ant caecum was separated in Figure 10 and then the microbial analysis of caecum was carried out for *Campylobacter jejuni*, *Lactobacillus reutri* and *Enterococcus faecium.*

A **B**

Figure 10: Slaughtering of Birds for caecum Microbiological Analysis

4.3.1 Enumeration of *Campylobacter jejuni*

results of microbiological analysis of the caecum for *Campylobacter jejuni* are presented in Figure. On the first day of the trial, all samples tested negative for *Campylobacter jejuni*. Days 7, 14, 21, and 28 after the *Campylobacter* challenge showed a significant difference of ($P < 0.001$) in the cecal contents of *Campylobacter jejuni* populations

between the probiotic-treated group and the positive-challenged group, with a difference of 2 logs in Figure (11).

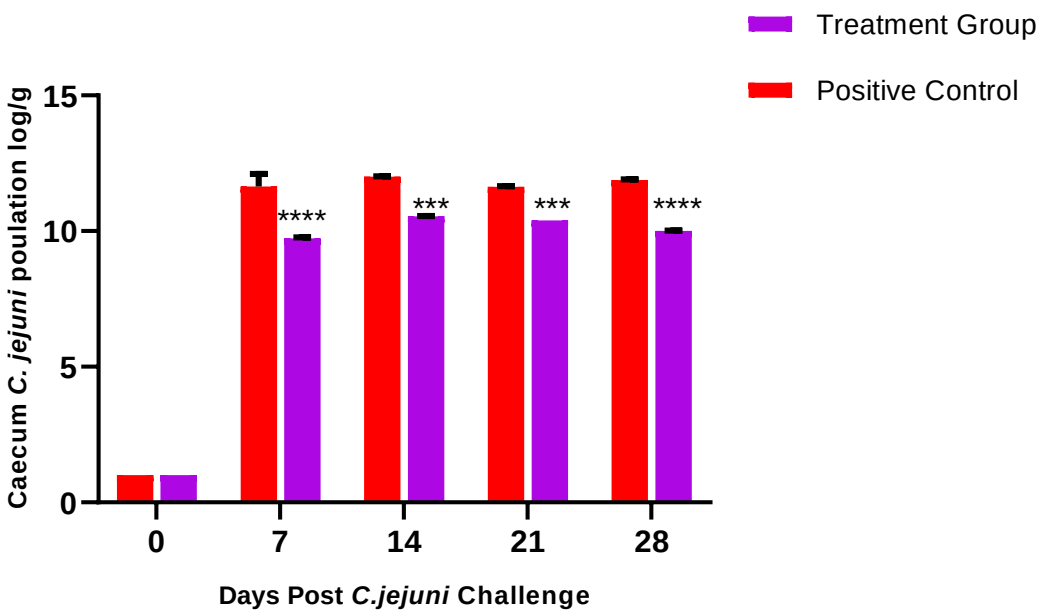


Figure 11: Effect of indigenous probiotics on the amount of Campylobacter digesta in the cecum following a *Campylobacter jejuni* challenge in Broiler chickens.

From the day 1st to 28 days of age, the chicks were fed with organic diet and probiotic supplemented in drinking water. Cecal contents were collected and reported as log values, and the contents were analyzed for *Campylobacter jejuni* using the plate count technique at 0, 7, 14, 21 and 28 days post-infection. Bars show a significant difference ($P < 0.001$).

4.3.2 Enumeration of total *Lactobacillus, and Enterococcus* spp

Results of microbiological analysis of the caecum for *Lactobacillus*, and *Enterococcus* spp are presented in Figure 12 and 13. In terms of *Enterococcus* and *Lactobacillus* count on Days 7, 14, 21, and 28 after the *Campylobacter jejuni* challenge showed a significant difference as the *Campylobacter jejuni* challenged (Positive Group) group had

considerably lower ($P < 0.001$) count as compared to the treatment group, with a difference of 2 log fold.

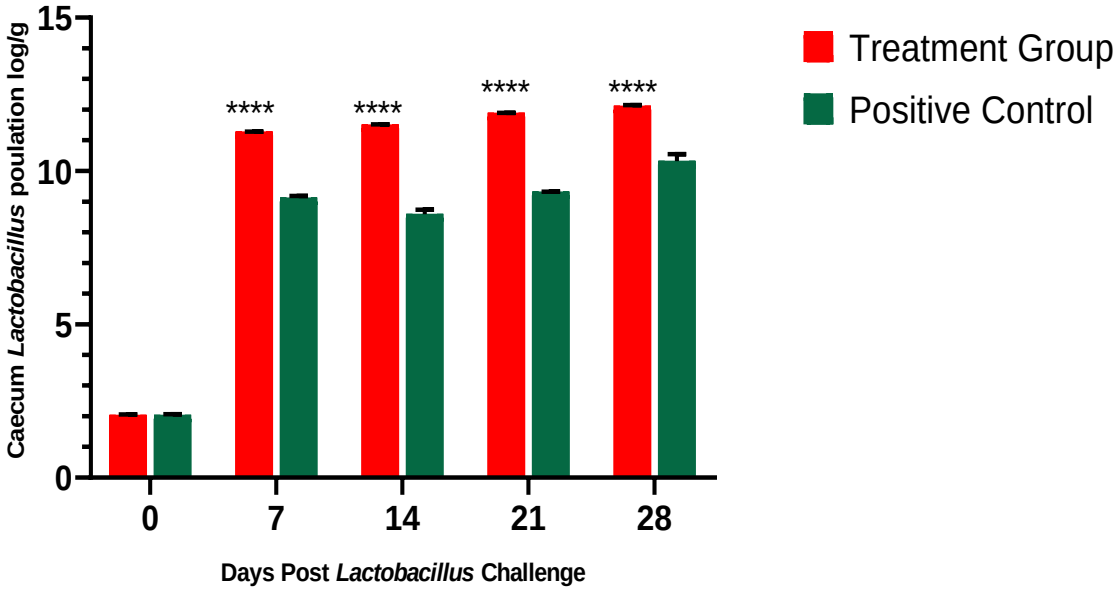


Figure 12: Effect of indigenous probiotic on the two groups of chicks, the Positive Control and Treatment Group, on their cecum's' average logarithm counts of *Lactobacillus*.

From the day first to 28 days of age, the birds were fed with the organic diet and probiotic supplemented in drinking water. Using the plate count technique, the cecal contents were examined at 0, 7, 14, 21, and 28 days to check for *Lactobacillus.* Values within a bird experiment differed substantially ($P < 0.001$). All results are provided as (±SEM) and are in log CFU/g contents.

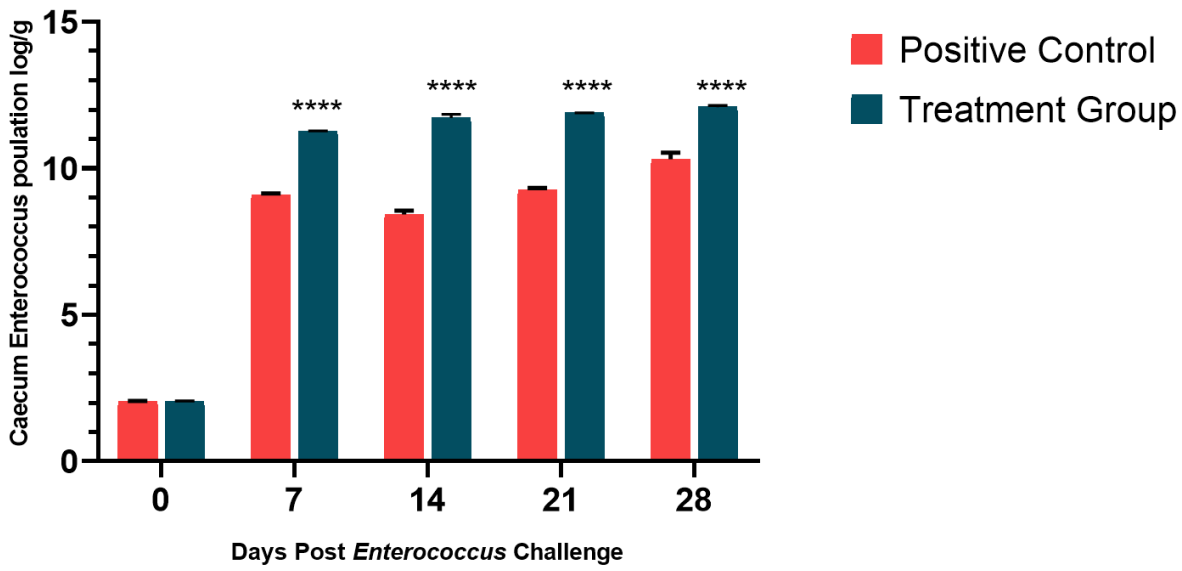


Figure 13*:* Effect of indigenous probiotics on the two groups of chicks, the Positive Control and Treatment Group, on their cecum's' average logarithm counts of *Enterococcus feacuim.*

From the day of hatch to 28 days of age, the birds were fed feed supplemented with the control diet and probiotic supplemented in water. Using the plate count technique, the cecal contents were examined at 0, 7, 14, 21, and 28 days to check for *Enterococcus faecium*. Values within a bird experiment differed substantially ($P < 0.001$). All results are provided as (±SEM) and are in log CFU/g contents.

CHAPTER 5

5 DISCUSSION

Campylobacter jejuni is highly motile, flagellated, microaerophilic bacteria, and may strongly colonise cecal crypt mucus. When cocultured with LAB strains, the bacterial isolates show the capacity to suppress *Campylobacter jejuni* growth. Biofilms made up of both pathogenic and non-pathogenic bacteria are in charge of preserving similar microbes in vivo in healthy habitats. One advantageous characteristic of probiotic strains that facilitates the settlement and long-term persistence on the mucosa of host is their capacity to produce biofilms. Both two of the LAB isolates are capable of preventing the pathogenic biofilm according to our results. Images captured by a scanning electron microscope (SEM) showed that *Campylobacter jejuni* cells adhered firmly and aggregated. Additionally, our SEM pictures demonstrated that following CFS therapy, cell adhesion was reduced. Comparable results were noted in an earlier investigation, when the Cell free supernatant of several probiotic strains reduced and inhibited the production of biofilms in previous studies by (Javed et al., 2013).

Our LAB isolates demonstrated significant adhesion abilities and the capacity to inhibit Campylobacter colonization in gut of poultry through a competitive bond and adhesion mechanism as demonstrated by the findings of the in vitro mucin adhesion assay, cell surface hydrophobicity, the biofilm formation capability and aggregation ability. Our discoveries lay the groundwork for comprehending the processes of adhesion of *L. reuteri*

and *E. faecium,* as well as for forecasting attaching in diverse host models. According to another study (Gharib-Naseri et al., 2012). *L. reuteri,* which has a strong mucin adhesion profile and coaggregation capacity, significantly inhibited the development of Campylobacter in chicken birds . Diacetyl, ethanol, organic acids, hydrogen peroxide, and antimicrobial agents are among the antimicrobial compounds that lactic acid bacteria create. Probiotic-induced inhibition and destruction of pathogenic bacterial biofilms is a compelling target for beneficial intervention. The probiotics have gained a lot of interest lately, which has resulted in the development of biofilm inhibitors to combat food-borne infections like *Campylobacter jejuni.*

According to our findings, the parabiotics (dead cells of probiotics) did not significantly reduced the number of *Campylobacter jejuni* when time kill assay was performed using 96 well microtiter plate as comparable to the other studies (Tareb et al., 2013). So the probiotics were used in in-vivo model as they significantly reduced the *Campylobacter jejuni* number in time kill assay by 96 well microtiter plate and by plating method.

Indigenous probiotics both alone and in combination *L. reuteri PFS1* and *E. faecium* PFS15 dramatically decreased the production of Campylobacter jejuni biofilms in comparison to the control. Previous research have reported on the anti-biofilm property of Cell free supernatant of *E. faecium* and *L. reuteri* against *Campylobacter jejuni* (Al-Megrin et al., 2022). Cell-free supernatants (CFS) include extracellular antibiotic chemicals that some bacteria have antagonistic action against, which makes them an effective tool for treating foodborne infections like *Campylobacter jejuni.* These antagonistic activity results against *Campylobacter jejuni* may be the consequence of different antibacterial compounds generated by probiotic strains. Numerous antimicrobial

compounds, including lactic acid, bacteriocins, acetic acid, hydrogen peroxide, and bacteriocin-like repressive chemicals, were generated by *E. faecium* and *L. reuteri* (Schneitz & Hakkinen, 2016)

Our research has demonstrated that CFS of probiotics is effective against MDR *Campylobacter jejuni* and that it considerably slows the number of *Campylobacter jejuni* in poultry. There is a noticeable growth difference in the birds receiving probiotics in our in-vivo investigation. Compared to the positive control birds, they were more active and heavier. Additionally, the microbial contents of the ceca were examined and plated on MRS and M17 for *Lactobacillus reuteri* and *Enterococcus Faecium* growth, as well as on mccda agar for the count of *Campylobacter jejuni*. When probiotics were also given to the treatment group, the growth of *Campylobacter jejuni* was found to be reduced by two times when compared to the positive control as compared to treatment group. (Ghareeb et al., 2012) . Additionally, the cecal contents were examined at 0, 7, 14, 21, and 28 days using the plate count technique to check for *Lactobacillus* and *Enterococcus*, and the treatment group had log 2 fold more probiotics than the positive control (Santini et al., 2010)

Two LAB isolates in combination were used in the current investigation and they were able to consistently lower cecal *Campylobacter jejuni* numbers, but they were unable to completely eradicate Campylobacter colonization in poultry. The reason for these isolates' efficacy in liquid culture and their failure to entirely eradicate *Campylobacter jejuni* colonization in chicks remains unknown. It has been suggested that probiotic bacteria have beneficial effects by producing different bacteriocins and organic acids, by competing with them for substrates or attachment sites, or by enhancing macrophage-

mediated phagocytosis, though the exact mechanism by which they do so remains unclear (Prabhurajeshwar & Chandrakanth, 2019). Additional ways in which they have positive impacts include the synthesis of antibacterial compounds and volatile fatty acids (Santini et al., 2010).

Probiotics must also be able to colonize in the ceca, live in the low pH environment of the proventriculus or gizzard (2.5–3.5), and pass through bile salt in the small intestine. Probiotics can actually stimulate the immune system and increase the activity of enteric bacterial enzymes in goods when they contain more than 10^7 CFU/ mL of probiotics. Even Lactobacillus species that are resistant to acidity are vulnerable to acidic pH levels (pH 2) and exhibit reduced feasibility throughout the tract of gastrointestinal (Santini et al., 2010). The gut is a very competitive habitat because of the huge, active, and complicated microbiota found in the gastrointestinal system. Complex interactions exist between the different kinds of bacteria in the gut lumen, and these connections may potentially limit or impede the benefits of probiotics strains in the gastrointestinal system (Gharib-Naseri et al., 2012). Because fewer isolates were able to enter or penetrate the Campylobacter-containing cecal crypts in the chickens but these isolates decreased *Campylobacter jejuni* counts by one to two logarithmic units, although not completely eliminating *Campylobacter jejuni* colonization. According to risk assessment, a two log reduction in the amount of *Campylobacter jejuni* on chicken carcasses can result in a 30 fold decrease in human occurrence. Thus, bacterial isolates exhibiting the decline in counts generated in the present investigation may considerably lower the prevalence of this illness in people (Willis & Reid, 2008).

CHAPTER 6

6 Conclusion and Future Prospects:

Campylobacteriosis is a serious issue worldwide, and in Pakistan, where food-borne pathogen surveillance is lacking, this problem is so neglected. The primary cause of diarrhea is Campylobacter species, as stated by the Centers for Disease Control and Prevention. Targeted antibiotic treatment is greatly concerned about the advent of zoonotic MDR *Campylobacter jejuni*. The food industry and the general public are quite concerned about these isolates' ability to build biofilms. This finding paves the door for an global strategy utilizing probiotic strains to limit the amount of *Campylobacter jejuni* in chicken. This study explores the potential of using native LAB strains as probiotics in opposition to this infection. Our research shows that indigenous probiotic strains from the gut of chicken have antagonistic action in vitro against resistant strains of extended-spectrum tetracycline and ciprofloxacin *Camylobacter jejuni*, which are important medications for the treatment of campylobacteriosis. But the research shows that parabiotics did not significantly reduce the *Campylobacter jejuni* count when time kill assay was performed. So, we excluded the parabiotics and used the probiotics in poultry as they had significantly reduce the *Campylobacter jejuni* count in all the in-vitro experiments. Thus, after undergoing in vivo investigations, *E. faecium* PFS15 and *L. reuteri* PFS1 were the potential and best candidates to manage *Campylobacter jejuni* species resistant to ciprofloxacin and extended spectrum tetracycline. According to our research the birds challenged with *Campylobacter jejuni*, the indigenous probiotic supplements increases body weight and feed intake while decreasing MDR *Campylobacter jejuni* colonization. This finding is interesting since it regulates the

amount of *Campylobacter jejuni* in chickens. To examine the influence of our indigenous probiotics strain on the growth performance of chicks and campylobacter control in chicken, a whole farm should be chosen.

CHAPTER 7

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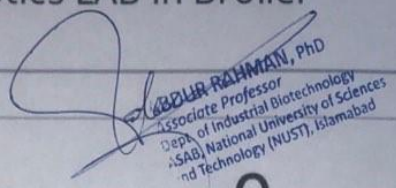

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