ABSTRACT

Title of Dissertation: PROBIOTIC, PREBIOTIC, AND SYNBIOTIC APPROACHES IN SUSTAINABLE POULTRY PRODUCTION THROUGH MICROBIOME MODULATION

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*Campylobacter* is one of the prominent causative agents of acute gastroenteritis in the US and more than 70% of *Campylobacter* infections, known as *Campylobact*eriosis, are occurred through raw or undercooked poultry consumption or improper handling of contaminated poultry products. Moreover, reports show that the antibiotic resistance pattern of *Campylobacter* is persistent and in the absence of sub-therapeutic antibiotic growth promoter, colonization of this bacterial pathogen in poultry gut or on skin and the potential risk of cross-contamination of the finished poultry products are increasing, Therefore, both conventional and organic/pasture poultry farmers are searching for sustainable alternative to synthetic antibiotics which can reduce colonization and cross-contamination of poultry products with poultry-borne bacterial pathogens specifically *Campylobacter*. On the other hand, due to the consumers' demand, majority poultry farmers are growing their chicks without sub-therapeutic growth promotor which leads to slow growth and higher mortality rates. Therefore, to make the poultry farming sustainable, farmers need alternative

feed or water supplement which can promote poultry health and growth. Probiotics, prebiotics, or a combination of these two referred to as synbiotics, have emerged as a promising natural and alternative approach to sustainable animal farming. Probiotics and their metabolites such as conjugated linoleic acids (CLAs) play a crucial role in improving host health and act as antimicrobials against enteric pathogens. Our lab developed a genetically engineered probiotic, **LC$^{+mcra}$** that can convert more CLA by over-expressing the *mcra* (myosin-cross-reactive-antigen) in *Lactobacillus casei* (LC). Further, prebiotic-like components such as bioactive phenolic extracts (**BPEs**) from berry pomace can stimulate the growth of beneficial microbes including LC, competitively inhibit growth of enteric bacterial pathogens, and promote the growth of chickens in a concentration-dependent manner when applied throughout the growth period. In our previous study, we observed that LC$^{+mcra}$ effectively eliminated *Campylobacter jejuni* (**CJ**) in co-culture condition as well as the cell free culture supernatants (CFCS) of LC$^{+mcra}$ was effective in growth reduction of CJ. LC$^{+mcra}$ and its CFCSs also reduced the adherence and invasion ability of CJ to both HD-11 and HeLa cells. Physicochemical properties and gene expressions related to CJ virulence were also altered by CFCSs treatments. These findings suggested, LC$^{+mcra}$ can be an alternative in controlling CJ growth along with other beneficial attributes of LC. Then, we aimed to enhance the efficiency of antimicrobial/beneficial activities of LC$^{+mcra}$ by combining BPEs. In mixed culture condition, LC$^{+mcra}$ in the presence of BPE reduced the growth of CJ more efficiently as well as the CFCS of LC$^{+mcra}$ in the presence of BPE. Interaction of CJ with cultured DF-1, HD-11, and HeLa were altered significantly. Further, combined treatments altered the physicochemical properties and expression of multiple virulence genes such as *ciaB*, *cdtB*, *cadF*, *flaA*, *flaB* of CJ. This finding indicates that BPE and LC$^{+mcra}$ in combination might be able to prevent colonization of CJ in poultry. So, in our present study at simulated gut conditions, we evaluated combined effect

of LC$^{+mcra}$ and BPE in reducing growth of *Campylobacter* in cecum contents. Cecum contents were collected from chickens pre-inoculated with kanamycin resistant CJ (CJ-Km), incubated over 48h time period, while being supplemented with either BPE, CFCS from LC$^{+mcra}$, or their combination. It was found that combined treatments were able to reduce both inoculated and naturally colonized *Campylobacter* more effectively. Microbiome analysis using 16S rRNA sequencing also revealed that combined treatments were capable of altering natural microflora positively within chicken cecum contents. Then, the effect of sustainable probiotics on CJ colonization and gut microbiome composition was evaluated using chicken as a model. A total of 120 chickens were used in duplicate trials to investigate the effectiveness of LC$^{+mcra}$ in decreasing CJ colonization by means of CJ-Km compared to the control group. We observed that LC$^{+mcra}$ could efficiently colonize various parts of the chicken gut and competitively reduce colonization of natural and challenged *Campylobacter*. Furthermore, 16S rRNA compositional analysis revealed lower abundance of Proteobacteria, higher abundance of Firmicutes, along with enriched bacterial genus diversity in gut of LC$^{+mcra}$ fed chicken. Outcomes of this study reveal high potential of LC$^{+mcra}$ as sustainable approach to decrease colonization of *Campylobacter* in poultry gut along with other beneficial attributes. So, we further evaluated the combined effect of LC$^{+mcra}$ and a low dose of BPE on *Campylobacter* colonization in chicken gut using a day-old chick model. Colonization of CJ-Km as well as the natural colonization of *Campylobacter* was reduced by the combined effect of LC$^{+mcra}$ and BPEs significantly at all time points. In the cecum contents of the LC$^{+mcra}$ and BPEs treatment group, there was notable change at phylum level microbiome compared to the control group. At genus level colonization of *Lactobacillus* was significantly higher (1.7 folds), *Campylobacter* colonization was reduced significantly (6.3 folds), and other microflora remained balanced due to the combined treatment of LC$^{+mcra}$ and BPEs. Therefore,

LC$^{+mcra}$ with BPEs could be an alternative to improve the safety of poultry products and reduce campylobacteriosis in humans sustainably. The application period of this synbiotic compositions could be extended to improve the poultry growth rate as an additional benefit of the LC$^{+mcra}$ and BPEs.

# PROBIOTIC, PREBIOTIC, AND SYNBIOTIC APPROACHES IN SUSTAINABLE POULTRY PRODUCTION THROUGH MICROBIOME MODULATION

by

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## Preface

This dissertation is an original and independent work by Zajeba Tabashsum under the supervision of Professor Dr. Debabrata Biswas

# Dedication

To my father (Abbu).

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There is no language to show my gratitude to my parents. The least I can say about my parents is that I am grateful to the Almighty for giving me ‘my parents’. My mother, Nasima Akhter (Ma) has supported me in all my life ventures, however illogical, irrational it seems to her. I am always there to make her life a little more difficult, she is taking it so far and I hope she would take it for my lifetime. Lastly, I want to give a very special tribute to my father, Md. Asadullah (Abbu), who left me 17 years ago to the unknown, for everything good I am today.

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## Scholary Articles

Findings from this dissertation have been published or under process of publication in the following peer-reviewed articles-

1. Tabashsum, Z., Peng, M., Alvarado-Martinez, Z. Aditya, A., Bhatti, J.,Romo, P. B. & Biswas, D. (2020). Competitive reduction of poultry-borne enteric bacterial pathogens in chicken gut with bioactive *Lactobacillus casei*. *Scientific Reports, 10,* 16259.
2. Tabashsum, Z., Alvarado-Martinez, Z., Wall, M. J., Aditya, A., & Biswas, D. Combined effect of metabolites produced by modified *Lactobacillus casei* and berry phenolic extract on Campylobacter and microbiome in chicken cecum content. (Accepted in Journal of Food Science).
3. Tabashsum, Z., Scriba, A., & Biswas, D. Alternative approaches to therapeutics and sub-therapeutics in sustainable poultry production. (Under review in Poultry Science).
4. Tabashsum, Z., Alvarado-Martinez, Z., Aditya, A., Mijar, S., Peng, M., & Biswas, D. Combined effect of conjugated linoleic acid over converting *Lactobacillus casei* and berry phenolic extracts on *Campylobacter* colonization in chicken gut. (Ready to submit in Microbiology Spectrum)

List of Abbreviations

AGP, antibiotic growth promoter

BPE, berry pomace extract

CFCS, cell free culture supernatant

CFU, colony forming unit

CJ, *Campylobacter jejuni*

CLA, conjugated linoleic acid

FBS, fetal bovine serum

GAE, gallic acid equivalence

GBS, Guillain-Barre syndrome

IBD, irritable bowel syndrome

LA, linoleic acid

LC, *Lactobacillus casei*

$LC^{+mcra}$, conjugated linoleic acid over converting *Lactobacillus casei*

MCRA, myocin cross reactive antigen

PBS, phosphate buffer saline

PUFA, poly unsaturated fatty acid

SCFA, short chain fatty acid

# Chapter 1

## Literature review

### *Campylobacter, as a foodborne pathogen*

*Campylobacter* is a spiral-shaped microaerophilic Gram-negative bacterium. *C. jejuni* and *C. coli* are the most common species of the reported 22 species of *Campylobacter*. *Campylobacter* has been reported to grow best at 42$^{o}$C temperature and low environmental oxygen level. These specific growth conditions are also very important and in some instances, necessary for the metabolic efficiency (energy and nutrient balance) and virulence-associated characteristics of *Campylobacter*. Due to these factors, *Campylobacter* generally inhabits in warm-blooded animals, preferably in the mucosal layer of the intestine. This phenomenon makes *Campylobacter* a big threat to human, as it is easy to transmit from animal to human. Chicken is considered the major reservoir of *Campylobacter*. The increased number of *Campylobacter* in chicken gut increases the chances of transmission of this bacteria to humans and ultimately causes diseases. *Campylobacter* shedding in chickens starts about 2 weeks after hatching, and all the birds in a flock can get contaminated overtime. After initial colonization, the load of *Campylobacter* can reach $10^7$ colony-forming unit (CFU) per gram of cecum content naturally (Stern et al. 1995). It has been noticed that the genotypes of isolated *Campylobacter* from both humans and chickens are similar (Nadeau et al., 2002). *Campylobacter* can also reside in other warm-blooded animals like cattle, sheep, and goats. Due to the host specificity of *Campylobacter*, the occurrences of common serotype in human, chicken, and/or other animals is limited. Not only domesticated animals but also wild animals have been reported as the source of *Campylobacter* and been traced back to the human

clinical isolates. This makes the wild animals, the source and means of transmission of *Campylobacter* to the farm animals (Hald et al., 2004; Meerburg et al., 2006). Farm animals can be a definite vector for the transmission of *Campylobacter* to food products, animal handlers in the farms, and the environment (Peng et al., 2016; Salaheen et al., 2016).

*Campylobacteriosis and its consequences*

The disease caused by *Campylobacter* is campylobacteriosis, and campylobacteriosis is one of the leading foodborne diarrheal diseases in the US. This disease is the cause of at least 1.5 million hospitalizations and hundreds of deaths each year reported in 2018 (CDC, 2018). Campylobacteriosis's common symptoms include inflammatory/bloody diarrhea or dysentery, cramps, fever, and pain. Not only are these symptoms, but campylobacteriosis is also the cause of many post-infection complexities. Guillain-Barrie syndrome (GBS), irritable bowel syndrome (IBS), reactive arthritis, and immune-proliferative small intestinal diseases are known to be the post-infection complexities associated with campylobacteriosis (Chen et al., 2010; Garin et al., 2012). As chickens are considered the main source of *Campylobacter*, handling or consuming raw or partially cooked poultry meat is considered the primary means of getting campylobacteriosis (Garin et al., 2012; Salaheen et al., 2016; Wingstrand et al. 2006). Handling raw or consuming raw or partially cooked meat can also transfer *Campylobacter* to other food products and environments. This transfer is mainly horizontal and is prevalent in organic mixed crop-livestock farms as no synthetic chemicals and antimicrobials are permitted in these farms (Peng et al., 2016; Salaheen et al., 2016). With the higher chances of survival and transmission in the organic farming system at the pre-harvest level, there are also chances of survival of *Campylobacter* in post-harvest products. On the other hand, in the conventional farming system, there is the possibility of growing antibiotic

resistance and cross-contaminating different farm products in pre-harvest and post-harvest levels (DeWaal et al., 2013). With the risk of contamination in both organic and conventional farming systems, it is crucial to have strategies to reduce cross-contamination, antibiotic resistance, and improve farm animals' production.

The prevalence of *Campylobacter* with or without antibiotic resistance is a big issue, but limited understanding of the infection and virulence factors of this bacterium is yet another bigger issue. In general, *Campylobacter* can survive in various environmental conditions and the mucosal layer of the animal gut. The understanding is that *Campylobacter* penetrates the intestinal mucosal layer of animal gut and releases cytolethal distending toxin, cdt A, B and C or proteins called *Campylobacter* invasion antigen, cia. The release of the toxins or proteins is through the Type III secretion system like the flagellar apparatus (Pope et al., 2007; Uzoigwe, 2005; Campieri and Gionchetti, 2001; Sasaki et al., 2012). The protein modulates the epithelial engulfment of *Campylobacter*, which ultimately disrupts the integrity of the epithelial lining. *Campylobacter* antigens induce the host's innate immunity through pro-inflammatory cytokines, chemokines, and other effector molecules. When innate immunity is induced by *Campylobacter*, complications like pancreatitis, cholecystitis, peritonitis, and gastrointestinal hemorrhage arise. There are also chances of transient bacteremia in immunocompromised patients. Though these complications like food poisoning and local *Campylobacter* enteritis are not trivial, the post complexities, including Guillain-Barre syndrome (GBS), reactive arthritis, cardiac problem, and uncreative colitis, are more clinically and economically important. The symptoms of *Campylobacter*-induced diarrhea include abdominal cramp, fever, nausea, vomiting, loss of appetite, mucus, and bloody stool. GBS is considered as the disease of the peripheral nervous system and causes demyelination. *Campylobacter*-associated reactive arthritis has the symptoms of chronic or revert

musculoskeletal. Transitory acute pain in the chest with concomitant electrocardiogram variations and increased secretion of cardiac enzymes in association with antecedent and coincident enteritis are the symptoms of myocarditis and myopericarditis. Ulcerative colitis is an inflammatory condition of the large intestine with bloody diarrhea, cramp. This colitis also disrupts the immune function and epithelial cell barrier function and alters gut microbiota (Pope et al., 2007; Uzoigwe, 2005; Campieri and Gionchetti, 2001; Sasaki et al., 2012).

*Major sources of Campylobacter*

Poultry is considered the main natural reservoir of *Campylobacter,* causing 73.6% of *Campylobacter*-related foodborne illnesses. This 73.6% includes chicken, turkey, and other poultry meat. Though poultry is the major source of foodborne illnesses related to *Campylobacter,* the bacteria does not cause any diseases in the chicken itself. The possibility of transmission of *Campylobacter* from chicken to human increases as the number of *Campylobacter* increases in the gut or fecal shedding of chicken and the number of increased interactions between chicken and human. It has been reported that only two-log less *Campylobacter* in chickens can lead to 30-fold less campylobacteriosis in humans (Johnson et al., 2017; Rosenquist et al., 2003). Generally, shedding of *Campylobacter* starts about 2-3 weeks after hatching. Overtime, all the birds in a flock naturally can be contaminated with a bacterial load of more than $10^7$ colony-forming unit (CFU) per gram of cecum contents (Stern et al., 1995).

*Campylobacter* can also reside in other warm-blooded animals like cattle, sheep, and goats. Wild animals have also been reported as the source of *Campylobacter,* and this makes the wild animal a source and means of transmission of *Campylobacter* to the farm animals (Hald et al., 2004; Meerburg et al., 2006). Farm animals can be a definite vector for the transmission of

*Campylobacter* to food products, animal handlers in the farms, and the environment (Peng et al., 2016; Salaheen et al., 2016). In one study, it has been reported, 87.5%, 71.43%, and 33.33% prevalence of *Campylobacter* in meat samples collected from farmers' markets, organic supermarkets, and retail supermarkets, respectively in Maryland- DC metropolitan area (Salaheen et al., 2016). In the big picture all over the US, the prevalence of *Campylobacter* is 43% to 89% in the poultry meat obtained from organic and conventional retail supermarkets (Smith et al., 1999; Zhao et al., 2001; Cui et al., 2005; Price at al., 2005; Luangtongkum et al., 2006; Price at al., 2007; Han et al., 2009). These statistics make it prominent that poultry products in the regular market, regardless of production procedures, are contaminated with *Campylobacter* and with an even higher percentage of organic poultry products.

*The trend of farm animal production and increased consumption of poultry*

Both broiler and layer production continue to grow and industrialization in many parts of the world and increasing population, greater purchasing power and urbanization have been strong drivers of this growth (Statista.com, 2022). Further, the availability and the inexpensiveness of chicken meat and eggs are also making the poultry products popular and, the affordable choice for protein requirement (USDA, 2022). Not only the developing countries but also the developed countries, including the US, are becoming dependent on poultry for their major protein source (USDA, 2022). The US is the largest poultry producing and second largest egg-producing country (next to China) in the world (Poultry world news, 2020; Statista.com, 2022). In 2019, the US poultry industry had produced around 23 million tons of poultry meat, which was little over 500 million of chickens (Poultry world news, 2020; Statista.com). Currently, poultry meat consumption in the US is more than 113.7 lbs. per person yearly (Poultry world news, 2020). This

is higher than any other single red-meat consumption yearly. Not only in respect of consumption or meeting the internal demand, poultry and poultry products have also become one of the notable exporting goods for the US. Further, the US exports a huge quantity of poultry, the second largest of the total poultry-producing countries (Statista.com, 2022). The US produces almost 18% of the total poultry products produced worldwide (Poultry world news, 2020; Statista.com, 2022). With the booming demand and economy poultry production entity has become huge but with increasing obstacles. There are different modes of poultry production including the conventional, organic and fairly recent approach of pasture production. Different production systems face different levels of difficulties including the antibiotic resistance, microbial contamination, poultry growth rate, ban on the use of synthetic antimicrobials and so on. With all these problems in focus, farmers are trying to get suitable solutions and meet the expectations of the consumers. To deal with these issues different farming systems are approaching towards different solutions like use of bacteriophages, vaccinations, probiotics, prebiotics and the newest venture of using synbiotics.

Various obstacles along with the current trend of poultry production

With the increasing population, demand, and consumer awareness, animal production trends have been changing towards efficiency and sustainability (Thornton, 2010). Though there is a demand for a higher production rate, the concern over animal welfare and environmental sustainability is more imperative than ever. In the US, chicken is produced by both conventional farming and organic farming systems, but the popularity of the organic farming system is increasing with consumers' awareness. Both the systems have their pros and cons and can be argued for their place in one of the biggest poultry production systems of the world. In the current situation, along with the conventional and organic farming systems, mixed/pasture farming practices are becoming significant for the production of chickens. In a mixed/pasture farming

system, different animals are grown together along with or without vegetables and corn. Pasture farming practice may contribute to the transmission of bacterial pathogens from animal to animal or animal to produce and is a big concern over cross-contamination especially by bacterial pathogens (Peng et al., 2016; Salaheen et al., 2016).

With the ever-growing population, it is a challenge to meet the food demand. To meet the challenges, farmers are going into cheaper option of commercial production, more often known as the conventional farming system. Sustainable farming is important, but with the growing population and thus demand, farmers are looking for effective ways to produce more at a lower cost and within a reasonable time. Feed efficiency is very important, and at least 1:2 feed conversion ratio is desirable. Conventional poultry farming has its own merits of meeting the demand at affordable prices but by doing so, it is increasing the risk of antibiotic-resistant pathogens (Peng et al., 2016; Salaheen et al., 2016). In a conventional poultry production system, poultry is produced in a controlled environment with specific temperature, humidity, light and feeds. Though FDA bans sub-therapeutic use of antibiotics, feeds in this system might contain synthetic antimicrobials and supplements for growth promotion. Along with that, antibiotics can be used in this farming system for therapeutic use. If an antibiotic is used, the chickens have a withdrawal period before slaughtering but residual antibiotics are tracked down frequently. This leads to multi-drug-resistant bacterial pathogens (Price et al., 2005). On the other hand, in the organic poultry farming system there is more chances of environmental exposure by the means of grazing. No synthetic chemicals can be used in the organic farming system, leading to a slower growth rate and increased bacterial contamination from the environment. In the pasture farming system, there is yet more chances of bacterial contamination from the environment, other animals and crops but there is limited scope of using antibiotics and synthetic growth promoters. With the

pros and cons of conventional, organic, and pasture farming systems, farmers are looking for a sustainable poultry farming scheme where sustainability can be achieved along with the demand, quality and cost.

Shift of poultry farming trend with consumers' choice

The growing conditions of broiler chicken and their slaughter age/weight differ substantially depending on the farming system. These factors have a great influence on consumers' choice along with the factors like price and demand of the poultry products. In a conventional farming system, the expected slaughter age is 5 weeks, but some broilers are kept till week 9. At week 9, the conventional poultry flocks reach 7-9 lbs. In the conventional poultry farming system, the farmers use grow-out houses for growing poultry which have feed and water delivery systems along with controlled heating and ventilation systems. In conventional poultry farming, the feed may include vitamins and minerals along with the regular feeds like corn and soybean. Space-wise in conventional farming, there should be a one-half square foot of space per bird, and for bedding woodchips, peanut shells, rice hulls, and dry litter are usually used (Animal Welfare for Broiler Chickens, 2012). Nowadays, the cage-free pasture poultry farming system is getting popularity, and in cage-free poultry farming system, outdoor space for each broiler has to be 3 square feet or more (Compassion in World Farming, 2011). In this farming system, poultry reaches the slaughter age by 8 weeks (Compassion in World Farming, 2011). Generally, the feeds in cage-free farming systems are similar as in conventional farming system without the added supplements. On the other hand, in an organic farming system, the space requirement per broiler is at least six and a half square feet and has to be free-range strictly without any synthetic growth promoters and antimicrobials. In organic farming system, poultry reaches the age of slaughter by 12 weeks. Choice of poultry farming system vary with the consumers and depend on the economic standpoint

and/or awareness mostly. Though, some consumers prefer cage-free or organic poultry, due to the higher price, the choice may go further than their budget.

Safety concern over pasture poultry production

Consumer awareness has lead the poultry industry to look for organic or cage-free pasture or otherwise called mixed production practices. Organic or cage-free pasture production practices can ensure animal welfare and sustainability but with the risk of lower production rate, higher price, and potential risk of increased pathogenic bacterial contamination compared to conventional poultry production system (Cui et al., 2005; Luangtongkum et al., 2006; Meerburg et al., 2006; Peng et al., 2016; Salaheen et al., 2016). The risk of increased pathogenic contamination arises from the open environment of the farms, and the practice of raising multiple livestock within close vicinity (Peng et al., 2016; Salaheen et al., 2016). In most pasture farming practices, the use of chemical sanitizers and/or synthetic antimicrobials are also restricted. So, the probability of contamination with different zoonotic pathogens increases significantly. It has been observed in different studies that different zoonotic pathogens including *Salmonella* spp., *Campylobacter* spp., and *Staphylococcus* spp. were more prevalent in organic or pasture farms in comparison to the conventional farms. The rate of the higher prevalence of zoonotic pathogens were prominent in both pre- and post-harvest levels (Peng et. al. 2016; Salaheen et al., 2016; Zhao et al., 2001). With these facts in mind, it is of utmost importance to figure out the alternatives that can mitigate the problems associated with all production procedures.

*The use of antibiotics/the chemicals and their effect*

The introduction of antibiotics in the early twentieth-century reduced morbidity and mortality in humans dramatically, and even now the phenomenon of using antibiotics is considered very

important. Now, the use of antibiotics is not only confined to humans but also used in farm animal productions (Wise, 2002; Anderson and Hughes, 2010). The widespread but inappropriate use of broad-spectrum-antibiotics in human medication and animal farming resulted in antibiotic resistance in different bacterial pathogens (Salyers and Shoemaker, 2006). The mechanism of antibiotic resistance may be the reduced antibiotic uptake by bacteria, elimination of antibiotic binding receptors, enzymatic inactivation of antibiotic by cleavage or modification of antibiotic targets, and so on (Todar, 2003). The antibiotic resistance can expand both horizontally and vertically by transferring the antibiotic-resistant genes. The resistance can also transfer through host ranges, from animal to human and vice versa (Marshall and Levy, 2011). It has been reported that almost all the known bacterial pathogens are getting resistant to one or more antibiotics, and with the antibiotic resistance, pathogens are becoming more virulent. Infections are getting more severe with the virulent species, and antibiotic resistance is making the infections untreatable (Marshall and Levy, 2011; Todar, 2003).

The use of antibiotics in animal production was introduced in the mid-nineteenth century for growth promotion. Due to the use of antibiotics, the feed conversion ratio was improved and in the poultry industry, this improved feed conversion has led to as much as 50 percent faster weight gain (Fouad et al., 2014). Again, antibiotics are cheaper than the available vitamins, and now as the growth hormones are banned, it is feasible to use antibiotics even with the risk of antibiotic resistance. The use of antibiotics at the pre-harvest level is a common practice in developing countries and it is not long before this practice was also popular in the developed countries like US. Approximately 3,345,022 kg of antimicrobials (therapeutic and sub-therapeutic) were sold and used in the US poultry industry in 2016. Among the above-mentioned antimicrobials, 1,265,420 kg were 'medically important' in human medical therapy (CDC, 2016).

Flavophospholipol and virniamycin are the two most commonly used antibiotics in the US for poultry production. There is a 'withdrawal' period for the chickens given therapeutic antibiotics before slaughtering, still there are chances of residual antibiotics. The use of target non-specific antibiotics may also result in disruption of gut microbiome, impairing animal health and ultimately hampering food safety. Due to these phenomena, the European Union (EU) has banned antibiotics in animal production for non-therapeutic purposes. Following the EU, the Food and Drug Administration (FDA) of the US has also taken steps to ban antibiotics for the purpose of non-therapeutic use in all types of poultry farming systems (CDC, 2016).

*Potential alternatives to therapeutic and sub-therapeutic*

With the increased concern about antibiotic resistance and other concerns related to food safety, several non-antibiotic antimicrobials have been introduced to tackle and reduce foodborne bacterial pathogens. The development of alternatives to antibiotic growth promoters has also advanced. The major potential agents include bacteriophages, vaccination, plant or animal-derived products, bacteria, and their derivatives (Peng et al., 2017; Peng et al., 2015a; Peng et al., 2015b; Peng et al., 2015c; Salaheen et al., 2014b).

Application of bacteriophage and its limitations

Bacteriophage is one of the most easily available entities and can be very effective against specific bacterial strains (Van Belleghem et al., 2019). Bacteriophages can be found everywhere in the environment, but special skill is needed to isolate the bacteriophages from the environment. Specificity is a unique property of bacteriophages and can be beneficial in controlling the targeted pathogens, thus a very promising alternative to antibiotics. The phenomenon of specificity allows the bacteriophages to be used against targeted pathogens in a mixed population without disturbing

the composition of normal microflora (Inal et al., 2003; Yongdi et al., 2021). Phage-based products are generally developed against different poultry pathogens and occasionally against zoonotic pathogens those can cause disease to the farmers, handlers and consumers. The targeted zoonotic pathogens include but not limited to *Salmonella* spp., *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Staphylococcus aureus (*Katarzyna et al., 2020). Bacteriophage can also modify the host immunity by reducing the cytokines and inflammations, making it a good candidate for animal growth promoters (Yongdi et al., 2021). For the method of application, aerosol administration against different bacterial pathogens have been studied both at farm level and post-production /processing level and found beneficial (Huff et al., 2002; Huff et al., 2004; Grant et al., 2017). With all the above-mentioned pros in hand, it is worth mentioning that strain specificity sometimes leads to the use of cocktail bacteriophages, making the application of bacteriophages more demanding. Further, horizontal and vertical gene transfer to and from the environment has been a huge risk factor of using the bacteriophages.

Available vaccination and efficacy

Vaccines can inhibit the pathogens by inducing the defense mechanisms of the host's immune systems, decreasing infections, and improving animal health. Vaccines are widely used in poultry industry to prevent different poultry diseases and in most of the cases, vaccines are very effective against the poultry pathogens including bacterial and viral pathogens (Marangon et al., 2006). However, vaccines made from any particular bacterial serovar cannot confer cross-protection against another serovar, therefore, they have super-high specificity (Singh et al., 2009). The development of vaccines needs skill, resources and time. It is not cost effective or regular practice to develop vaccines against any organism if not pathogenic/disease causing to the host. For example poultry is a natural reservoir of *C. jejuni* and do not cause diseases in poultry. So,

even if *C. jejuni* is a human pathogen and decreasing the load of *C. jejuni* in poultry will reduce the campylobacteriosis in human, it is not reasonable in terms of resources to develop a vaccine for poultry against *C. jejuni*. So, along with *Campylobacter* sp., different zoonotic pathogens including most of the *Salmonella* spp., many of the *Staphylococcus* spp. fall into the categories against which vaccines are not developed for poultry. Moreover, vaccines are mainly used as prophylactics and the additional costs, makes it difficult to motivate the farmers to use vaccinations as a common alternative to antibiotics for zoonotic pathogens.

Gut microbiome modulation

Products derived from plants are natural, considered less toxic than antibiotics, and generally residue-free and might positively modulate intestinal microbiota. The term 'phytobiotic' includes various substances differing from biological origin, chemical formulation, and purity (Windisch et al., 2006). Some phytobiotics reduce microbial toxins by altering the microbiome (Periˇc et al., 2010; Steiner et al., 2006; Windisch et al., 2008) and can be beneficial in growth promotion and beneficial health properties of animals. These positive effects of phytobiotics are mainly linked to the plant constituents, including terpenoids (mono- and sesquiterpenes, steroids), phenolics (tannins), glycosides, alkaloids (present as alcohols, alheydes, ketones, esters, ethers, and lactones), flavonoids, and glucosinolate (Barug et al., 2006; Wenk et al., 2006). Plant extracts, especially essential oils (EOs), are another new class, and their modes of action in modulating the gut microbiome are still not completely understood (Windisch et al., 2008). Yet again, berries of different kinds are considered a generic source of bioactive compounds (Moussa et al., 2014). Proanthocyanidins from cranberry are one of the popular options (Foo et al., 2000). Flavonoids of cranberry have been shown to reduce or inhibit atherosclerosis by preventing the oxidation of low-density lipids (Reed et al., 2002). The plant tannins with proanthocyanidins, as potential

alternatives to growth promoters in poultry, have recently been reviewed by Redondo et al. (2014). Further studies have revealed that extracts from these sources can affect various bacterial structures and functions, including disruption of bacterial cell envelope, which parallels some antibiotics widely used as growth promoters in the poultry industry (Salaheen et al., 2014b). It has also been reported that the mixtures of bioactive compounds have the ability to modulate the gut microbiome by reducing different enteric pathogens (Salaheen et al., 2016; Salaheen et al., 2014a; Salaheen et al., 2017b).

Several animal-derived products have also been shown to be effective in foodborne-pathogen inhibition by the modulation of the gut microbiome. Chitosan has been observed to inhibit mold growth and several foodborne pathogens, including *L. monocytogenes, S*. Enteritidis, and *E. coli* (Leleu et al., 2011). Chitosan is an animal derived product and isolated from the exoskeletons of crustaceans and arthropods, (Leleu et al., 2011). Pleurocidin is another animal derived product and is heat-stable and salt-tolerant peptide (Jung et al., 2007). It has been isolated from myeloid cells and mucosal tissue of both vertebrates and invertebrates, which has an inhibitory effect against foodborne pathogens as *L. monocytogenes* and EHEC (Jung et al., 2007). Other products like defensin, lactoferrin, lactoperoxidase, lysozyme, and ovotransferrin have shown their possible effectiveness in meat or milk products preservation and in reducing multiple foodborne pathogens, though their application in pre-harvest level and in farm animals needs more investigation.

Probiotic, prebiotic and synbiotic

Probiotics are living cells, which are beneficial to the host, and some examples include *Lactobacillus, Bifidobacterium, Bacillus*, and *Streptococcus* (Dunne et al., 2001; Guo et al., 2006). A probiotic strain should be non-pathogenic, resistant to the stomach acids and bile, have the

potential to colonize in the host, produce some forms of nutrients, be free of antibiotic resistance genes, or have reduced gene transferability and antagonistic to pathogens. Probiotics can be considered as the bacterial alternatives with the ability of competitive exclusion and additional benefits. By modulating the gut microbiome for a better microbial balance, probiotics are believed to improve an animal's overall health. The actual mechanism of the action of probiotics has not been yet fully established, though through different studies, it has been hypothesized that their actions can be potted in three ways. The first hypothesis is, by colonizing the gut in large numbers, probiotics exclude pathogens and thus protect the gut from infection. This is similar to the principle of competitive exclusion to some extent. The second hypothesis is to act as an incitement for the host immune system. When the host immune system is exposed with the probiotics, pathogenic bacteria otherwise unnoticeable are also brought to notice. Thus, by the amplified scrutiny of the immune system, probable pathogens are withdrawn. The third hypothesis is related to the metabolites produced in the intestine by the gut microbiome. It is suggested that probiotics may impact the production of different metabolites like polyunsaturated fatty acids (PUFAs), short-chain fatty acids (SCFAs), vitamins, and others (Peng et al., 2016); ultimately favoring the benificial bacteria and opposing the pathogens. Different studies have also proposed other hypothesis but those are yet to be elaborated.

Probiotics are effective in various cases to develop gut microbiome, especially in newborn animals and animals which have gone through the treatment of antibiotics. Feed conversion rates and the weight gain are other two factors those may be improved by the use of probiotics. Comparing with antibiotic growth-promoters, which have plenty of proof and are also supported by excellent field results, probiotics are yet to prove their efficiency but in both *in vitro* and *in vivo* experimental conditions, probiotics have shown efficiency (Peng et al., 2015a; Peng et al., 2015b;

Shi et al., 2016). Nevertheless, in the farm level, the efficacy of the probiotics is not consistent due to variable results in different experiments (Gaggı`a et al., 2010; Vondruskova et al., 2010). The variability in the farm level experimental results may be due to differences in experimental parameters like experimental conditions, age, genetics, health status of the animals, and so on. In addition, a lack of understanding of the mechanism of action of probiotics, complex and undefined interactions within the metabolites produced by probiotics, the host, and the gastrointestinal (GI) microbiome poses yet another issue. There are studies to reveal the mechanism of actions, like the studies that reveal the response of the GI microbiome of host in quantitative aspects after the supplementation of probiotics (Modesto et al., 2009; Tabashsum et al., 2020 ). However, the microbial community have not been yet completely characterized by those studies, thus leaving the accurate consequences on the microbiome by the probiotic and vice versa fundamentally unidentified. Next-generation sequencing technologies combined with the approaches of systems biology together can be a considerable effort in defining the gut microbiome along with the host responses due to the addition of probiotics and its consequences in humans and animals. Along with all these issues related to the effect of probiotics on gut, delivery method of probiotics is yet another issue to be settled upon.

It is also known that probiotics, especially lactic acid bacteria (LAB), play important roles in modulating the gut microbial ecosystem of farm animals and thus reduce pathogens including zoonotic bacterial pathogens from farm animal gut (Campana et al., 2012). This phenomenon has been getting popularity as alternative therapeutics in recent years, and multiple research groups are looking into it. *Lactobacillus casei* is recognized as an effective probiotic in alleviating gastrointestinal pathogenic bacterial infections both *in vitro* and *in vivo* (Peng et al., 2015b; Peng et al., 2014; Salaheen et al., 2014b; Shi et al., 2016), and there is no documentation of any

pathogenic trait of *L. casei* on human and animals. Antimicrobial compounds produced by LAB include organic acids, hydrogen peroxide, diacetyl, short-chain fatty acids (SCFAs), polyunsaturated fatty acids (PUFAs), small peptide inhibitors, bacteriocins, and bio-surfactants (O'Shea et al., 2012; Peng et al., 2016a; Sharma et al., 2014). SCFAs, particularly linoleic acid (LA) are known as one of the most beneficial compounds produced by probiotics due to their enormous benefits in host health and anti-pathogenic activities (O'Shea et al., 2012). The positional and geometric isomers of LA (C18:2, c9, c12) in mixture is known as conjugated linoleic acids (CLA). CLA productivity varies with many factors, including bacterial species, temperature, oxygen availability, and substrate concentration. The rumen anaerobic bacteria *Butyrivibrio fibrisolvens* was the first one to be recognized as a CLA producer (Kepler et al., 1996). Since then, various genera of dairy and human/animal intestinal bacteria, including *Lactobacillus* and *Bifidobacterium*, have been found which are also able to produce CLA during normal metabolic activities. The production of these antimicrobial derivatives suggests the possible usability of different *Lactobacillus*, *Bifidobacterium* as favorable biological alternatives against bacterial pathogens (Alonso et al., 2003; Lin et al., 2005; Ogawa et al., 2001).

Among the species of *Lactobacillus, L. rhamnosus* possesses the highest CLA conversion ability, but *L. rhamnosus* has a relatively low anti-pathogenic activity. On the other hand, CLA conversion ratio of *L. casei* is relatively low (4.8%), but *L. casei* has shown remarkable antimicrobial activity against enteric pathogens, including *Salmonella* and *Escherichia coli.* Considering these facts, over-expression of 1720 bp myosin-cross-reactive antigen (*mcra*) gene encoding linoleate isomerases from *L. rhamnosus* GG was performed in *L. casei* resulting in 7.15 folds over-expression of *mcra* gene. It was also observed that total linoleic acid production per ml of cell-free supernatant was 4.48 folds more, and total linoleic acid production per bacterial cell

was 21.06 folds more. We named the *mcra* over-expressing *L. casei* as $LC^{+mcra}$ (Tabashsum et al., 2018; Peng et al., 2019).

The term prebiotic is defined as 'selectively fermented ingredients that allow specific changes both in the composition and/or activity in the GI microflora that confer benefits upon host well-being and health' (Gibson et al., 2004). Inulin, lactulose, phenolics, and oligosaccharides are common prebiotics, which are fermentable by the colonic microflora and are also resistant to digestive enzymes of the human gut (Kolida et al., 2002; Bielecka et al., 2002). Several studies have revealed in their preliminary results that prebiotics can provide energy and nutrition to the gut microbiome for fermentation. One way of providing the energy and nutrition may be by supplying the vitamins, SCFAs, and antioxidants for modulating the intestinal microbiome composition; another way may be the impartation of antimicrobial products for competitive advantages by excluding the bacterial pathogens (Videla et al., 2001; Cummings and Macfarlane, 2002; Fukuda et al., 2002). Stimulating the mineral absorption and improving the effectiveness of the host immune system are some other beneficial traits of prebiotics (Geier et al., 2006; Scholz-Ahrens and Schrezenmeir, 2007; Lomax and Calder, 2009; Lohner et al., 2014). The sources of prebiotics generally are different food elements, and whole wheat, raw oats, beans, banana, berries, nuts, cocoa, coffee have been mentioned in different literatures to be the food sources of prebiotics (Kolida et al., 2002; Bielecka et al., 2002).

The use of dietary plant phenolic extracts is becoming another attractive option, and many research teams have mentioned different berries as being used (Feng et al., 2017; Muthaiyan et al., 2012; Sudjana et al., 2009). Blackberry (*Rubus fruticosus*) and blueberry (*Vaccinium corymbosum)* derivatives, commonly known as pomace, contain many bioactive phenolics including flavan, flavanone, flavones, glucuronides, glucosides, quinolones, catechol, coumarin, phenols, luteolines,

tannins, quercetin, gallic acid, vanillic acid, chlorogenic acid, ellagic acid, xanthoxic acid. These compounds have been revealed to possess antimicrobial effects against a wide variety of enteric bacterial pathogens (Salaheen et al., 2014a; Salaheen et al., 2014b; Salaheen et al., 2015; Yang et al., 2014). Furthermore, stimulation of growth of beneficial bacterial/probiotic in the presence of prebiotic like supplements also increases the production of bioactive metabolites and result in an improved host immune system and thus create an adverse environment in the gut for the colonization of bacterial pathogens (Callaway et al., 2008; O'Shea et al., 2012).

Different kinds of berries are considered as a common source of bioactive compounds (Moussa et al., 2014). Proanthocyanidins from cranberry are one of the popular bioactive compounds (Foo et al., 2000). Another class of bioactive compound, flavonoids of cranberry have been also revealed to reduce or even some cases inhibit the atherosclerosis by stopping the oxidation of low-density lipids (Reed et al., 2002). The plant tannins with proanthocyanidins have recently been reviewed by Redondo et al. (2014) as potential alternatives to growth promoters in poultry. Further studies have shown that purified or even crude extracts from these sources can affect various pathogenic bacterial structures and functions, including disruption of bacterial cell wall, which parallels with some antibiotics, those are widely used as growth promoters in the poultry industry (Salaheen et al., 2014b). Though the spectrum of activity or the mode of action of purified components is often very narrow or non-specific, crude berry extracts or pomace-containing mixtures of bioactive compounds have become an attractive alternative to create an added value to animal feeds, e.g., blueberry and blackberry pomace (Salaheen et al., 2014b). It has also been reported that the mixtures of bioactive compounds have the ability to modulate the gut microbiome by reducing different enteric pathogens and favoring beneficial bacteria (Salaheen et al., 2016; Salaheen et al.,

2014a; Salaheen et al., 2017b). On top of these, delivery system of prebiotics is fairly accommodable, though continuous supply of the prebiotic components is needed.

'Synbiotic' can be termed as the combination of 'probiotic' and 'prebiotic' used for the benefit of the host by providing the nutrients and other positive environmental factors for the beneficial gut microbiome of the host (Roberfroid, 1995). On the sections above we have discussed how the probiotic and prebiotic individually can be beneficial as alternatives to the antibiotics as sub-therapeutics and therapeutics. If these two individual alternatives can be combined together, they may be more effective than their individual applications in improving growth and product quality. Studies have demonstrated that metabolites of *L. casei* which is a probiotic, were more effective in the exclusion of *Salmonella*, *Listeria*, and pathogenic *Escherichia coli* in the presence of prebiotic-like components, including peanut fractions and cocoa (Peng et al., 2015a; Peng et al., 2014; Peng et al., 2015b; Peng et al., 2015c; Peng et al., 2016b). Recently, the ability to produce increased amount of multiple antimicrobial derivatives of *Lactobacillus* in the presence of prebiotic-like components has also been identified (Peng et al., 2016a; Sharma et al., 2014). Further, it was reported by Fooks and Gibson (2002) that in experimental laboratory conditions *B.bifidum* and *L. plantarum*, along with prebiotic inulin/oligofructose, are able to promote the growth of *Bifidobacteria* as well as lower the growth of different enteric pathogens including EHEC, *C. jejuni*, and *S.* Enteritidis. A similar study by Asahara et al. (2011) found if mice are fed with *Bifidobacteria* and trans-galacto-oligosacharides, they can survive from lethal infection by *S.* Typhimurium. It has also been reported that *L. paracasei* and oligo-fructose, in their synbiotic effects, can increase the number of *Lactobacillus* spp., *Bifidobacterium* spp., which are considered beneficial bacteria, in weaning piglet's feces (Bomba et al., 2002). In the same study, there was also an observation of decreased concentration of *Clostridium* spp. and *Enterobacterium* spp.

(Bomba et al., 2002). In one study by Baffoni et al. (2012) shows that a *Bifidobacterium* based synbiotic product can reduce the transmission of *Campylobacter* in food chain. Fructooligosaccharide and galactooligosaccharaide were used as the prebiotics and microencapsulated *Bifidobacterium longum* subsp. *Longum* PCB133 was used as the probiotic in that specific study (Baffoni at al., 2012). These findings make the synbiotic approach an attractive alternative. There are also studies focusing on the different concentration levels of synbiotic application and their effects on the overall composition of gut including microbiome of poultry. Dibaji et al. (2014) described the effect of different levels of concentrations of synbiotics on the gut microflora of chicken gut. The authors fed the chickens with different concentrations of synbiotics including manufacturer's recommended level of concentrations, also higher and lower concentrations than the manufacturer's recommendation level and compared the gut microflora among the groups by culture based techniques, targeting coliform and lactic acid bacteria. At genus level, *Escherichia* was found to be decreased in number and *Lactobacillus* were found higher in number with the increasing concentration level of synbiotics. Another study reported the effect of synbiotic preparations in the gut microbiome, fatty acid profile, lactic acid production and performance of chickens including mortality rate, feed conversion ratio, and also the European Production Efficiency Factor (Śliżewska, et al., 2020). The synbiotic preparations increased the count of beneficial bacteria, restricted the growth of pathogenic bacteria, increased the concentration of SCFAs and lactic acids, and decreased the concentration of branched chain fatty acids; all these phenomenon indicate improved performance of broiler chickens (Śliżewska, et al., 2020). Multiple studies have suggested, synbiotics are more effective than either probiotics or prebiotics alone in improving gut health by modulation of gut microbiome (Saulnier et al., 2008; Periˇc et al., 2010; Adebola et al., 2014).

*Hypothesis and specific aims*

$LC^{+mcra}$ and/or BPEs reduce CJ colonization and promote the weight gain towards sustainable poultry production.

For this hypothesis following aims is investigated-

Aim 1: Evaluate the effect of $LC^{+mcra}$ and/or BPEs on CJ in simulated gut condition.

Aim 2: Investigate the effect of $LC^{+mcra}$ on CJ colonization in chicken model.

Aim 3: Assess the synergistic effectiveness of $LC^{+mcra}$ and/or BPEs on CJ colonization and growth performance of chicken.

# Chapter 2

## Aim 1: Synergistic effect of metabolites produced by a modified *Lactobacillus casei* and berry phenolic extract on *Campylobacter* and microbiome in chicken cecum contents

### *Introduction*

Currently, chicken meat is the number one protein source in the US and around the world (Dohlman et al., 2022). An average, each person in the US consumes more than 120 lbs of chicken meat per year (USDA, 2021). Further, with the growing population, the demand for poultry products is increasing significantly each year (Dohlman et al., 2022). Recently, due to the consumers' awareness and inclination towards safer food products that are also environmentally sustainable, organic and/or pasture farming are gaining popularity in the US. Even though conventional poultry farm practices is important to meet the demand of huge amount of poultry products, this farming systems create a loophole or potential risk to contribute in antibiotic resistant bacterial pathogens, as well as antibiotic/chemical residues in the final product, while also having animal welfare and environmental sustainability issues (Hafez et al., 2020). On the other hand, organic/pasture farming systems suffer from higher prevalence of pathogenic bacteria and slower animal growth rate, thus leading to higher prices (Peng et al., 2016; Salaheen et al., 2016b). Different enteric pathogens are becoming antibiotic resistant, and it has been reported that *Campylobacter* became 20% more antibiotic resistant over the last 20 years leading to the ban of antibiotic use for sub-therapeutic use in 2017 by FDA (CDC, 2019). Antibiotic resistance is more closely associated with conventional farming practices (Salaheen at al., 2016b), however,

*Campylobacter* is known to be more prevalent in systems that implement organic poultry farming when compared to conventional farming practices (Salaheen at al., 2016b), with the source of 73% of the foodborne campylobacteriosis cases being attributable to poultry/poultry products, including chicken, turkey, and other birds (Williams et al., 2021).

With the growing antibiotic resistance patterns in the conventional farming system and after the ban of conventional antibiotics for sub-therapeutic use, along with the amount of pathogenic bacterial contamination in the organic farming system, alternatives for controlling bacterial growth/contamination are in increasingly high demand. As alternatives, prebiotics and probiotics are becoming popular due to their effectiveness as feed supplements, animal growth promoter, and as alternative antimicrobials (Carter et al., 2017; De Montijo-Prieto et al., 2015; Saint-Cyr et al., 2017). There are many probiotics in use, which includes *Lactobacillus, Streptococcus, Bifidobacterium* (Carter et al., 2017; Dunne et al., 2001; Guo et al., 2006) and many more which are still at the experimental level. The beneficial attributes of these probiotics include synthesis and metabolism of vitamins, short chain fatty acids, and bile acids, while also contributing to the reduction of pH in the host bowel system, development of mucosal barrier, and immune activation of the host, all of which aid in the protection of the host from incoming pathogenic bacteria (Li & Gu, 2016; Markowiak & Śliżewska, 2017; Peng et al., 2020a, 2020b; Vandenbergh, 1993). Probiotics have also been reported to be effective against multiple zoonotic and enteric pathogens at both *in-vitro* and *in-vivo* conditions (Carter et al., 2017; Peng et al., 2018; Saint-Cyr et al., 2017; Schoster et al., 2013). But all these beneficial effects of probiotics are vastly dependent on the number of the probiotics present, concentration of the beneficial metabolites produced, and combination of the beneficial attributes present for a specific probiotic. To address these issues, our lab developed a probiotic strain of *Lactobacillus casei* with the ability to over-

express the *mcra* (myosin-cross-reactive-antigen) gene ($LC^{+mcra}$), which has been proven to be notably effective in reducing the growth of *Campylobacter, Escherichia coli* and *Salmonella* in *in-vitro* and multiple animal models including chicken (Peng et al., 2020b; Tabashsum et al., 2018, 2020).

There are also many prebiotics in current use, found to be effective for the purposes of growth promotion of various farm animals as well as reducing the growth of zoonotic pathogens (Salaheen et al., 2016a, 2017; Tran et al., 2018). Extracts from different berry products/byproducts have been shown to contain a variety of phenolic compounds that have proven their efficacy in growth promotion of chickens at lower concentration, while also being able to reduce the bacterial load of zoonotic pathogens like *Campylobacter jejuni*, *Salmonella* in an *in-vivo* scenario at higher concentration (Al-Mnaser et al., 2022; Mahfuz et al., 2021; Mountzouris et al., 2011; Salaheen at al., 2017, 2018; Viveros et al., 2011).

Previously, we have observed that cell free cultural supernatant (CFCS) taken from $LC^{+mcra}$ and/or berry phenolic extract (BPE) can reduce growth of *Campylobacter*, *Salmonella* and *E. coli* in *in-vitro* conditions efficiently (Tabashsum et al., 2019a, 2019b, 2020) but their efficacy in a complex gut ecosystem has not yet been evaluated. The contents within the gut not only have a complex composition that can affect the potency of novel antimicrobials, but also harbors a wide diversity of the normal bacterial flora of the host, which is another major factor that can affect potency. Before advocating the use of this synbiotic composition in an *in-vivo* model, a better understanding of the combined effect of $LC^{+mcra}$ metabolites and BPE on the gut microflora is necessary. Generally, a simulated condition can be used to gain a better understanding of the microbial modulation patterns that can occur within a complex gut system as a result of an intervention through the addition of novel components, while remaining under a well-controlled

environment. It has been reported that simulated conditions are reliable to assess the alteration of gut microbiome in presence of prebiotics and/or probiotics, which is why the current study was designed implementing some of the core aspects of this kind of model (Astó et al., 2019; Venema & Abbeele, 2013).

The purpose of this study was to evaluate the effectiveness of the combination of CFCS of $LC^{+mcra}$ with BPE against pre-inoculated and naturally colonized *Campylobacter* in a simulated poultry gut condition. This was achieved by collecting and using cecum contents from euthanized chickens as the medium to be supplemented with the compounds being tested. In addition to assess the reduction of *Campylobacter*, experiments were performed to determine the effect of this combined treatment on the normal microbial composition of the microflora within the chicken cecum contents.

## *Materials and Methods*

### Bacterial strains and growth conditions

Modified *Campylobacter jejuni* RM1221 (ATCC BAA-1062) (CJ) and *Lactobacillus casei* (ATCC 334) (LC) were used in this study. To distinguish from other natural *Campylobacter* within chicken cecum content, a Kanamycin cassette was inserted into the chromosomal DNA of CJ and named 'CJ-Km' (Salaheen at al., 2017). CJ-Km was grown in Karmali agar (Himedia, India) with selective supplements (Himedia, India) and Kanamycin under microaerophilic conditions, in presence of mixed gas (10% $CO_2$, 5% $O_2$ and 85% $N_2$) at 37 °C. '$LC^{+mcra}$', conjugated linoleic acid over-converting LC was previously developed through the incorporation of the myosin cross reactive antigen gene (*mcra*) in the LC chromosome as described by Peng et al (2019). $LC^{+mcra}$

were grown at 37 °C for an overnight period in presence of 5% $CO_2$ (Thermo Fisher Scientific Inc., USA) on MRS (de Man Rogosa Sharpe) agar (EMD Chemicals Inc., USA).

Preparation of bacterial cell free culture supernatant

A liquid culture of $LC^{+mcra}$ was grown for 36 h in Dulbecco's modified Eagle's medium (DMEM) (Coring, USA) containing 5% fetal bovine serum (FBS) (Gibco, USA), which was later centrifuged at 4000 × g for 20 min (Thermo Fisher Scientific Inc., USA). Cell free culture supernatants was then collected without disturbing the bacterial pellet and filtered through a sterile syringe with a 0.2 μm filter (VWR Inc., USA) (Tabashsum et al., 2018). Filtered cell free culture supernatants from $LC^{+mcra}$ (CFCS) was collected and stored at 4 °C for further use.

Preparation of pomace extract

Commercial blackberry and blueberry pomaces were donated by Milne Fruit Products Inc., USA, stored at -20 °C, and were used to extract phenolics according to the protocol previously described by Salaheen et al. (2014) with slight modifications. Briefly, berry pomace was dissolved and incubated in 10% v/v ethanol solution for 24h at 37°C, solid and liquid portion was separated by centrifugation at 3000 × g for 20 min, and supernatant was passed through the 0.45 μm filter (VWR Inc., USA). The solution was then air-dried, stored at 4 °C, and dissolved in deionized water before use. Spectrophotometric method using Folin-Ciocalteu reagent (MP; Cat. No. 19586) was used to determine the total phenolic contents in blackberry and blueberry pomace extracts and expressed as Gallic Acid Equivalent (GAE) (Singleton et al., 1999). Berry pomace extract **(BPE**) was comprised of a combination of blackberry and blueberry pomace extracts at 1:1 (v/v) ratio.

Whole genome sequencing

Whole genome sequencing (WGS) was done using the Miseq for CJ with and without the treatments. In brief, CJ was grown alone as a control, or in presence of either CFCS or BPE or a combination of both, as treatment groups, for an overnight period under microaerophilic conditions as described above. Genomic DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen, USA) following the manufacturer's instructions. The quality and concentration of the genomic DNA were assessed through the Qubit (Illumina, USA). Prior to running on the Illumina Miseq, the sequencing libraries were prepared using the Illumina Nextera XT library preparation kit. Paired DNA sequencing raw reads were provided as FASTQ files in Miseq Basespace and analyzed through software tools SPAdes and Trimmomatic from GALAXY (Afgan et al, 2018). Mauve (Darling et al, 2010) was used for genome comparison at the DNA level, based on the genomic sequences available in the NCBI database for *Campylobacter jejuni* RM1221 (ATCC BAA-1062).

Sample collection and processing for enumeration of CJ-Km and *Campylobacter*

Cecum content was collected from euthanized chickens, which were previously inoculated with CJ-Km (IACUC no.R-16-33). Cecum samples were further individually supplemented with either 10% CFCS from $LC^{+mcra}$, BPE (0.1 mg GAE/mL), or a combination of 10% CFCS from $LC^{+mcra}$ with BPE (0.1 mg GAE/mL), in addition to an untreated control containing no additional supplementation with any treatment. No additional media was added to these samples and all of them were incubated at 42 °C under microaerophilic conditions (10% $CO_2$, 5%$O_2$, and 85% $N_2$). Sample aliquots were taken at the 0h, 24h and 48h time points, at which times they were homogenized and properly diluted in phosphate buffer saline (PBS) for further analysis. Diluted samples were plated on Karmali-kanamycin and Karmali agar plates for enumeration of CJ-Km

and *Campylobacter* at each time point of the treatment period, respectively following the methodology described above.

16S rRNA compositional analysis

For the comparison of the influence of CFCS and/or BPE on microbiome in simulated chicken gut condition, the incubated cecum contents with or without treatments were collected for 16S rRNA compositional analysis at 0h, 24h and 48h time points. Briefly, DNA was extracted using the QIAamp Fast DNA Stool Kit (QIAGEN, USA). The variable regions (V3) of 16S rRNA were targeted for phylogenetic classifications. Nextera DNA Library Preparation Kit and Nextera Index Kit (Illumina, USA) were used to prepare DNA libraries and pooled according to the protocol provided by the manufacturers into equimolar concentration. Sequencing (paired-end: 2 × 300 bp) was performed using MiSeq v3 600-Cycle Kit (Illumina, USA) by Illumina MiSeq. Sequencing data was processed by MiSeq Reporter-BaseSpace for FASTQ workflow generation (Greengenes database). Demultiplexing was conducted using the perfect index recognition (mismatch =0) and then by removing PhiX reads. The data was analyzed to determine the differences of relative abundance at phylum, genus levels and diversity (number of species, Simpson index, Shannon index and Margalef's richness) at species level among four groups (control, CFCS, BPE, and CFCS with BPE groups) following the standard protocol.

Statistical analysis

For statistical analysis, each control or treatment was considered as an experimental unit. For statistical analysis, ANOVA followed by Tukey's test was used to determine the differences between control and treatments based on a significant level of 0.05.

*Results*

Whole Genome sequencing of the treated and untreated *Campylobacter*

To compare the effect of probiotic-derived metabolites and BPE on *Campylobacter* genome, we treated CJ with 10% CFCSs collected from $LC^{+mcra}$ and/or BPE (0.1 mg GAE/ml) and aligned using the *Campylobacter jejuni* RM1221 (ATCC BAA-1062) sequence data from the GenBank database. The whole genome sequencing revealed that the treatments were able to affect the CJ at genomic level. In the Mauve comparison, the genome of the control CJ (without any treatment) had least number of fragmentations and was readily comparable GenBank genome, the treatment groups however had more fragmentations. The genome comparison of the control and treated *Campylobacter* with the GenBank genome is shown in Figure 2.1.

Reduction of *Campylobacter* in cecum contents treated with CFCS and/or BPE

Combined effect of 10% CFCS collected from $LC^{+mcra}$ and BPE (0.1 mg GAE/ml) on growth of CJ-Km, which was fed to chickens, and naturally colonized *Campylobacter* was more efficient than the each of these components individually both at 24h and 48h time points (Figure 3.2). After 24h of incubation, CFCS, BPE or their combination reduced growth of CJ-Km by 1.65 log CFU/ml, 1.5 log CFU/ml, by 1.85 log CFU/ml, respectively ($p<0.05$). When evaluating the results for total number of *Campylobacter* after 24h of incubation, a similar pattern of growth reduction was observed (Figure 2.2). Growth of *Campylobacter* was reduced by 2.8 log CFU/ml, 2.67 log CFU/ml, and by 3.3 log CFU/ml, in presence of CFCS, BPE, or combination of CFCS and BPE, respectively ($p<0.05$). At the 48h time point, CFCS, BPE, or their combination reduced the growth of CJ-Km and *Campylobacter* by 2.02 log CFU/ml and 3.34 log CFU/ml, by 1.9 log CFU/ml and 2.78 log CFU/ml, and by 2.14 log CFU/ml and 3.28 log CFU/ml, respectively ($p<0.05$).

Microbiome dynamics in cecum contents during treatment with CFCS and/or BPE

Analysis on the relative microbial abundance of the bacterial community at each time point and treatment group was assessed through 16S rRNA sequencing. There was noticeable change in the abundance of different phyla in the treatment groups at different time points compared to the control groups at 0h (Figure 2.3). The abundance of Firmicutes decreased in the control and treatment groups at both time points ($p<0.05$). More specifically, abundance of Firmicutes was reduced to 67.18% at 24h and to 55.17% at 48h, respectively from an initial 88.50% at 0h in the untreated control group. In the treatment groups, there were also reductions in the Firmicutes but comparatively less than the control group at the same time point. At 48h time point the Firmicutes level in the CFCS, BPE and in the combined group were 68.18%, 66.19% and 69.93%, respectively. For Proteobacteria, treatments reduced the level of increment in their respective groups compared to the untreated control group from 0h to both at 24h and 48h time points. Proteobacteria went from 2.52% for control group at 0h to 10.05%, 8.41%, 10.84%, and 4.75% for control, CFCS, BPE, and the combined groups, respectively at 24h; while at the 48h time point there was an increase in abundance to 14.8%, 10.53%, 12.42%, and 14.02% for control, CFCS, BPE, and the combined groups, respectively. Two other major phyla, Bacteroidetes and Actinobacteria, both increased in percentage of abundance for control group and treatment groups at both 24h and 48h, respectively when compared to the control group of 0h.

There was also notable change in the percentage of abundance of various genus at both 24h and 48h time points ($p<0.05$) (Figure 2.4). At the 24h time point, there was an observable significant increase of *Lactobacillus* in the presence of combined treatment group (1.2 folds) as compared to the control group of both 0h and 24h. Though, *Lactobacillus* was not altered in the CFCS treatment group, a slight increase in BPE treatment group at 24h was noticeable compared

to control of 0h. At 48h, the percentage of *Lactobacillus* was reduced slightly for control (0.92 folds), BPE (0.88 folds), and combined treatment group (0.76 folds) but remained similar for the CFCS treated group. There were also observations regarding *Campylobacter* at both 24h and 48h time points, as in the 24h time point, there were increases for the control (1.8 folds), BPE (2 folds) and combined treatment (2.5 folds) groups but no change for the CFCS treatment group when compared to the control group at 0h. At the 48h time point, *Campylobacter* decreased in the control (0.45 folds), CFCS (0.4 folds), and combined treatment (0.55 folds) groups except for the similar pattern of abundance observed in the BPE treatment group compared to the control group of 0h. Other noticeable changes in abundance were seen for the genera of *Ruminococcus*, *Faecalibacterium*, *Eubacterium*, and *Alkaliphilus*, as they were shown to decrease over time in the controls and treatment groups, whereas *Bifidobacterium*, *Acinetobacter*, *Anaeroplasma*, *Bacillus*, and *Desulfovibrio* increased in all the control and treatment groups at both 24h and 48h when compared to the control group of 0h. When compared to control of 0h, the genus *Blautia* decreased in all groups over the time points except for the combined treatment group of 24h, while the genus *Clostridium* decreased in all groups over the time points except for the CFCS group of 48h. *Escherichia/Shigella* increased in all the control and treatment groups except for the combined treatment group at 24h and *Pseudomonas* increased in all groups except for the combined treatment group at both 24h and 48h time points.

When using various indexes for determining distribution and diversity at the species level for each group, there was no statistically significant difference in the number of species, Simpson index, Shannon index, and Marglef's richness. Though there were numerical differences when comparing diversity at species level among the treatments and control groups at both 24h and 48h time points (Figure 2.5). Number of species and the Simpson index slightly reduced in the

combined treatment group compared to the control group at 24h, though Shannon index and Margalef's richness increased in the combined treatment group marginally. At 48h the Number of species, Simpson index and Margalef's richness increased for the combined treatment group, but Shannon index decreased when compared to the control group of same time point.

*Discussion*

This study explored the effectiveness and efficacy of the combined effect of probiotic metabolites and prebiotics on the growth of *Campylobacter* in a more complex environment than those normally used when testing in the *in-vitro* laboratory condition. For this study, chicken cecum content from birds pre-inoculated with CJ-Km was collected and treated with CFCS from an engineered probiotic and/or BPEs to evaluate their collective and individual effect within this new system. These treatment strategies were also studied through whole genome sequencing to determine any potential effects on the genomic DNA of *Campylobacter*. The whole genome sequencing revealed that the treatments were able to affect the CJ at genomic level. The whole genomic DNA comparison shows the control was readily assembled with the GenBank sequence of the *Campylobacter jejuni* RM1221 (ATCC BAA-1062) but the treatment groups were not. Previously, it has been mentioned that DNA might be affected by the probiotic/prebiotic treatments (Van Loo et al, 2005; Aditya et al., 2020).

The effectivity of different probiotic strains has been examined before in simulated gut conditions, in addition to being used for studying the effects of many natural antimicrobials, as seen in previous studies using simulated conditions (Stasiak-Różańska et al., 2021; Vermaak et al., 2009). Though in *in-vivo* system, the complex gut condition affects the survival and the effectivity of different probiotic strains (Stasiak-Różańska et al., 2021), there was still effect on

the enteric pathogens (Salaheen at al., 2018; Tabashsum et al., 2020). Similar effectiveness was observed in case of natural antimicrobials or prebiotic-like components; though there was alteration in the component structure, but effectiveness against bacterial pathogens was still noticed (Crittenden et al., 2009; Salaheen et al, 2018; Vermaak et al., 2009). In the current study, an increase in antimicrobial effect was observed in the case of the combination of metabolites from the modified probiotic strain and the BPE, as seen in the reduction of *Campylobacter* and CJMKm in the cultural study. The growth of CJ-Km and *Campylobacter* was also reduced in presence of CFCS or BPE in the simulated gut condition when determined by cultural techniques. Previously, it has been observed that BPE or $LC^{+mcra}$ can reduce the growth of CJ-Km and *Campylobacter* in chicken gut individually (Salaheen at al., 2017; Tabashsum et al., 2020). It has been also noted in multiple studies that synbiotic approaches more efficiently reduce the growth of different enteric bacterial pathogens in complex gut conditions (Asahara et al., 2011), which was also seen in the current study.

Understanding the effects of cell free metabolites from the probiotic, as well as those of the prebiotic-like components from the BPE, and their combination on the normal composition of the microflora in a simulated gut condition at phylum level, genus level, and at species level for diversity, was an important outcome of this research study. Previously, different studies have proposed that there is no significantly detrimental effect of probiotics or prebiotic like components on the normal gut microbiome in chicken or mice, though the treatments effectively reduced different enteric pathogens in the complex gut condition (Peng et al., 2019; Salaheen et al., 2018; Tabashsum et al., 2020). When analyzing the relative abundance of the most abundant phyla comprising the microbiome, this study observed that, overall, the reduction of Firmicutes was less noticeable in the treatment groups than in the control groups, at both time points. The increments

in the level of Proteobacteria were also less in the treatment groups when compared to control. Bacteroidetes and Actinobacteria were increased in percentage in all the groups including control group at the 24h and 48h time points, compared to control group of 0h. These results could be attributable to the limited nutrient availability within the model, as well as the chemical makeup of the cecum content, which contains multiple undefined compounds. Over time within the simulated gut conditions, there will be certain pressures and environmental factors that will contribute to the alterations seen in the microflora, on top of the effects that a given treatment will have and depending on how the bacteria interact with them. The alteration at the genus level, specifically the increase of *Lactobacillus* level in synbiotic treatment at 24h time point and then decrease at 48h can also be ascribed to the limited nutritional availability and environmental condition such as oxygen availability, pH (Astó et al., 2019; Venema & Abbeele, 2013). Another important factor to note with regards to the metabolites from $LC^{+mcra}$ is their half-life within this system, which could potentially be shortened as more extended bouts of time progressed. Potency of these compounds could be lost over time and since this experiment did not supplement with any additional probiotic bacteria, the metabolites that were initially added were not replenished. This could imply that if only given metabolites, these will only be effective in simulated gut conditions for a short period of time unless live probiotic bacteria are added in the beginning or throughout the experiment to provide a continuous supply of beneficial metabolites over the time. For *Campylobacter*, increase in the level of abundance at 24h and then decrease at 48h, can also be attributed to the similar factors mentioned above. In addition, a viable but non-culturable state of the bacteria could also result in the reduction of *Campylobacter* at both 24h and 48h time points, when observed by cultural methods, but might still appear in the microbiome analysis, as the genomic DNA will still be present in the sample (Keramas et al. 2004).

*Conclusion*

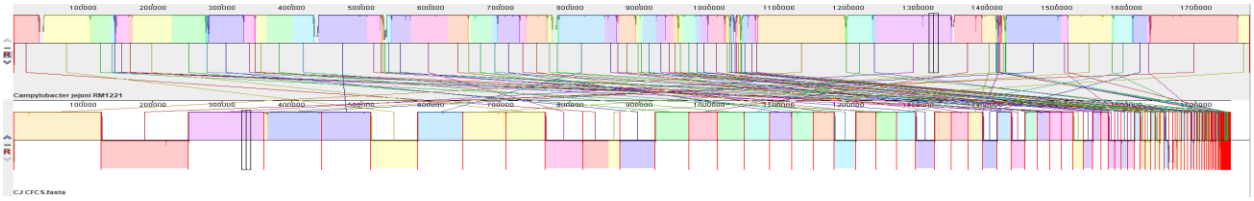
This study explored the effectiveness and efficacy of the combined effect of probiotic-derived metabolites and prebiotics specifically BPE on the growth of *Campylobacter* in a more complex environment than those normally used when testing in the *in-vitro* laboratory condition. Given the above mentioned observations in simulated chicken gut condition, in the future it will be recommended that a combination of CFCS and BPE be used as it has been shown in this study, that it can effectively reduce colonization of *Campylobacter*, as determined by cultural techniques. These results also suggest that these antimicrobial alternative can be implemented without any notable effect on the overall chicken gut microbiome composition as seen in the preservation of key bacterial phyla, genera, and species diversity indexes at the level of a simulated gut condition using cecum content. This also serves as an intermediate step for validating novel procedures and microbial control protocols within a controlled system before implementing them in a live animal model, which will lead to safer application and more predictable results of the protocol.

*Figures*

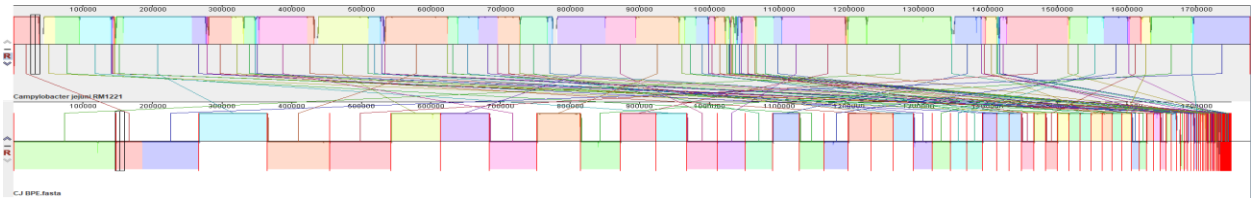
**Figure 2.1**: Comparison of whole genome sequence (WGS) of *Campylobacter jejuni* RM1221 (CJ) control and treatments with whole genome sequence of *Campylobacter jejuni* RM1221 obtained from GenBank using Mauve. (A) Comparison between WGS of *Campylobacter jejuni* RM1221 (GenBank DNA) and CJ without any treatment (control); (B) Comparison between WGS of *Campylobacter jejuni* RM1221 (GenBank DNA) and CJ treated with cell free cultural supernatant of $LC^{+mcra}$ (CFCS); (C) Comparison between WGS of *Campylobacter jejuni* RM1221 (GenBank DNA) and CJ treated with berry pomace extract (BPE); (D) Comparison between WGS of *Campylobacter jejuni* RM1221 (GenBank DNA) and CJ treated with combination of CFCS and BPE.

**(A)**


**(B)**




**(C)**




**(D)**

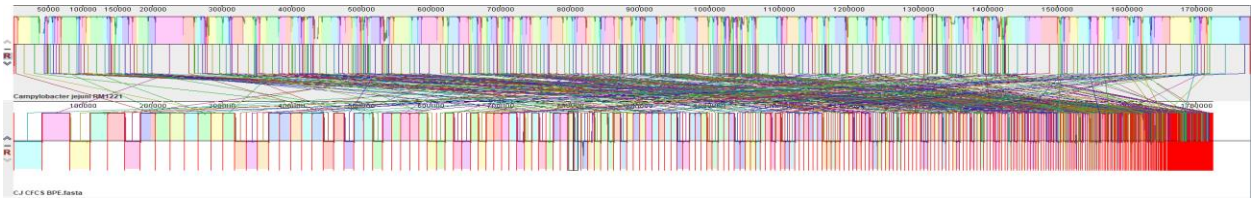
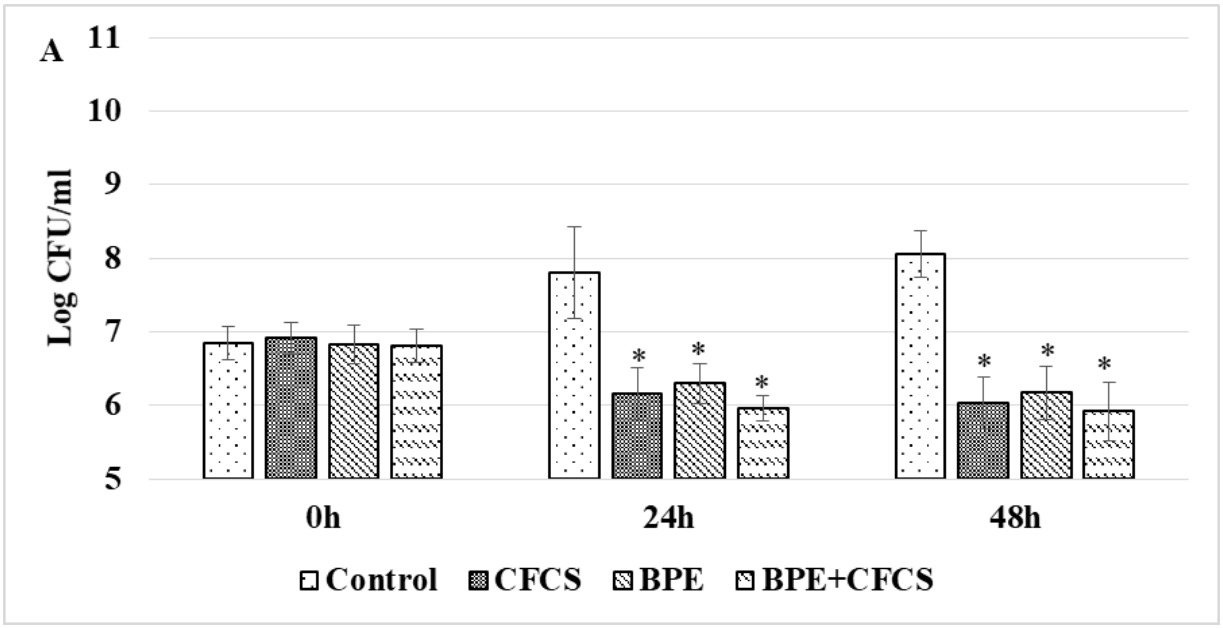


**Figure 2.2**: Effect of cell free cultural supernatant of LC$^{+mcra}$ (CFCS), berry pomace extract (BPE) and a combination of CFCS with BPE on the growth of CJ-Km, (CJ, with kanamycin gene, was fed to chicks) (A), and *Campylobacter* (*Campylobacter* which was naturally colonized) (B) in simulated chicken gut condition. Results are mean of at least three replications and '*' is used to denote significance difference at $p<0.05$ among the control and treatments at that specific time point.



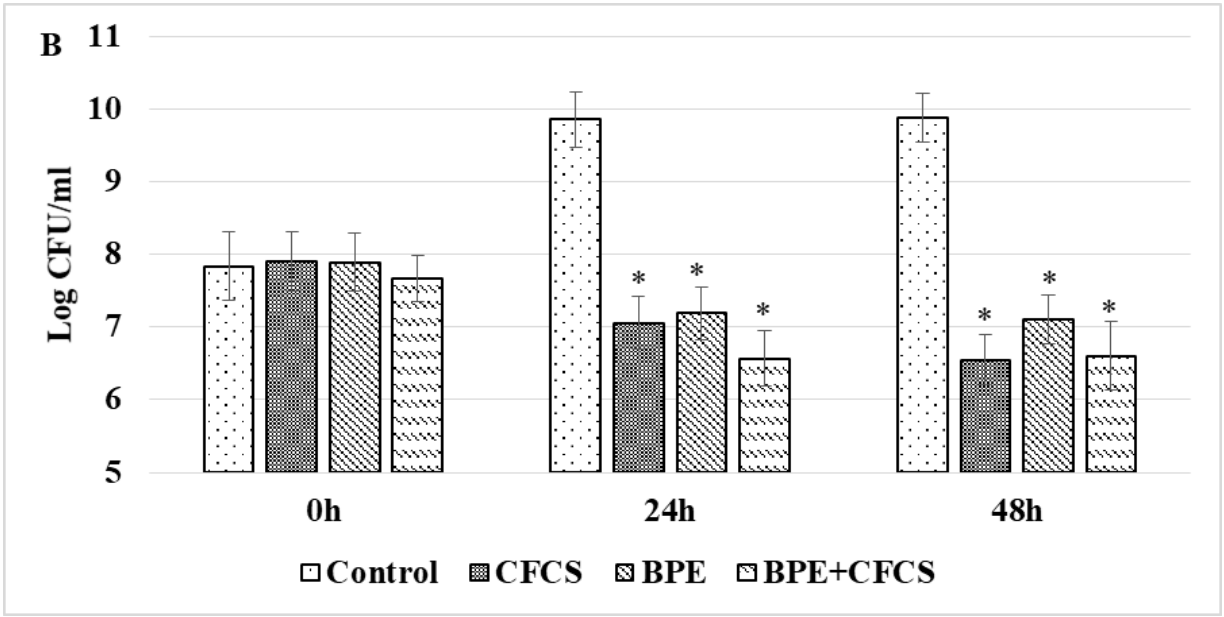



**Figure 2.3:** Effect of cell free cultural supernatant of LC$^{+mcra}$ (CFCS), berry pomace extract (BPE) or combination of CFCS and BPE on the chicken gut microbiome at phylum level in simulated chicken gut condition. Different panels show the abundance of microbiome at phylum level at 0h, 24h, and 48h and the effect of the respective treatments at similar time points.

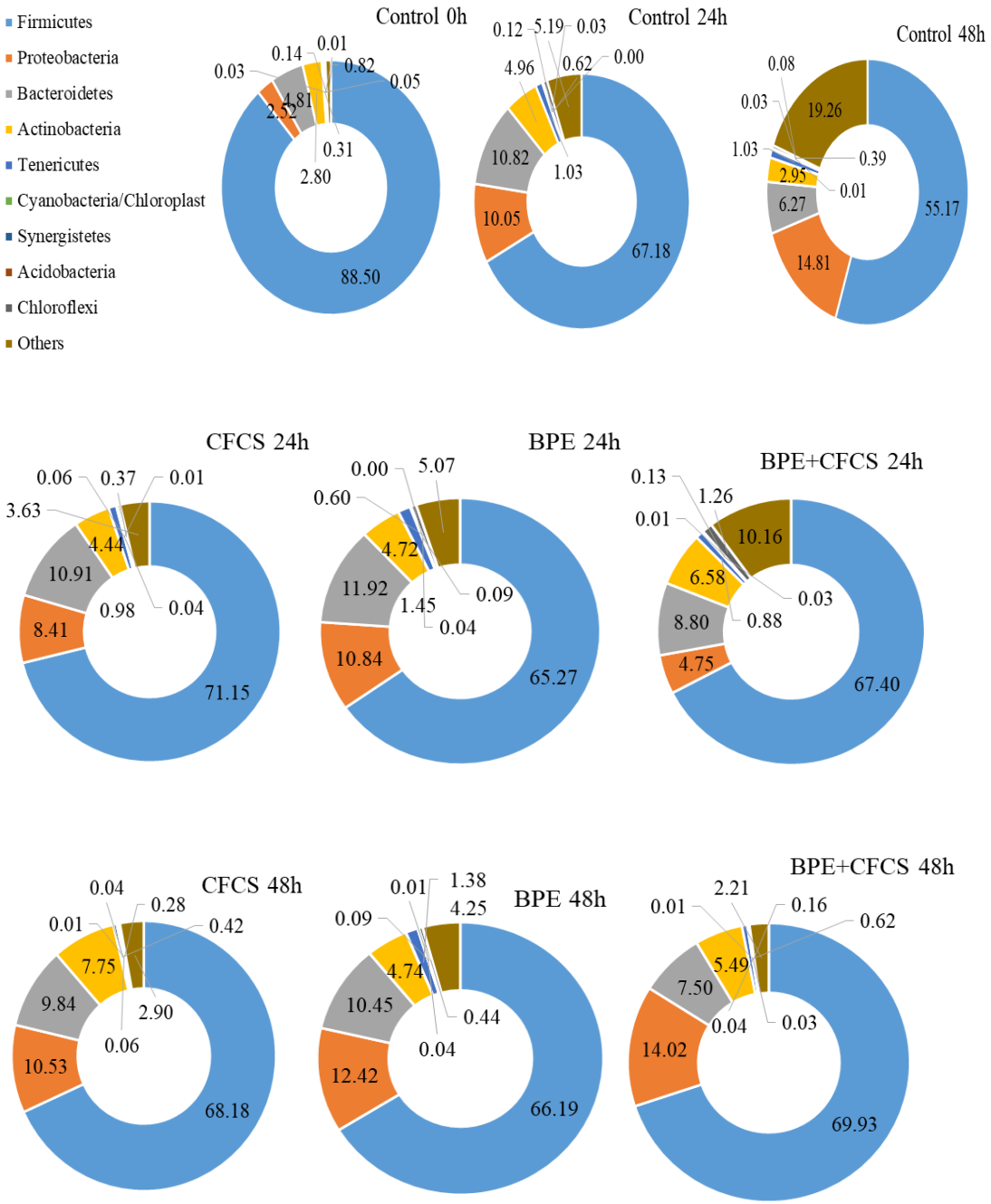


**Figure 2.4:** Effect of cell free cultural supernatant of LC$^{+mcra}$ (CFCS), berry pomace extract (BPE) or combination of CFCS and BPE on the chicken gut microbiome at genus level in simulated chicken gut condition. Different panels show the abundance of microbiome at genus level (selected) at 0h, 24h, and 48h and the effect of the respective treatments at similar time points.

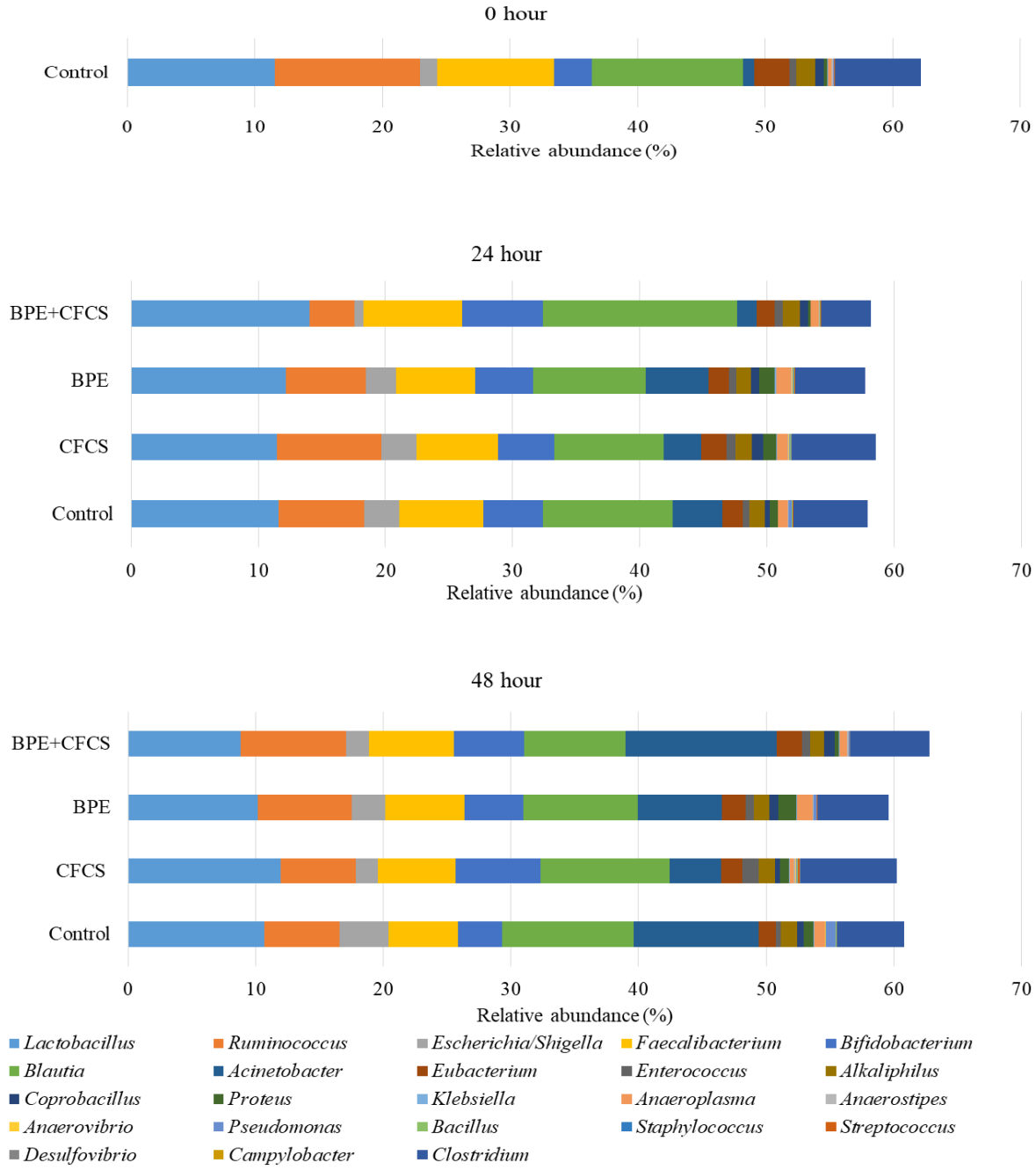
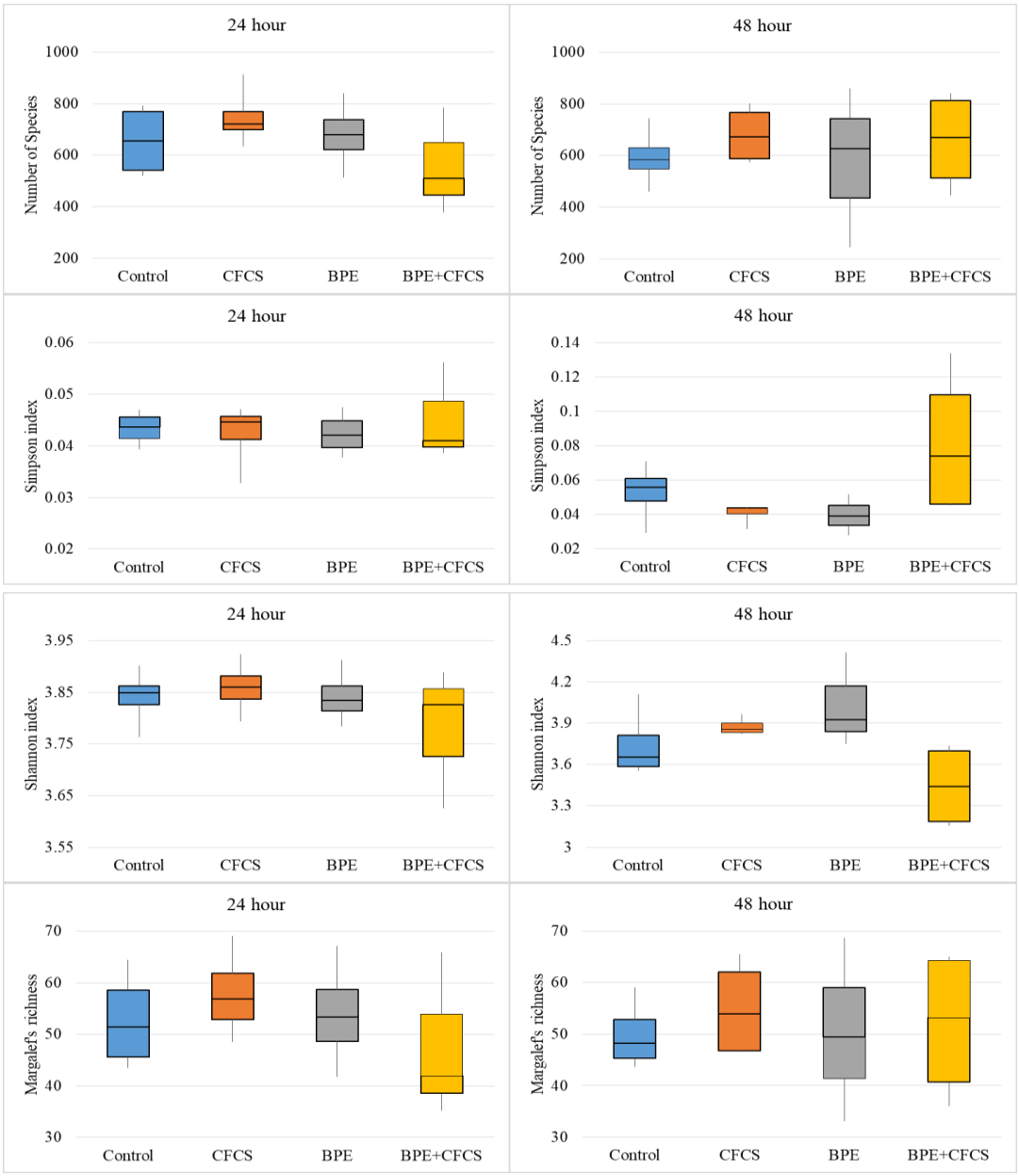


**Figure 2.5 :** Effect of cell free cultural supernatant of LC$^{+mcra}$ (CFCS), berry pomace extract (BPE) or combination of CFCS and BPE on the alpha diversity (total number of species, Simpson index, Shannon index, and Margalef's richness) of the chicken gut microbiome in simulated chicken gut condition. Different panels show the total number of species, Simpson index, Shannon index, and Margalef's richness of the control group and the effect of the respective treatments at 24h and 48h time points.



# Chapter 3

## Aim 2: Competitive reduction of poultry-borne enteric bacterial pathogens in chicken gut with bioactive *Lactobacillus casei*

### *Introduction*

*Campylobacter jejuni* causes foodborne enteric infection campylobacteriosis and is considered as one of the prominent zoonotic pathogens. During 2018, among over twenty-five thousand cases of foodborne infections reported by FoodNet, the incidence rate of *Campylobacter* infections was the highest, 19.5 in 1000 people (CDC, 2019). Poultry is the major reservoir of *C. jejuni* and cross-contamination of chicken meat during processing causes the majority of sporadic occurrences of campylobacteriosis (CDC, 2019; Facciolà et al., 2017). The current trend of white meat consumption, particularly chicken meat, has been increased significantly in the US and around the world, which is believed to be the reason for the increasing numbers of campylobcateriosis (Lin, 2009). *C. jejuni* is normally present in the gut microbiota of poultry and other birds, capable of growing optimally at 42°C, which is the body temperature of chicken (Williams et al., 2006). According to recent reports, the prevalence pattern of *C. jejuni* in pasture poultry and their sensitivity to various antibiotics indicate that animal farming without antibiotic and/or synthetic chemical growth promoters diminishes the prevalence of multiple antibiotic resistance *C. jejuni* in poultry, without altering colonization of other bacteria (Luangtongkum et al., 2006; Salaheen et al., 2016). Therefore, developing sustainable alternatives that can replace antibiotic growth promoters and synthetic chemicals is a priority in order to reduce multiple-drug resistant *C. jejuni*.

Probiotics can be considered as the prime candidates for the prevention and reduction of cross-contamination with foodborne bacterial pathogens in food products (Nagarajan et al., 2019; Peng et al., 2018; Peng et al., 2017; Hayes et al., 2016; Peng et al., 2016; Amalaradjou et al., 2012), however, their effectiveness is heavily dependent on the variety and total amount of produced and secreted metabolites/byproducts (Peng et al., 2019; Peng et al., 2017). Probiotics can break down undigested dietary components through colonizing in the gastrointestinal (GI) tract of the host and after reaching these undigested components in different portions of intestine, especially cecum, jejunum and ileum, the probiotics produce a remarkable quantity of metabolites or bacterial byproducts (Marcobal et al., 2013; Flint et al., 2012). Probiotics and their metabolites also modulate the composition of gut microbiome by competitive colonizing or through the secretion of bioactive compounds in different gut intestinal portions (Dahiya et al., 2017; Cammarota et al., 2014). For example, the increase of the beneficial microorganisms in gut microbial ecosystem ultimately decreases the detrimental microbiota, which can lead to a healthy gut state for the overall health of the host (Dahiya et al., 2017; Cammarota et al., 2014).

The amount and types of bioactive metabolites produced by beneficial microbes are vastly influenced by undigested dietary components and/or prebiotics (Peng et al., 2019; Peng et al., 2017). Any potential prebiotic intended for use has to comply with some regulations, like possessing the "Generally Recognized as Safe (GRAS)" status, being assessed for appropriate dose and potential side effects, must be free of contaminants and impurities and must not cause alterations to the intestinal microbiota that could negatively affect the host (FAO, 2017). According to Wang, it is reported that prebiotics must not be digested in the upper portion of the gastrointestinal tract before reaching large intestine, where they are selectively fermented by beneficial and/or commensal intestinal microbes (Wang et al., 2009). Such type of fermentation

leads to the changes in metabolic processes of gut microbes and nutrients availability in gut ecosystem, thus possessing a beneficial effect on the host. But these beneficial effects vastly depend on the selective stimulation of intestinal probiotics with prebiotic (Wang et al., 2009). With such regulations and conditions, it is difficult to find an ideal prebiotic. Instead of adding stimuli or prebiotic, designing a probiotic that can provide enhanced beneficial effects by itself is a better alternative approach (Peng et al., 2014). Bacterial strains usually used for synbiotic include but not limited to *Lactobacillus* spp., *Bifidobacteria* spp. and several prebiotics mostly form natural food sources like cocoa, peanut (Pandey et al., 2015).

The secondary metabolites produced by beneficial microbes include lipids, particularly short chain and poly-unsaturated fatty acids (Bassaganya-Riera et al., 2012; O'Shea et al., 2012; Louis et al., 2009; Serini et al., 2009). Among the short chain and poly-unsaturated fatty acids, linoleic acid (LA) is considered as one of the most functional metabolites produced by different probiotic species of *Bifidobacterium*, *Lactobacillus* and *Lactococcus* (Rizos et al., 2012).Several research groups, including our own, have focused on enhancing the productivity of LA and conjugated LA (CLA) from probiotic sources, both in the human/animal gut environment and the industrial production (Peng et al., 2019; Peng et al., 2017; Yadav et al., 2007). Our previous study showed a relative high number of *Lactobacillus* and their metabolites exhibited antimicrobial activities against various enteric pathogens such as *C. jejuni*, *S. enterica* and enterohemorrhagic *Escherichia coli* (Tabashsum et al., 2019; Tabashsum et al., 2018; Peng et al., 2015). *Lactobacillus* spp. also have the adherence and multiplying ability in the host (Reid et al., 1999). They can produce acids, hydrogen peroxides and bacteriocins, but with no pathogenic traits and can persist as normal flora (Reid et al., 1999). Among different species of *Lactobacillus*, *L. casei* has been reported as one of the crucial probiotic strain (Reid et al., 1999). Considering this, our research

team over-expressed the myosin-cross-reactive antigen (Mcra) ('*mcra*' gene obtained from *L. rhamnosus*, another probiotic strain) (Reid et al., 1999) for linoleate isomerase over-production in *Lactobacillus casei* (LC). The *mcra* over-expressing probiotic, named as "$LC^{+mcra}$", showed enhanced activities, including anti-inflammatory and antimicrobial effects both *in vitro* and *in vivo* animal model (mice) (Peng et al., 2019; Peng et al., 2018). It has also been reported that $LC^{+mcra}$ induced no detrimental effect on mice gut, rather it improved the murine gut health through the modulation gut microbiome (Peng et al., 2019).

Here, we intended to evaluate the effectiveness of $LC^{+mcra}$ on the colonization of a marker-containing *C. jejuni* strain (CJ-Km) as well as the natural colonization of *C. jejuni* in different sections of chicken gut. We also aimed to compare the gut microbial composition of the control and $LC^{+mcra}$ fed group chickens through 16S rRNA compositional analysis. This study may help to evaluate the effect of $LC^{+mcra}$ in controlling the colonization of *C. jejuni* in chickens at the pre-harvest level, along with the effect of $LC^{+mcra}$ on modulation of gut microbiome.

## *Materials and Methods*

### Bacterial strains and growth conditions

*Lactobacillus casei* (ATCC 334) (LC) and *Campylobacter jejuni* RM1221 (ATCC BAA-1062) were used in this study. To distinguish from other *C. jejuni*, a marker-containing Kanamycin cassette was inserted into the chromosomal DNA of *C. jejuni* and named as 'CJ-Km' (Salaheen et al., 2018; Salaheen et al., 2017). CJ-Km was grown in Karmali agar (Himedia, India) with selective supplements (Himedia, India) under microaerophilic conditions, in presence of 10% $CO_2$, 5% $O_2$ and 85% $N_2$ at 37 °C. Over-production of conjugated linoleic acid in LC, was achieved by incorporating the myosin cross reactive antigen gene (*mcra*) in LC chromosome giving it the name

of 'LC$^{+mcra}$' (Peng et al., 2019; Tabashsum et al., 2019; Peng et al., 2018; Tabashsum et al., 2018; Peng et al., 2015). Both LC and LC$^{+mcra}$ were grown at 37 °C for 18 h in presence of 5% $CO_2$ (Thermo Fisher Scientific Inc., USA) on MRS (de Man Rogosa Sharpe) agar (EMD Chemicals Inc., USA).

*In vivo* study design and chicken model

Experiments in the chicken model were carried out up to 4 weeks, in duplicate trials, using 934-1 Isolator Units (Dye Sheet Matter, USA), which were equipped with HEPA filter and air circulation system. Each animal trial consisted with 60 SPF chicks (one day-old), obtained from Charles River, USA. Institutional Animal Care and Use Committee (IACUC, protocol number R-16-33) recommended guidelines were followed for chicken husbandry. Commercially available crumbles without antibiotic supplementation (Purina Animal Nutrition, USA) were used as chicken feed. Chickens were divided into 3 groups (20 chickens per group) which corresponded to their respective 934-1 Isolator Units (Dye Sheet Matter, USA) (Table 3.1 and Table 3.2). For each treated/control group, chickens were further divided into 2 separate sub-groups (10 chickens in each case) and housed in individual 934-1 Isolator Unit (Dye Sheet Matter, USA). Water was given using Gravity-fed water containers.

Feeding probiotics and *C. jejuni* challenge to chicken model

Both the probiotic strains LC and LC$^{+mcra}$ were grown at 37 °C overnight in shaking incubator (at 120 rpm) on MRS broth (EMD Chemicals Inc., USA) and suspensions were prepared using PBS. LC and LC$^{+mcra}$ solutions (100 µl) containing $10^{10}$ CFU/ml were fed to chicken groups as shown in Table 4.2. On day 8, all chickens were challenged with CJ-Km which was grown in Bolton broth (Himedia, India) in presence of 5% fetal bovine serum (FBS) (Corning, USA) with shaking (at 120 rpm) for 30 h under microaerophilic condition (10% $CO_2$, 5% $O_2$ and 85% $N_2$).

Suspension of CJ-Km (100 µl) containing $10^9$ CFU/ml was fed to the 8-day-old chicks through oral gavage. Chickens were routinely checked for any physical injury after the gavage administration and then moved back to their respective cages.

Sample collection from chicken carcass and processing

Euthanization was performed on day 14 and day 21 for 6 chickens per group and on day 28 for the rest of the chickens, with the purpose of evaluating the degree of colonization of probiotics and CJ-Km in the chick cecum, ileum and jejunum. To determine the colonization pattern of probiotics, homogenized cecum, ileum or jejunum contents (~ 200 g) were diluted and then plated on MRS agar (EMD Chemicals Inc., USA) for enumeration. To test the CJ-Km colonization level, cecum, ileum, or jejunum contents (~ 200 g) were homogenized in 1 ml of phosphate buffer saline (PBS) and then plated on Karmali Agar containing selective supplements (Himedia, India) and Kanamycin (100 µg/ml) (Thermo Fisher Scientific, USA) following serial dilution techniques for enumeration. Colonization of *C. jejuni* (challenged and natural) was also determined following standard methods. For enumeration of *C. jejuni*, intestinal contents from cecum, ileum or jejunum (~ 200 g) were collected and homogenized in PBS solution (1 ml), diluted and then plated on Karmali Agar with selective supplement (Himedia, India). Water samples were also collected from the drinking containers of cages. Water samples (50 ml) were collected in centrifuge tubes (VWR, USA) using a sterile 25 ml pipette (VWR, USA). Then the samples were centrifuged (3000 × g) for 20 min, bacterial pellets were re-suspended in PBS (1 ml) and plated on Karmali Agar with selective supplement for enumeration.

16S rRNA compositional analysis

For comparison of the chicken gut microbiome, the cecum contents of six 28-day-old chickens from each group were randomly collected for 16S rRNA compositional analysis

following the methods previously described by Peng et al (2019). Briefly, DNA was extracted with QIAamp Fast DNA Stool Kit (QIAGEN, USA). The variable regions (V3 and V4) of 16S rRNA were targeted for phylogenic classifications. DNA libraries were prepared using Nextera DNA Library Preparation Kit and Nextera Index Kit (Illumina, USA) and pooled into equimolar concentration according to the protocol provided by the manufacturers. Sequencing (paired-end: 2 × 300 bp) was preformed using MiSeq v3 600-Cycle Kit (Illumina, USA) by Illumina MiSeq. Sequencing data were processed by MiSeq Reporter-BaseSpace for FASTQ workflow generation (Greengenes database). Demultiplexing was conducted using the perfect index recognition (mismatch =0) and then by removing PhiX reads. A total 8509169 quality-filtered reads were used for analysis of the 16S rRNA composition. The data were analyzed to determine the differences of relative abundance at phylum, genus and species (number of species, Simpson index, Shannon index and Margalef's richness) levels among three groups (control, LC or LC$^{+mcra}$ fed groups) following standard protocol.

Statistical analysis

For statistical analysis, cecum, ileum or jejunum from a bird was considered as an experimental unit. The data were ranked, and ANOVA was used to differentiate the level of colonization (CFU/g cecum, ileum or jejunum contents). For comparison of the mean ranks, Tukey's test was used. Differences in the water samples were compared by performing ANOVA and Tukey's test was used for comparison of mean ranks.

*Results*

Efficient colonization capability of LC$^{+mcra}$ in chicken gut

To compare the colonization capability of LC and LC$^{+mcra}$ in various sections of chicken gut, the colonization of *Lactobacillus* was enumerated and compared in our three groups of chicks from two separate trials (Fig. 4.1). The administration with LC$^{+mcra}$ showed higher and more stable cecal colonization level of *Lactobacillus,* above 10 log CFU/g throughout 4 weeks, which were substantially higher than wild-type LC fed group as well as natural colonization group (Figure 3.1A). A similar pattern was found in LC$^{+mcra}$ fed chicken jejunum, which showed a significantly ($p< 0.05$) higher number of *Lactobacillus* at day 28 (Figure 3.1B). Though this difference was only numerically higher in ileum when compared to wild type LC fed group (Figure 3.1C). The number of *Lactobacillus* in the fecal shedding of probiotic-fed group were observed to rise when compared to the control group throughout the study period (Figure 3.1D). Specifically, in the chicken group fed with LC$^{+mcra}$, colonies of *Lactobacillus* in fecal shedding gradually increased, whereas fecal shedding of *Lactobacillus* in the wild-type LC fed group and control group (group fed with no probiotic) were observed to be noticeably lower than that in the LC$^{+mcra}$ fed group (Figure 3.1D).

Control of colonization of *C. jejuni* strain in probiotic fed chickens

The chickens were orally administered with LC or LC$^{+mcra}$ in order to evaluate their roles in competitively reducing the colonization of the marker-containing strain CJ-Km. Even though, the colonization of CJ-Km was decreased notably by both LC and LC$^{+mcra}$ compared to the control group, in various parts of chicken gut, chickens that were fed with LC$^{+mcra}$ demonstrated a higher reduction in the colonization of CJ-Km than the LC fed groups. Chickens colonized with LC$^{+mcra}$ strain showed a notable reduction in CJ-Km colonization in different portions of intestine specifically ileum and jejunum by at least 1 log or more as compared to the chickens which were fed with wild type probiotic (LC) strain at day 14, 21 and 28 (Figure 3.2). When comparing the colonization of CJ-Km in the cecum, we observed that chickens pre-administered with LC or

LC$^{+mcra}$ showed lower CJ-Km colonization in cecum contents (Figure 3.2A). Likewise, both probiotics (LC and LC$^{+mcra}$) reduced the number of CJ-Km colonization in ileum contents compared to control group (Figure 3.2B). The same pattern was found in jejunum contents compared to control group (Figure 3.2C). The notable reduction on CJ-Km gastrointestinal colonization was also found in form of a lower number of CJ-Km fecal shedding when fed with LC or LC$^{+mcra}$ (Figure 3.2D). Notable effectiveness of LC$^{+mcra}$ was observed in chickens after 1st week post-challenged with CJ-Km, at which there was an approximate 0.9 log CFU/g reduction in chicken feces compared to control group. In the following two weeks, CJ-Km fecal shedding of LC$^{+mcra}$ fed chickens continuously reduced by more than 1.0 log CFU/g and 1.10 log CFU/g at day 21 and 28, respectively, compared to the control group (Figure 3.2D).

Reduction on natural colonization of *C. jejuni* in probiotic fed chickens.

Chickens pre-treated with either LC or LC$^{+mcra}$ showed resistance against the natural colonization of *C. jejuni* in cecum, jejunum and ileum, but the chickens pre-treated with LC$^{+mcra}$ had a much effective influence on reduction of colonization of both bacterial pathogens (Figure 3.3). Specifically, chickens fed with LC$^{+mcra}$ showed remarkable reduction in the level of natural colonization of *C. jejuni* in cecum contents, ileum contents and in jejunum contents at days 14, 21 and 28, respectively when compared to the contents of the control groups (Figure 3.3A, 3.3B, 3.3C). Meanwhile, a consequential decrease in fecal shedding of *C. jejuni* was also detected in LC$^{+mcra}$ fed chickens (Figure 3.3D).

Reduced level of contamination in water provided to the probiotic fed chickens

At different time points (day 14, 21 and 28), water samples were collected to determine the cross-contamination *C. jejuni*. The contamination level of *C. jejuni* in water was reduced approximately by 1.0 log CFU/50 ml and more than 1.0 log CFU/50 ml in the groups of chickens

fed with LC and LC$^{+mcra}$, respectively, compared to the control group throughout the experimental period (Figure 3.4A).

Gut microbiome alteration in the probiotic fed chickens

At the phylum level, we observed all three groups of chickens (control, LC fed and LC$^{+mcra}$ fed groups) were colonized with abundant number of Firmicutes. Over all 83.47%, 82.38% and 84.16% of Firmicutes were found in control, LC fed group and LC$^{+mcra}$ fed group, respectively (Fig. 3.5). Increased numbers of both Bacteroidetes and Tenericutes was also observed in the LC and LC$^{+mcra}$ fed groups compared to the control group. Higher number of Bacteroidetes was also observed in the gut of LC (21.72%) and LC$^{+mcra}$ (19.82%) fed chickens compared to the control group. The percentage of Tenericutes was approximately double for both LC and LC$^{+mcra}$ fed groups of chickens (Figure 3.5). On the other hand, a decrease of the abundance of Proteobacteria in both LC (38.3%) and LC$^{+mcra}$ (39.8%) fed groups was observed. In fact, the lowest abundance of Proteobacteria was observed in the LC$^{+mcra}$ fed group (Figure 3.5). In case of Verrucomicrobia, there was not much difference between control group and LC fed group, but there was a noteworthy reduction of Verrucomicrobia, approximately 80%, in the abundance in LC$^{+mcra}$ fed group. For Actinobacteria and other phyla, no remarkable differences were observed among the groups (Figure 3.5).

At the genus level, the distribution of bacteria in the three groups of chickens fed with LC, LC$^{+mcra}$ or control showed a distinct variation. For example, there was a decreased abundance in the LC or LC$^{+mcra}$ fed groups compared to the control for *Ruminococcus, Anaerovibrio, Intestinimonas, Papillibacter, Fusibacter, Eubacterium, Erysipelotrichaceae, Subdoligranulum* and most importantly for *Campylobacter* (Figure 3.6). For *Alistipes, Streptococcus, Clostridium, Alkaliphilus, Parasutterella, Eisenbergiella, Anaeroplasma* and *Pseudoflavonifractor*, there was

an increase of the abundance in either the LC or LC$^{+mcra}$ fed group compared to the control group (Figure 3.6). There was also an increase of *Lactobacillus* in both LC and LC$^{+mcra}$ fed groups of chickens as expected. *Anaeroplasma* (2.31 and 2.71-fold increase in LC and LC$^{+mcra}$ fed groups, respectively) and *Streptococcus* (2.43 and 2.07-fold increase in LC and LC$^{+mcra}$ fed groups, respectively) were found as the foremost differences in the abundance at the genus level compared to the control group. *Pseudoflavonifractor* was also detected with approximately 1.40-fold increase in both LC and LC$^{+mcra}$ fed groups compared to the control group. *Lactobacillus* was observed with around 1.16-fold increase in the LC fed group and around 1.17-fold increase in the LC$^{+mcra}$ fed group in comparison with the control group. Then, the abundance of *Papillibacter* was decreased by 1.86 and 4.85-fold in LC and LC$^{+mcra}$ fed groups, respectively; followed by 1.54 and 1.71-fold decrease of *Erysipelotrichaceae* in LC and LC$^{+mcra}$ fed groups, respectively (compared to the control group). Colonization of *Salmonella* was decreased by 2.13-fold in the LC fed group and 3.54-fold in the LC$^{+mcra}$ fed group chickens. The decrease of *Campylobacter* colonization was approximately 3.16-fold in the LC$^{+mcra}$ fed group of chickens compared to the control group (Figure 3.6).

We have also compared the diversity of bacterial species among the three groups using alpha indices (Shannon index, Simpson index and Margalef's richness). There was no significant difference observed in bacterial diversity among the three (control, LC and LC$^{+mcra}$ fed) groups. For instance, the number of observed species in both LC and LC$^{+mcra}$ fed groups was only numerically higher than the control, with LC$^{+mcra}$ group having the highest, an average of 1592 species (Figure 3.7A). There was also only numerical difference in Simpson index for control, LC and LC$^{+mcra}$ fed group (Figure 3.7B). The average of Shannon index of LC$^{+mcra}$ fed group was numerically the highest followed by LC fed group and control group (Figure 3.7C). The Margalef's

richness was also numerically the highest for LC$^{+mcra}$ fed group followed by LC fed group and control group (Figure 3.7D).

*Discussion*

Previously we showed the antagonistic effect of conjugated linoleic acid over-producing probiotic, LC$^{+mcra}$, on enteric bacterial pathogens through *in vitro* studies (Peng et al., 2018; Tabashsum et al., 2018; Peng et al., 2015). Here, we verified the effectiveness of CLA over-producing LC$^{+mcra}$ against the colonization of the marker-containing CJ-Km strain, as well as on the natural colonization of *C. jejuni* in a chicken model. According to our *in vitro* study, the novel probiotic strain LC$^{+mcra}$ demonstrated at least 21 folds more CLA conversion rate in bacterial cell free cultural supernatant and it could also survive longer in comparison with the wild-type LC strain (Peng et al., 2018). We also observed a remarkable decrease of *C. jejuni* growth in co-culture with LC$^{+mcra}$ and in the presence of the cell-free cultural supernatant collected from LC$^{+mcra}$. LC$^{+mcra}$ could also alter the host cells-*C. jejuni* interactions, expression of virulence genes and other virulence properties (Peng et al., 2018; Tabashsum et al., 2018).

Though, according to literature, efficiency of probiotics in reducing colonization of enteric pathogenic bacteria is yet inconclusive, several groups of research have proposed that the increased production of metabolites from probiotics (e.g., CLA) might improve the overall health benefits of probiotics on host, in addition to the exclusion of enteric pathogens through colonization competition in gut ecosystem (Tabashsum et al., 2019; Peng et al., 2018; Tabashsum et al., 2018; Peng et al., 2017). In this study, we used a CJ-Km strain containing kanamycin resistance gene which was inserted into *cdt* of *C. jejuni* chromosome as shown in a previous study demonstrating that deletion of *cdt* did not alter the colonization capability of *C. jejuni* in chicken gut (Biswas et

al., 2006). The 7-day-old chicks challenged with CJ-Km showed a persistent resistance to CJ-Km colonization in their gut through the effect of probiotics. Specifically, probiotic treatments reduced the colonization of CJ-Km from chicken cecum, ileum and jejunum in a time-dependent manner at two, three and four weeks of age. The microbial balance of fecal shedding in the beneficial, commensal and detrimental microbes serves as the crucial indicator of gastrointestinal health (Lee et al., 2013), correspondingly we observed the decreased colonization of CJ-Km in both fecal contents and various intestinal contents, as well as an increased colonization trend of probiotic strains. Probiotics themselves were able to limit gastrointestinal bacterial pathogens through competitive exclusion and colonization resistance (McKenney et al., 2015; Peng et al., 2015a; Peng et al., 205b; Buffie et al., 2013; Amalaradjou et al., 2012).

From the *in vivo* findings of this study, we observed that both probiotics (LC and $LC^{+mcra}$) remarkably diminished *C. jejuni* colonization in gastrointestinal portions including cecum, jejunum and ileum, which supported our previous studies (Peng et al., 2019). In addition, $LC^{+mcra}$ showed more aggressive reductions on the colonization of CJ-Km and *C. jejuni* as a result of the effects achieved from its increased amount of CLA conversion (Peng et al., 2019; Peng et al., 2018). Previously, CLA has been reported and associated with antibacterial effect against different enteric pathogens including *C. jejuni*, though the distinctive mechanisms of interaction between CLA and bacterial cells are still under investigation (Peng et al., 2018; Tabashsum et al., 2018; Meraz-Torres et al., 2012). Furthermore, our *in vivo* examination based on chicken model also confirmed the protective functions of $LC^{+mcra}$ against gastrointestinal bacterial pathogens, supporting the previous *in vitro* outcomes (Tabashsum et al., 2019; Peng et al., 2018; Tabashsum et al., 2018). In mice model, CLA was observed to provide a protective mechanism through both stimulating beneficial bacterial colonization and maintaining the balance in microbial composition

in gut ecosystem, which served as the first line defense against foodborne pathogenic bacterial colonization (Peng et al., 2019).

The investigation of the chicken normal floral distribution was another goal of this study. Several research groups also reported that CLA-rich diets could stimulate the fatty acids metabolism and preserve homeostatic gut microbiota (Kho et al., 2018; Marques et al., 2012). The balanced gut microbial ecosystem, along with the diversity of microbes, was modified by the introduction of probiotic capable of producing CLA. The researchers also suggested that higher abundances of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* could influence the gut homeostasis in a positive way that contributes to defending against enteric pathogens. In this study, we found that the most abundant bacterial phyla in both probiotic fed and non-fed group of chickens were Firmicutes and Bacteroidetes. When the chickens were given probiotic either wild-type LC or bioactive $LC^{+mcra}$ strain, the abundance of the Firmicutes and Bacteroidetes were increased notably. Previously, Wang et al. also reported a similar finding (Wang et al., 2017). When they treated the chickens with probiotics specifically *Lactobacillus*, they also observed that Firmicutes and Bacteroidetes were colonized notably higher in the cecum of the broiler. We also observed the relatively a smaller number of Proteobacteria in both groups of chickens treated with probiotics, specifically in the gut of the $LC^{+mcra}$ fed group. Proteobacteria compose with a large number of Gram-negative enteric bacteria including pathogens. By feeding probiotics, several research groups also observed reduced number of Proteobacteria in the animal models (Peng et al., 2019; Oakley et al., 2014; Luo et al., 2013). We also found that *Clostridium, Faecalibacterium, Ruminococcus* and *Alistipes* were the predominant genera in all three groups of chickens which is in agreement with the previous reports of Shaufi et al (2015) and Luo at al (2013). These *Clostridium, Faecalibacterium, Ruminococcus* and *Alistipes* genera are also found to involve in

the production of different beneficial metabolites in poultry gut, including SCFA (Luo et al., 2013). In our study, we observed that these *Clostridium, Faecalibacterium, Ruminococcus* and *Alistipes* genera were highly abundant in the probiotic fed group chickens specifically in the gut of LC$^{+mcra}$ fed chickens.

As two groups of chickens were given orally either LC or LC$^{+mcra}$, higher abundance of the *Lactobacillus* species was observed in those groups. Previously Wang et al. also observed that probiotic feeding increased the abundance of the probiotics in poultry (Wang et al., 2017). Furthermore, we observed that there was reduced number of Gram-negative bacteria including *Fusibacter, S. enterica* and *C. jejuni* in the gut of probiotic fed chickens. We also found that the decreasing pattern of the Gram-negative bacteria was much more noticeable in the LC$^{+mcra}$ fed group than the LC fed group which is agreed with the previous findings of Peng et al (2019). The abundance and diversity of the available species were also compared in this study. Though there was no statistically significant difference, numerical differences among control, LC and LC$^{+mcra}$ fed groups of chickens were observed. Similar finding was also reported by Wang et al (2017). The diversity of the species largely varies with the heredity, environment and diets, which might be responsible for the nonsignificant difference among the groups of chickens.

In addition to this, reduced numbers of *C. jejuni* were observed in the drinking water collected from the water containers in the probiotic-fed groups. This result is supported by our previous studies where we reported LC$^{+mcra}$ inhibited *C. jejuni*, *S. enterica* and *E. coli* (Peng et al., 2019; Tabashsum et al., 2019; Peng et al., 2018; Tabashsum et al., 2018; Peng et al., 2015b). The survival and persisting ability of *C. jejuni* in water has been reported, which means that reducing survivability in drinking water of chicken may limit both the horizontal transfer of this bacterial pathogen to other chicks and the colonization of *C. jejuni* in flocks (Newell et al., 2003).

It has been reported that antibiotic growth promoter limits both the prevalence of *C. jejuni* at the pre-harvest level in conventional poultry farms as well as the poultry carcass cross-contamination with *C. jejuni* in conventionally processed chicken meat when compared to organic processors (Salaheen et al., 2014; Luangtongkum et al., 2006). Previously, we observed that berry pomace extracts decreased the natural colonization of *C. jejuni* and colonization of marker-containing CJ-Km strain in chicken cecum remarkably with positive modulation of chicken gut microbiome (Salaheen et al., 2018; Salaheen et al., 2017; Salaheen et al., 2014b). Similarly, here we also observed noteworthy decrease in CJ-Km and *C. jejuni* in different portions of chicken gastrointestinal tract in the probiotics fed chicks (both wild-type and CLA over-producing LC) with the $LC^{+mcra}$ having a substantially better performance. In water samples of both probiotics fed groups, there was reduction in the cross-contamination with *C. jejuni*. Therefore, the combination of berry pomace extract and $LC^{+mcra}$ can be a potential approach for reducing the colonization of *C. jejuni* as well as improving the growth of chickens, but further investigation is needed.

*Conclusion*

Conclusively, this study indicated the effectiveness of CLA over-producing probiotic strain, $LC^{+mcra}$, on the colonization of both marker-containing CJ-Km strain and naturally colonized *C. jejuni* in various sections of chicken intestine along with the modulation of gut microbiota towards healthier gut. Specifically, chickens fed with $LC^{+mcra}$ daily for one week showed a notable reduction on the colonization of CJ-Km. Natural colonization of chicken gut and fecal shedding with *C. jejuni* was also observed to be notably reduced by probiotics feeding. Though the study was conducted in controlled laboratory environment, the outstanding roles of $LC^{+mcra}$ provides a

promising option for reducing the colonization of the most predominant zoonotic pathogens, specifically *C. jejuni* in chicken gut, which may eventually lead to lower incidence of cross-contamination in poultry products. Thus, $LC^{+mcra}$ may have a beneficial effect on the quality of the poultry products and retain consumer satisfaction with regards to the safety of the poultry products and reducing the risk of zoonotic infections.

*Figures and Tables*

**Figure 3.1:** Colonization of wild-type probiotic, LC and CLA over-producing probiotic, LC$^{+mcra}$ in different intestinal portions of chickens and their fecal shedding pattern compared to control in terms of *Lactobacillus* count. Colonization of LC/LC$^{+mcra}$ in cecum (A), ileum (B), jejunum (C) and fecal shedding of LC/ LC$^{+mcra}$ (D) in terms of *Lactobacillus* count. Results are mean ± SD and letters (a-c) are used to mark the significant differences at various time points compared with control and among the treatment groups at $p < 0.05$.

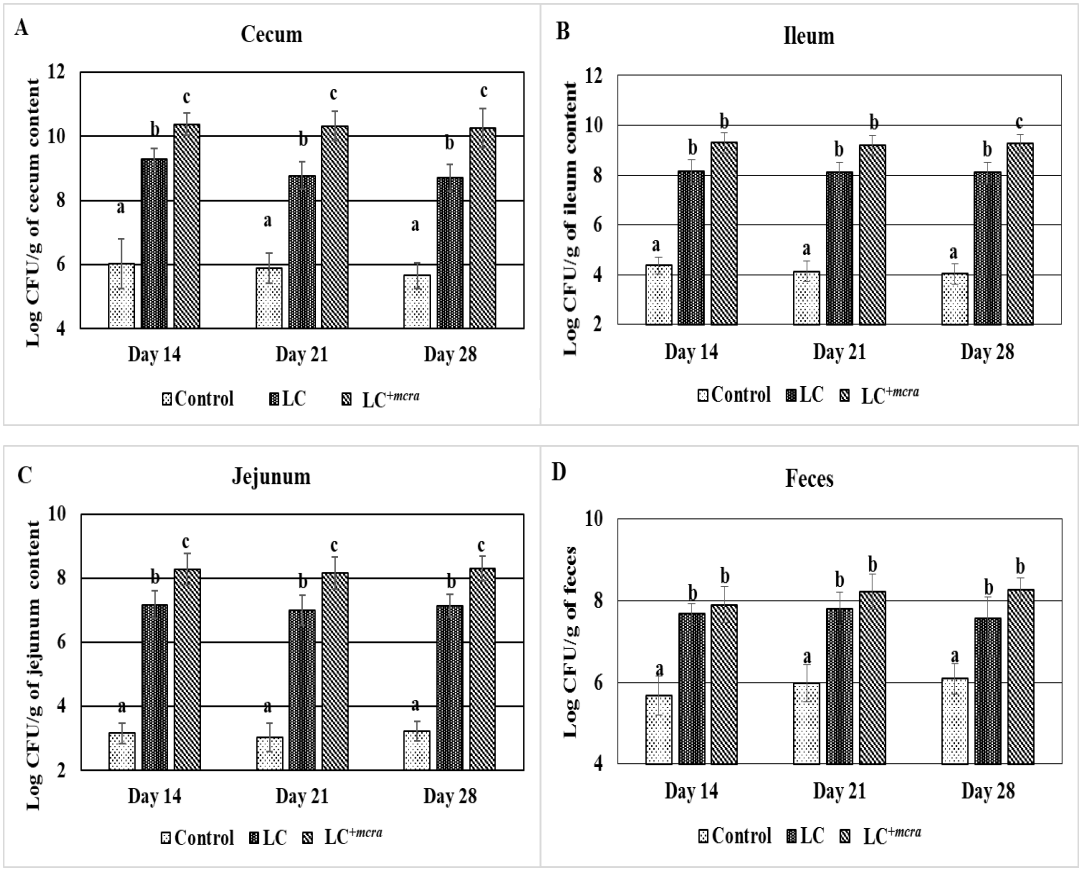


**Figure 3.2.** Effect of wild-type probiotic, LC and CLA over-producing probiotic, LC$^{+mcra}$ on colonization and fecal shedding pattern of CJ-Km in different intestinal portions of chickens at days 14, 21 and 28 of infection compared to control. Colonization in cecum (A), ileum (B), jejunum (C) and fecal shedding of CJ-Km (D). Results are mean ± SD and letters (a-c) are used to mark the significant differences at various time points compared with control and among the treatment groups at $p < 0.05$.

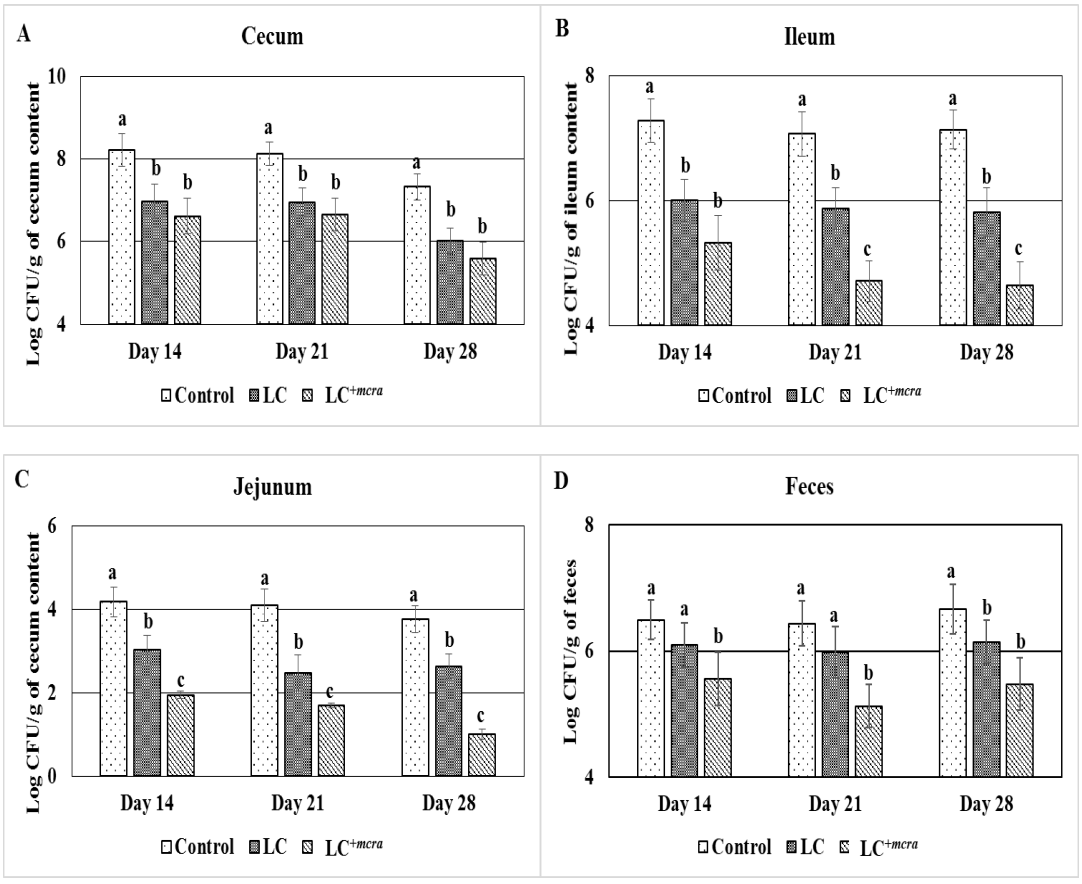


**Figure 3.3.** Effect of wild-type probiotic, LC and CLA over producing probiotic, LC$^{+mcra}$ on colonization and fecal shedding pattern of *C. jejuni* in different intestinal portions of chickens at days 14, 21 and 28 compared to control. Colonization in cecum (A), ileum (B), jejunum (C) and fecal shedding of *C. jejuni* (D). Results are mean ± SD and letters (a-c) are used to mark the significant differences at various time points compared with control and among the treatment groups at $p < 0.05$.

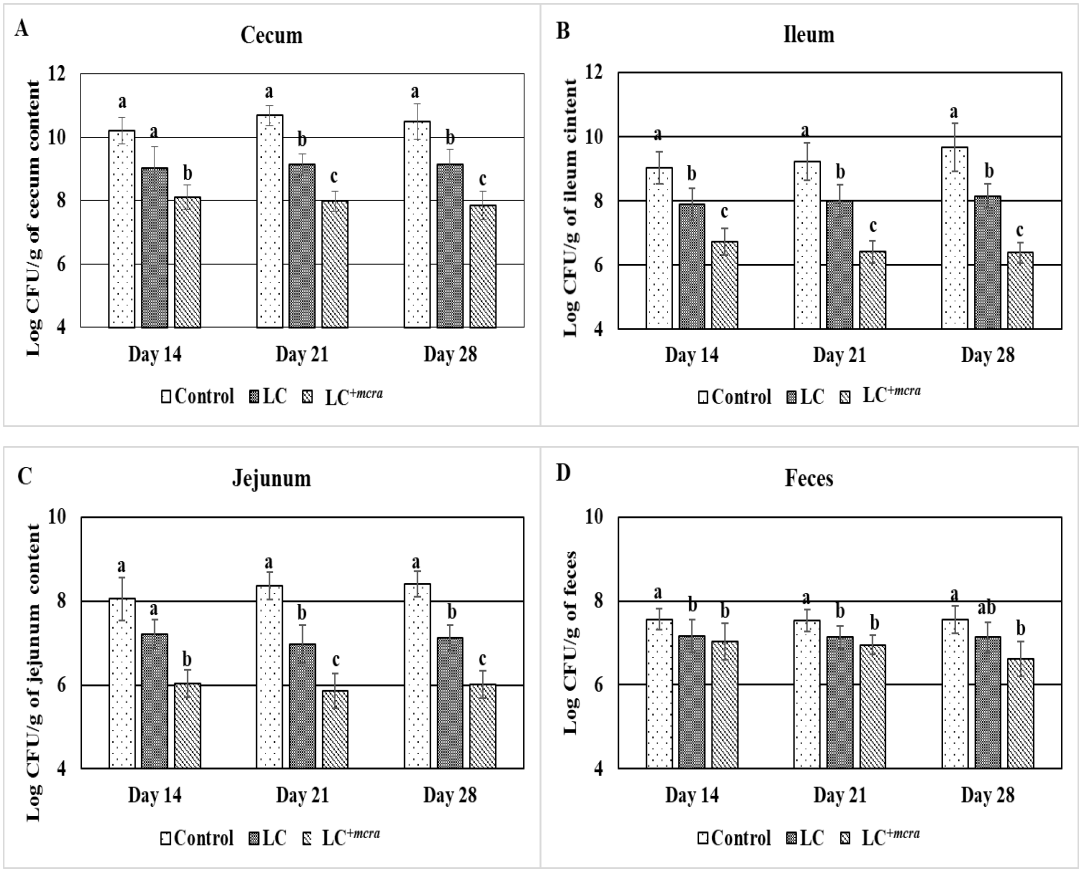


**Figure 3.4.** Comparison of water contamination level among the probiotics fed (wild-type probiotic, LC and CLA over-producing probiotic, LC$^{+mcra}$) and control groups of chickens. Contamination level of *C. jejuni* in water bottles. Results are mean ± SD and letters (a-b) are used to mark the significant differences at various time points compared with control and among the treatment groups at $p < 0.05$.

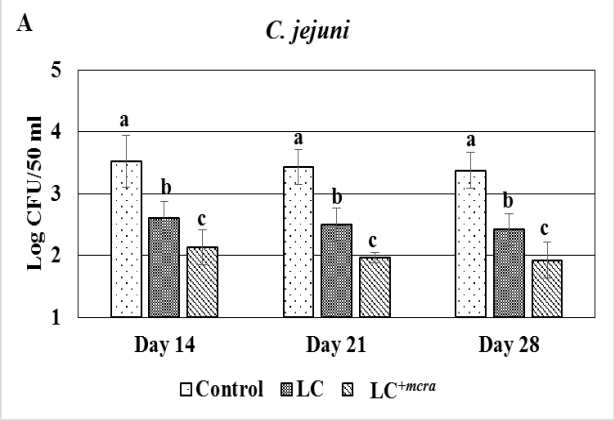


**Figure 3.5.** Relative abundance of sequences representing the chicken gut microbiota at phylum level. Comparison of bacterial population of chicken cecal contents collected from different groups at day 28, (A) control group, (B) wild-type probiotic, LC, fed group and (C) CLA over-producing probiotic, LC$^{+mcra}$, fed group.

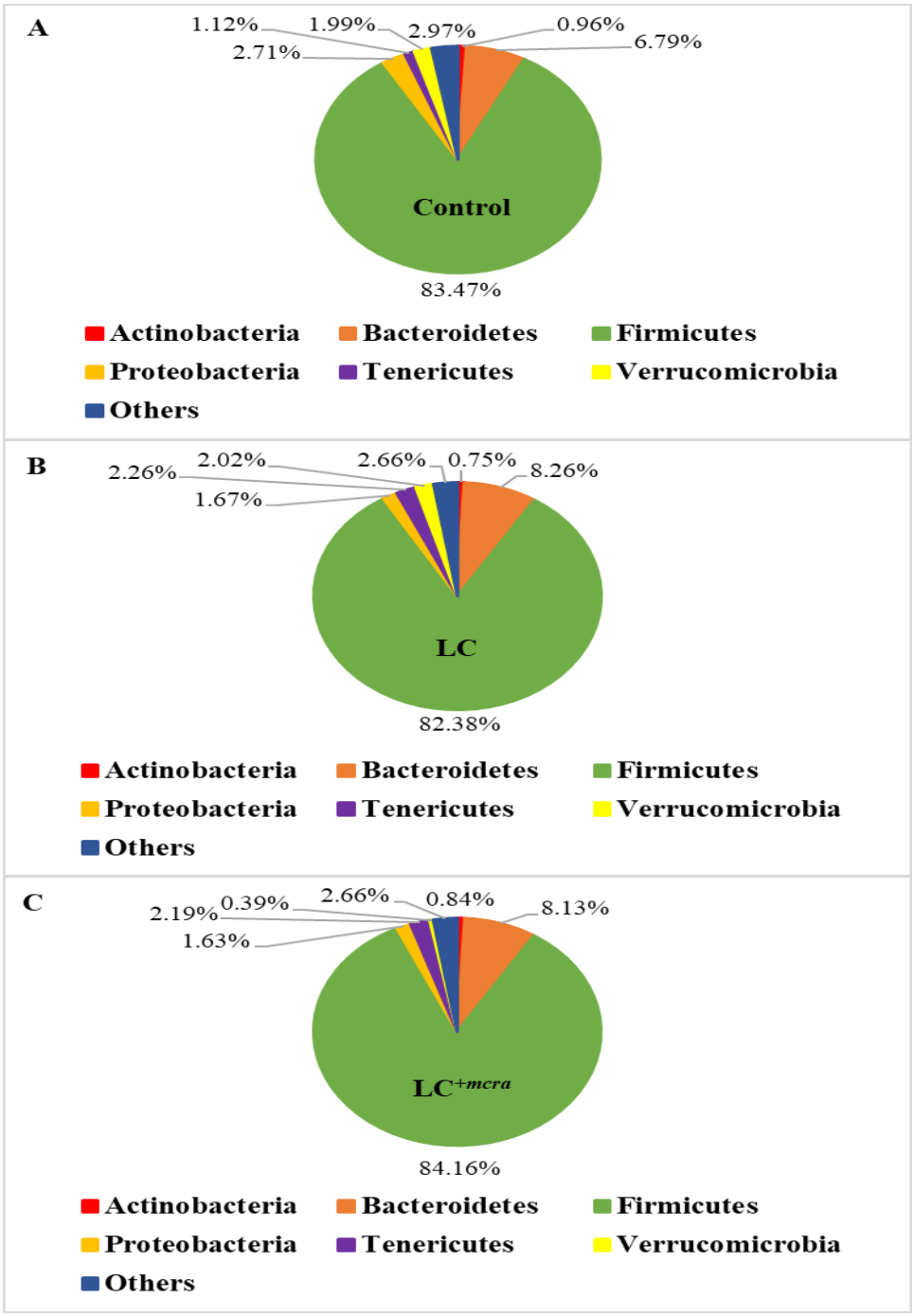


**Figure 3.6.** Comparison of bacterial composition at genus level of chicken cecal contents collected at day 28, among control group, wild-type probiotic, LC, fed group and CLA over-producing probiotic, LC$^{+mcra}$, fed group.

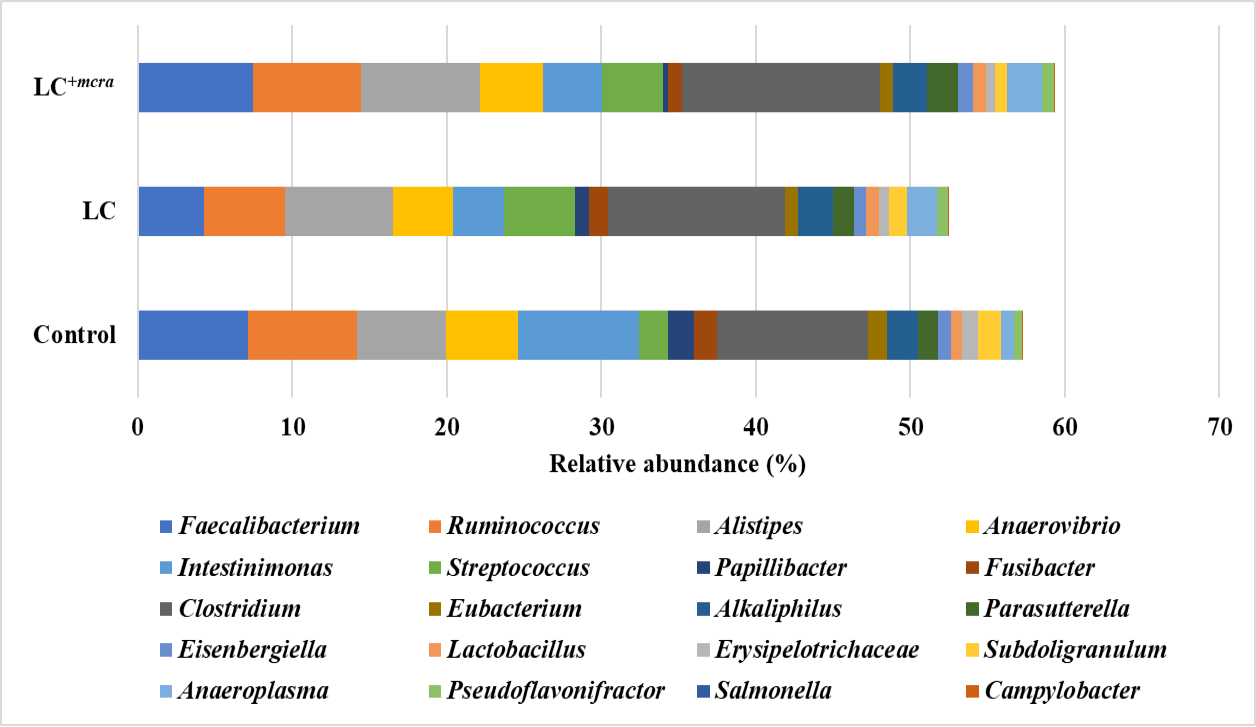


**Figure 3.7.** Comparison of chicken gut microbiome at species level. Chicken cecal contents were collected from control, wild-type probiotic, LC, fed group and CLA over-producing probiotic, LC$^{+mcra}$, fed groups at day 28. Bacterial diversity at species level (A) Number of species, (B) Simpson index, (C) Shannon index and (D) Margalef's richness was compared. Results are mean ± SD.

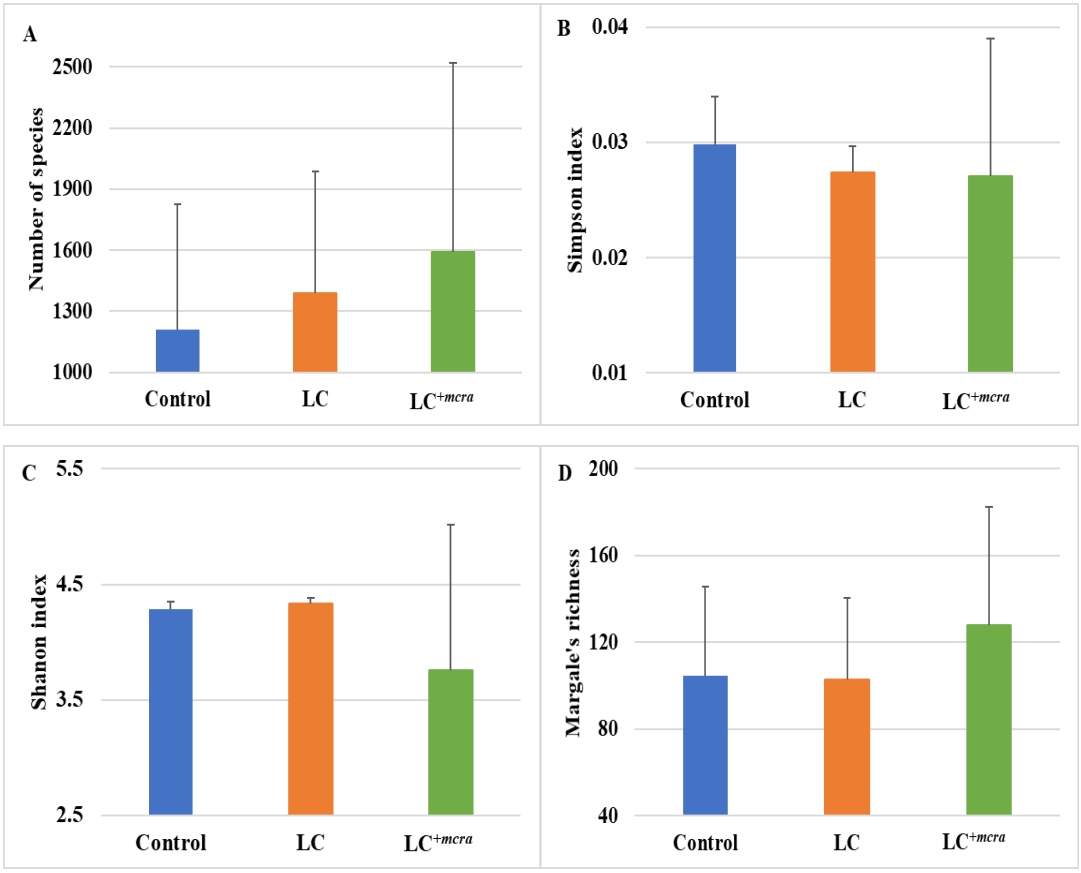


**Table 3.1.** Number of chickens allocated in the experimental groups with treatments.

| **Treatments** | **Trial 1** | **Trial 2** | **Total** |
|---|---|---|---|
| Phosphate buffered saline (A) | 20 | 20 | 40 |
| *L. casei* ATCC334 (B) | 20 | 20 | 40 |
| Mcra over-expressing *L. casei* (C) | 20 | 20 | 40 |
| Total | 60 | 60 | 120 |

**Table 3.2.** Experimental procedures with timeline (per trial).

| Timeline | Number of Chickens | | | | | |
|---|---|---|---|---|---|---|
| | **Daily Probiotic Colonization** | **Enteric Bacterial Challenge** | **Fecal Sample Collection** | **Euthanization** | **Cecum Sample Harvest** | **Remaining Live** |
| Day0 | - | - | 60 (1/chicken) | - | - | 60 |
| Day1-Day7 | 40 (B, C)* | - | - | - | - | 60 |
| Day8 | - | 60 (A, B, C)* | 60 (1/chicken) | - | - | 60 |
| Day14 | - | - | 60 (1/chicken) | 18 (6/group) | 18 (6/group) | 42 |
| Day21 | - | - | 42 (1/chicken) | 18 (6/group) | 18 (6/group) | 24 |
| Day28 | - | - | 24 (1/chicken) | 24 (8/group) | 24 (8/group) | 0 |

*A, corresponds to 'control group'; B. corresponds to the 'group fed with LC' and C, corresponds to 'group fed with LC$^{+mcra}$'

# Chapter 4

## Aim 3: Combined effect of conjugated linoleic acid over converting *Lactobacillus casei* and berry phenolic extracts on *Campylobacter* colonization in chicken gut

*Introduction*

*Campylobacter* was first recognized in the late nineteenth century, characterized and named in 1963, is now one of the prominent causative agents of diarrheal diseases in the US (Silva et al., 2011). The disease caused by *Campylobacter* is called campylobacteriosis and the infectious dose may be as less as 500 colony forming unit (CFU) (Kaakoush et al., 2015; Janssen et al., 2008). The common symptoms of campylobacteriosis are diarrhea, vomiting, and abdominal cramp and can lead to several post infection complexity like irritable bowel syndrome (IBD), Guillain-Barre syndrome (GBS) etc. (Kaakoush et al., 2015; Janssen et al., 2008,). Campylobacteriosis is mostly spread through consumption of raw or under cooked poultry or improper handling of contaminated poultry products, which attributes to more than 70% of the total disease outbreaks (Hakeem and Lu, 2021). There are 22 species of *Campylobacter* but *Campylobacter jejuni* is one of the important species to infect human (Facciolà et al., 2017). *C. jejuni* is a normal inhabitant in chicken and in many other animals, making it one of the leading zoonotic pathogens in the US with increased infection rate over past years (Hakeem and Lu, 2021; Masila et al., 2020).

In absence of sub-therapeutic antibiotic or antimicrobial and appropriate biosecurity measure, farm animals including chicken may expose to various pathogens (Roth et al., 2019, Peng et al, 2018; Salaheen et al., 2016). In such situation, farmers may need to use more antibiotic as therapeutic to treat their sick chickens that may lead to residual antibiotics in poultry products and

the organic poultry may suffer with production loss or loss their organic status. Moreover, it has been reported that the antibiotic resistance pattern of *C. jejuni* is persistence all over the world and more so in the conventional poultry production systems (Rivera-Mendoza et al., 2020; Salaheen et al., 2016). Different synthetic chemicals are in use for sub-therapeutic purposes of poultry production but that may lead to chemical contaminations as well as organic poultry farming systems are searching for solution (McEvoy, 2019). In this scenario, alternatives to antibiotics or synthetic chemicals in the poultry farming systems are in demand and synbiotic compositions may be used for the purpose (Angiolillo et al, 2014; Cardarelli et al., 2008).

Probiotic or prebiotic, individually are beneficial to different poultry production systems as growth promoter and/or antimicrobials (Tarradas et al., 2020; Tabashsum et al., 2020b; Ferdous et al., 2019; Salaheen et al., 2018; Tran et al., 2018; Carter et al., 2017; Saint-Cyr et al., 2017). Probiotics has been reported to be effective in growth promotion of chicken as well as in reduction of different enteric pathogens including *C. jejuni* (Tabashsum et al., 2020b; Tarradas et al., 2020; Ferdous et al., 2019; Carteret al., 2017; Saint-Cyr et al., 2017). The beneficial effect of the probiotic can be limited by the colonization and/or metabolic ability of the specific probiotic. In our laboratory, we have developed a probiotic, $LC^{+mcra}$ with the ability to over-convert conjugated linoleic acid (CLA) by over-expressing the *mcra* (myosin-cross-reactive-antigen) in *Lactobacillus casei* (Peng et al., 2018; Tabashsum et al., 2018). $LC^{+mcra}$ can colonize, as well as reduce the colonization of different enteric pathogens in host gut more efficiently than *L. casei* (Peng et al., 2020; Tabashsum et al., 2020b). Among different prebiotics in current use, berry phenolic extracts (BPEs) have shown their efficacy in promoting the growth of probiotic *in vitro*, reducing the growth of different enteric pathogens in host gut, and also was able to promote growth of chickens in concentration dependent manner when applied throughout the growth period (Al-Manaser et al.,

2022; Mahfuz et al., 2021; Tabashsum et al., 2019a; Salaheen et al., 2018; Salaheen et al., 2017). Previously, we have observed that combination of probiotic LC$^{+mcra}$ and berry phenolic extracts (BPEs) can reduce the growth of different enteric pathogens including *Escherichia coli*, *Salmonella* Typhimurium, *C. jejuni* in *in vitro* conditions (Tabashsum et al., 2020a; Tabashsum et al., 2019a; Tabashsum et al., 2019b). Now, if this probiotic, LC$^{+mcra}$ and prebiotic, BPEs are used as a synbiotic in chicken model, they may be more efficient than their individual counterparts in terms of application period and/or concentration.

In this study, we have evaluated the combined effect of LC$^{+mcra}$ and BPEs when applied for shorter period rather than continuous application in reducing the colonization of *C. jejuni* using a day-old chicken model. Further, their combined effect on normal chicken gut microbiome as well as growth performance of the chickens were also determined.

## *Materials and Methods*

### Bacterial strains and their growth condition

A conjugated linoleic acid (CLA) over-converting strain of *Lactobacillus casei* (LC$^{+mcra}$) was used as probiotic in this study (Peng et al., 2019; Tabashsum et al., 2018). LC$^{+mcra}$ was grown in De Man, Rogosa and Sharpe (MRS) agar (EMD Chemicals Inc., USA) medium at 37º C overnight in presence of 5% $CO_2$. A Kanamycin resistant *Campylobacter jejuni* strain (CJ-Km), in which kanamycin cassette was inserted in the chromosome to differentiate from other *Campylobacter*, was used to challenge the chickens (Tabashsum et al., 2020; Salaheen et al., 2019). CJ-Km was grown in Karmali agar (Himedia, India) with supplements (Himedia, India) and kanamycin (Thermo Fisher Scientific, USA) at microaerophilic condition in presence of 10% $CO_2$, 5% $O_2$ and 85% $N_2$ at 37 °C.

Number of chickens, their distribution, and treatments

Total 160 a day-old chickens were used in duplicate trials and for each study, 80 chickens were divided into four groups (20 chickens/group), named group A (Grp-A), group B (Grp-B), group C (Grp-C) or group D (Grp-D), respectively (Table 4.1). These groups were treated with the respective treatments up to 7 days to evaluate *Campylobacter* colonization trend and overall microbiome on the gut of chickens in both trials (Table 4.2). For both trials 934-1 isolator units (Dye Sheet Matter, USA) were used. SPF chickens were purchased from Charles River, USA (80 for each trial), commercially available feed without antibiotic supplementation (Purnia Animal Nutrition, USA), and gravity fed water containers were used in both trials. For animal husbandry and stocking density, Institutional Animal Care and Use Committee (IACUC, protocol number R-FEB-20-04) recommendations were followed. Chickens of Grp-A were given PBS, chickens of Grp-B were given antibiotic growth promoters (AGPs) consisting of Oxytetracycline (1mg/L), Erythromycin (2mg/L), Tylosin (2mg/L), Bacitracin (4mg/L), and Neomycinsulfate (32mg/L) (Salaheen et al., 2017; Salaheen et al. 2018), chickens of Grp-C were given $LC^{+mcra}$ (~log 10 CFU/ml), and chickens of Grp-D were given combination of $LC^{+mcra}$ (~log 10 CFU/ml) and BPEs (0.1 mg/ml GAE) up to day 7 as treatments. To maintain the stocking density, each group were further divided in to two subgroups and put into the cages (10 chickens per cage). At day 8, all the chickens were challenged with CJ-Km (~log 8 CFU/ml). Chickens were euthanized at day14, 21, and 28 and cecum, jejunum or ileum were collected to study the contents (Table 4.2). Fecal samples were also collected to study the shedding pattern (Table 4.2). To evaluate the growth performance, the weight of the individual live chickens was measured at day 28 for both trials.

Culture and enumeration of *Lactobacillus*, $LC^{+mcra}$, CJ-Km and natural *Campylobacter*

To evaluate the colonization pattern of overall *Lactobacillus* in different intestinal portions or fecal shedding pattern, around 200 g of the cecum, jejumn, ileum contents or feces were homogenized, serially diluted and plated on MRS agar (EMD Chemicals Inc., USA) following the incubation conditions mentioned previously. To determine the reduction level of the challenged CJ-Km in different intestinal portions or fecal shedding, similarly 200 g of the cecum, jejunum, ileum contents or feces were plated on Karmali agar (Himedia, India) with supplements (Himedia, India) and kanamycin (Thermo Fischer Scientifc, USA) and incubated at microaerophilic condition in presence of 10% $CO_2$, 5% $O_2$ and 85% $N_2$ at 37 °C. Similar homogenization and serial dilutions were also used for the enumeration of the overall *Campylobacter* (natural and challenged) in the cecum, jejunum, ileum contents or feces, and plated on Karmali agar (Himedia, India) with supplements (Himedia, India) and incubated at standard conditions.

Metagenomic study

16S metagenomics approach was used to compare microbiome profiles of the different groups of chickens. At day 28, the cecum content of the euthanized chickens were collected and DNA was isolated using the QIAamp Fast DNA Stool kit (Qiagen, USA). Variable region V3 was targeted, and DNA libraries were prepared using the Nextera DNA Library preparation kit and Nextera Index Kit (Illumina, USA). According to the manufacturer's recommendation libraries were pooled into equimolar concentration and sequencing was performed in Illumina MiSeq system using MiSeq v3 600-Cycle Kit (Illumina, USA). The data was obtained from MiSeq Reporter-BaseSpace for FASTQ workflow generation (Greengenes database) and de-multiplexing was conducted by perfect index recognition and PhiX reads removal. Then, the data was analyzed for taxonomy by comparing relative abundance at phylum and genus level, and for diversity at

species level by comparing number of species, Simpson index, Shannon index and Margalef's richness among the control/treatment groups following standard protocol.

Statistical analysis

Cecum, jejunum, ileum contents or feces from a single bird was considered as an experimental unit in the cultural enumeration study (CFU/g), for chicken weight individual chicken was considered as an experimental unit, and for metagenomics study, each sub-group of chickens were considered as an experimental unit. ANOVA followed by Tukey's HSD was used to determine the differences at a single time point among different control/treatment groups.

*Results*

Colonization of *Lactobacillus* in chicken gut

The colonization of *Lactobacillus* was significantly ($p<0.05$) higher in the cecum, jejunum, and ileum of Grp-C (fed with $LC^{+mcra}$) or Grp-D (fed with combination of $LC^{+mcra}$ and BPEs) compared to the Grp-B (fed with AGPs) or Grp-A (fed with PBS). At day-14, we found that the *Lactobacillus* colonized in cecum only 6.47 log CFU/g in Grp-A, whereas the cecum of probiotic fed groups, Grp-C and Grp-D had 10.5 log CFU/g and 10.7 log CFU/g of *Lactobacillus*, respectively which included both natural and fed $LC^{+mcra}$ (Figure 4.1). This trend of higher number of *Lactobacillus* in the cecum contents of Grp-C or Grp-D continued till day 28 (Figure 5.1). In the jejunum and ileum contents, colonization of *Lactobacillus* was also higher in Grp-C or Grp-D throughout the experimental trail period when compared to Grp-A or Grp-B (Figure 4.1). At day-28, the colonization of *Lactobacillus* in jejunum content of the probiotic fed groups, Grp-C and Grp-D were 8.67 log CFU/g and 8.83 log CFU/g, respectively. Similarly, in the ileum, the colonization of *Lactobacillus* in Grp-C and Grp-D were 9.18 log CFU/g and 9.2 log CFU/g,

respectively at day-28. For the fecal shedding pattern of *Lactobacillus*, in the combination treatment group, Grp-D, there were 8.2 log CFU/g, 8.3 log CFU/g, and 8.23 log CFU/g of total fed and natural *Lactobacillus* in the feces collected at day-14, day-21, and day-28, respectively. In the feces collected from Grp-C, there were 8.06 log CFU/g, 8.04 log CFU/g, and 8.1 log CFU/g of *Lactobacillus* at day-14, day-21, and day-28, respectively. In Grp-A and Grp-B, which were not supplemented with probiotics, there were at least 2.0 log CFU/g less *Lactobacillus* in the fecal shedding at all the time points when feces sample were collected (Figure 4.1).

Effect of $LC^{+mcra}$ and/or BPEs on reduction of CJ-Km colonization and shedding

Colonization of CJ-Km in chicken gut compartments of Grp-D was reduced significantly ($p<0.05$) at all time points compared to control group, Grp-A (Figure 4.2). Specifically, at day-14, the reduction of CJ-Km colonization in cecum of antibiotic fed group, Grp-B was 1.24 log CFU/g whereas in the probiotic fed groups, Grp-C and Grp-D, the colonization of CJ-Km was 1.13 log CFU/g and 1.21 log CFU/g less, respectively (Figure 4.2). At day-21 and day-28 in cecum, CJ-Km colonization was 1.3 log CFU/g and 1.39 log CFU/g less, respectively in Grp-B, 1.23 log CFU/g and 1.15 log CFU/g less, respectively in Grp-C, while 1.25 log CFU/g and 1.33 log CFU/g less, respectively in Grp-D. In the jejunum of the chicken gut, CJ-Km colonization was reduced by the treatments, particularly by1.26 log CFU/g, 1.04 log CFU/g and 1.25 log CFU/g in Grp-B, Grp-C, and Grp-D, respectively compared to control group, Grp-A at day-14 (Figure 4.2). At day-21, in the jejunum, the colonization of CJ-Km was reduced by 1.4 log CFU/g, 1.1 log CFU/g, and 1.27 log CFU/g in Grp-B, Grp-C, and Grp-D, respectively. Similarly, at day-28, the reduction of the CJ-Km colonization in jejunum content of Grp-B, Grp-C or Grp-D were respectively 1.41 log CFU/g, 1.16 log CFU/g, and 1.3 log CFU/g, compared to Grp-A. In another compartment of chicken gut, in the ileum, the reduction of CJ-Km colonization at day-14, day-21 and day-28 were

respectively 1.34 log CFU/g, 1.37 log CFU/g and 1.39 log CFU/g in Grp-B compared to Grp-A (Figure 4.2). For the probiotic fed groups, the reduction in the colonization of CJ-Km in ileum at day-14, day-21 and day-28 were respectively 1.06 log CFU/g, 1.17 log CFU/g and 1.16 log CFU/g in Grp-C compared to Grp-A, while respectively 1.21 log CFU/g, 1.25 log CFU/g, and 1.29 log CFU/g in Grp-D compared to Grp-A. The fecal shedding of CJ-Km was also reduced in the treatment groups. At day-14, day-21 and day-28, the reduction of CJ-Km shedding in the feces collected from Grp-B were respectively 1.01 log CFU/g, 0.94 log CFU/g, and 1.25 log CFU/g. Similarly, at day-14, day-21 and day-28, the reduction of CJ-Km shedding in the feces collected from Grp-C and Grp-D were 1.09 log CFU/g and 0.94 log CFU/g, 1.1 log CFU/g and 1.02 log CFU/g, 0.98 log CFU/g and 1.1 log CFU/g, respectively.

Reduction of natural *Campylobacter* colonization and shedding

The combined treatments (Grp-D) reduced the natural colonization of *Campylobacter* in different intestinal portions of the chicken gut at all time points (Figure 4.3). In the cecum, the natural colonization of *Campylobacter* was reduced by 1.41 log CFU/g, 1.2 log CFU/g and 1.33 log CFU/g, respectively in Grp-B, Grp-C, and Grp-D at day-14 (Figure 4.3). At both day-21 and day-28, the similar pattern of reduction in the colonization of *Campylobacter* was followed in the cecum of Grp-B, Grp-C, and Grp-D. Specifically, the treatments reduced *Campylobacter* colonization by 1.23 log CFU/g, 1.23 log CFU/g, and 1.34 log CFU/g in the cecum of Grp-B, Grp-C or Grp-D, respectively at day-21, and 1.41 log CFU/g, 1.24 log CFU/g and 1.41 log CFU/g in the cecum of Grp-B, Grp-C or Grp-D, respectively at day-28. In the jejunum, the reduction of natural colonization of *Campylobacter* were 1.3 log CFU/g, 1.35 log CFU/g, and 1.4 log CFU/g at day-14, day-21, and day-28, respectively by the AGPs in Grp-B (Figure 4.3). In Grp-C, the $LC^{+mcra}$ treatment reduced the natural colonization of *Campylobacter* in jejunum by 1.18 log CFU/g, 1.33

log CFU/g, and 1, 12 log CFU/g at day-14, day-21, and day-28, respectively. The combined treatment of Grp-D was most efficient at day-14 and day-21 in reducing the *Campylobacter* colonization in jejunum by 1.4 log CFU/g and 1.42 log CFU/g, while only 1.32 log CFU/g at day-28. In the ileum content, the natural colonization of *Campylobacter* was reduced by 1.27 log CFU/g, 1.17 log CFU/g, and 1.19 log CFU/g in Grp-B, Grp-C, and Grp-D, respectively at day-14 (Figure 4.3). Similarly at day-21, the natural colonization of *Campylobacter* in the ileum were reduced by 1.65 log CFU/g, 1.59 log CFU/g, and 1.76 log CFU/g, respectively in Grp-B, Grp-C, and Grp-D and at day-28, the natural colonization of *Campylobacter* in the ileum were reduced by 1.28 log CFU/g, 1.06 log CFU/g, and 1.24 log CFU/g in Grp-B, Grp-C, and Grp-D, respectively. The fecal shedding of *Campylobacter* were also reduced in all the treatment groups (Figure 4.3), notably by 1.2 log CFU/g, 0.84 log CFU/g, and 1.14 log CFU/g in the feces collected from Grp-D at day-14, day-21, and day-28, respectively.

Effect of various treatment on gut microbiome

To observe the effect of the treatments of Grp-C or Grp-D on the gut microbiome compared to Grp-A or Grp-B, we studied the changes at phylum and genus level for taxonomy and species level for diversity. The most abundant phylum in all the groups were Firmicutes, accounting for 86.55%, 89.25%, 91.6%, and 83.8% in Grp-A, Grp-B, Grp-C or Grp-D, respectively. Proteobacteria reduced significantly in the treatment groups compared to Grp-A (control group). There were 3.84%, 1.77%, and 0.88% Proteobacteria in Grp-A, Grp-B, or Grp-C respectively, while only 0.41% in Grp-D. Bacteroidetes in Grp-A, Grp-B, Grp-C or Grp-D were 5.68%, 5.85%, 5.27% or 5.02%, respectively. Actinobacteria and Cyanobacteria increased in Grp-D compared to Grp-A, Grp-B or Grp-C, while Tenericutes, Synergistetes and Chloroflexi decreased (Figure 4.4).There was alteration at the genus level in the treatment groups compared to Grp-A. At genus

level *Lactobacillus* was 1.7-fold higher in Grp-D, which was significantly higher ($p<0.05$) than the *Lactobacillus* of Grp-A, and also higher than both Grp-B and Grp-C. *Campylobacter* was significantly lower (6.3-fold) in Grp-D compared to Grp-A ($p<0.05$) and was also lower than either Grp-B or Grp-C. *Escherichia*, *Faecalibacterium*, *Blautia*, *Acinetobacter*, *Enterococcus*, *Coprobacillus*, *Proteus*, *Klebsiella*, *Fusibacter*, *Pseudomonas*, *Bacillus*, *Desulfovibrio* were reduced in the Grp-D compared to Grp-A, Grp-B or Grp-C (Figure 4.5). *Ruminoccocus, Bifidobacterium, Eubacterium, Anaeroplasma, Anaerostipes, Staphylococcus, Clostridium* were increased in Grp-D compared to Grp-A, and in the other two groups, Grp-B or Grp-C these genera altered differently (Figure 4.5).For the diversity at species level, we compared the number of species, Shannon index, Simpson index, and Margalef's richness among control and different treatment groups (Figure 4.6). There were numerical differences in these indices but there were no significant differences statistically ($p<0.05$).

Growth performance

The weight of the chickens at day 28 varied numerically (Figure 5.7), though there were not statistically significance differences ($p<0.1$). At day 28, the weight of the chickens in Grp-B, Grp-C or Grp-D compared to the Grp-A (control group) was respectively 0.5%, 4.7% or 3.2% increased due to this short-term (1$^{st}$ week only) treatment with antibiotic and either probiotic or combination of both probiotic and BPEs

*Discussion*

In previous studies at *in vitro* conditions, probiotic LC$^{+mcra}$ could grow more efficiently in presence of trace amount (0.1 mgGAE/mL) of BPEs, and combined effect of LC$^{+mcra}$ and BPEs could reduce the growth of *Campylobacter* more competently than their individual effects

(Tabashsum et al., 2019b; Tabashsum et al., 2018). In this study, we observed the ability of LC$^{+mcra}$ to colonize in different portions of chicken gut when combined with BPEs compared to LC$^{+mcra}$ alone using day old chickens in duplicate trials. Here, we found that the effectiveness of the combined treatment of LC$^{+mcra}$ and BPEs (Grp-D) in reducing the colonization of challenged CJ-Km besides naturally occurring *Campylobacter* in cecum, jejunum, and ileum of chickens. We also observed that the overall microbiome shifts in the cecum of the chickens at the end of our study. The group (Grp-D) of chickens, which was treated with both probiotic and berry pomace extracts until day-7, were protected against colonization of *Campylobacter*. Previous studies have shown that probiotic, prebiotic or synbiotic can be efficient in concentration dependent manner (Salaheen et al., 2018; Salaheen et al., 2017; Dibaji et al., 2014). In this study, combined treatments with the genetically modified probiotic and natural phenolic extract for first seven days of the chicken growth period, showed best combination of synbiotic in improving safety of the poultry products and their growth performances. In both trials of this study, we observed the efficient colonization ability of LC$^{+mcra}$, in poultry gut and BPEs, treatments increased the count of *Lactobacillus* significantly within the different intestinal parts of chickens. This result was expected as both Grp-C and Grp-D, which were fed LC$^{+mcra}$. This finding reassured that the supplementation with probiotics increases the probiotic colonization in the chicken gut and agreed with previous findings (Tabashsum et al., 2020a; Wang et al., 2017).

In case of fecal shedding, we noticed higher *Lactobacillus* count in Grp-C or Grp-D as we observed in the probiotic fed groups of animals of previous studies (Tabashsum et al., 2020a; Peng et al., 2019). We have also noticed that colonization of CJ-Km was reduced in cecum, jejunum, and ileum of Grp-C or Grp-D which were fed either probiotic or both probiotic and berry phenolic extracts compared to Grp-A which was given only PBS, at all specific time points. At all the time

points in Grp-D, the colonization of CJ-Km was reduced more efficiently than Grp-C. This result has been supported by multiple *in vitro* and *in vivo* studies where it was reported that the combination or synbiotic treatment is more efficient than individual probiotic or prebiotic treatments (Śliżewska et al., 2020; Tabashsum et al., 2019a; Tabashsum et al., 2019b; Tabashsum 2020b; Dibaji et al., 2014; Baffoni et al., 2012; Periˇc et al., 2010; Saulnier et al., 2008; Bomba et al., 2002). Here, it is noteworthy to mention that the introduction of kanamycin cassette in *C. jejuni* chromosome did not hamper the colonization ability of CJ-Km, observed in previous studies and also in this study in terms of CJ-Km colonization in different intestinal portions of Grp-A (Tabashsum et al., 2020a; Salaheen et al., 2018; Biswas et al., 2006). When we observed total *Campylobacter* which includes both challenged CJ-Km and naturally colonized *Campylobacter* in different gut portions of chickens, we observed the colonization/growth was reduced notably in Grp-B, Grp-C or Grp-D than Grp-A. The trend of total *Campylobacter* reduction also followed the pattern of CJ-Km reduction. The fecal shedding of CJ-Km or total *Campylobacter* also reduced in the treatment groups compared to control as observed in our previous studies (Tabashsum et al., 2020a; Peng et al., 2019). The reduction of CJ-Km or total *Campylobacter* in Grp-B was comparable to the reduction in the Grp-D in this study. These results indicate that the synbiotic, combination of BPEs and LC$^{+mcra}$, might be a potential viable option to reduce colonization of zoonotic pathogens, specifically *Campylobacter* in both conventional and organic poultry farming (Brown et al., 2017; Luangtongkum et al., 2006). Ultimately, this pre-harvest practice will improve product safety and reduce environmental contamination and pressure in post-harvest processing.

In this study, we also deliberated the normal flora of the chicken gut in terms of microbiota present in the cecum contents. The most abundant phylum in all groups of chickens were Firmicutes as observed in previous studies (Tabashsum et al., 2020a; Wong et al., 2017). We have

observed that in Grp-D, the percentage of Firmicutes was slightly lower than Grp-A, Grp-B or Grp-C. Firmicutes mainly comprise of Gram positive bacteria and further observation revealed that lower percentage Firmucutes was compensated by Gram positive bacteria phylum Actinobacteria in Grp-D. Bacteroidetes was the second most abundant phylum and the changes in this phylum across different groups was minor. The phylum Proteobacteria comprise with Gram negative bacteria including a variety of pathogenic bacteria. The percentage of this phylum was reduced in the Grp-B, Grp-C or Grp-D compared to Grp-A, but the highest reduction was in Grp-D. Similar pattern of reduction for the phylum Proteobacteria was observed in previous studies (Śliżewska et al., 2020; Asahara et al., 2011; Bomba et al., 2002). Two bacterial genera *Lactobacillus* and *Bifidobacterium*, both Gram positive and representative of the phyla Firmicutes and Actinobacteria, respectively increased in Grp-D than any other groups. It has been mentioned across the literature that both these genera help to maintain the homeostasis in the gut (Oakley et al., 2014; Luo et al., 2013), confirming the positive impact of the $LC^{+mcra}$ and BPEs combined treatment over other treatments. *Ruminococcus* and *Clostridium* are two representing genera of Firmicutes and are responsible for the production of the metabolites leading to the healthier gut (Shaufi et al., 2015; Luo et al., 2013). Both *Ruminococcus* and *Clostridium* increased in Grp-D more than any other groups, again showing positive impact of the combined treatment on chickens. *Campylobacter* was reduced in all three treatment groups but more efficiently in Grp-D. This pattern of reduction was observed for other genera of the phylum Proteobacteria, including *Escherichia*, *Proteus*, *Klebsiella*, and *Pseudomonas*. Similar findings were also confirmed by Dibaji et al (2014), Buffie et al (2013), and Bafffoni et al. (2012) for the other synbiotic or combined treatments. It was observed in previous studies that the species diversity vary by heredity and environmental factors more than the probiotic or synbiotic treatments (Tabashsum et al.,

2020a; Wang et al., 2017). Similarly, we found that number of species or other indices did not vary significantly among the treatment groups of this study. For the chicken growth, treatments of Grp-B, Grp-C or Grp-D increased the weight of the chickens marginally. Similar pattern of increase was found previously but at a higher rate as treatments were given throughout the growth period of chickens rather than only at the beginning of the chicken growth (Salaheen et al., 2017; Ferdous et al., 2009).

*Conclusion*

In conclusion, we can say that the colonization of *Campylobacter* in poultry gut can be controlled efficiently by combination of $LC^{+mcra}$ and BPEs without interfering normal gut flora adversely. Therefore, $LC^{+mcra}$ with BPEs could be an alternative to improve safety of poultry products and reduce campylobacteriosis in human in a sustainable way. For improving the poultry growth rate as an additional benefit of the combined effect of $LC^{+mcra}$ and BPEs, and this effects may be amplified with extended period of application of this synbiotic compositions.

*Figures and Tables*

**Figure 4.1.** Comparison of colonization pattern of overall *Lactobacillus* in different intestinal contents including cecum, jejunum or ileum, and fecal shedding pattern of Grp-A (control), Grp-B (treated with AGPs), Grp-C (treated with $LC^{+mcra}$) or Grp-D (treated with $LC^{+mcra}$ and BPEs) chickens at day 14, 21, and 28. Results are shown as mean ± SD, and '*' is used to denote the statistically significant ($p < 0.05$) differences in the *Lactobacillus* count among control and treatment groups at a specific time point.

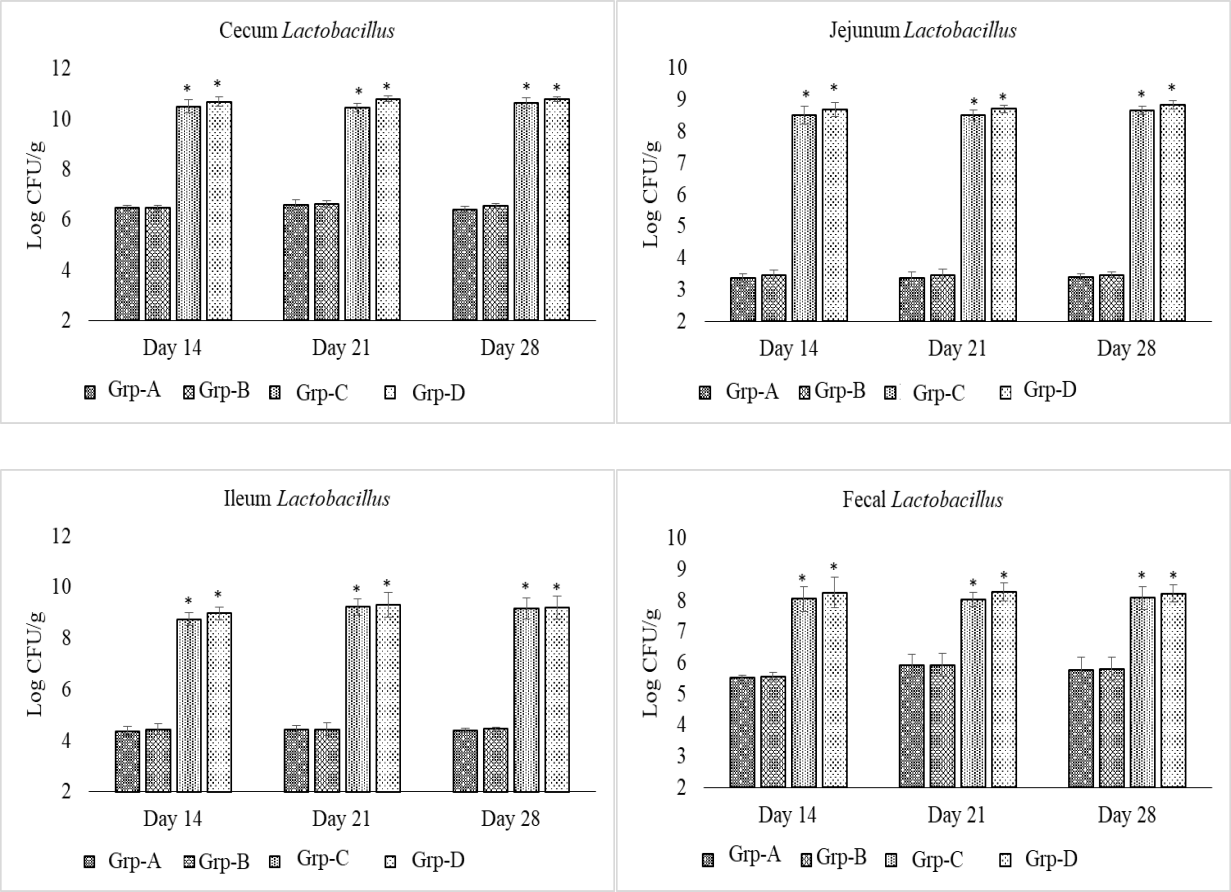


**Figure 4.2.** Comparison of reduction pattern in the colonization of challenged CJ-Km in different intestinal contents including cecum, jejunum or ileum, and fecal shedding pattern of Grp-A (control), Grp-B (treated with AGPs), Grp-C (treated with LC$^{+mcra}$) or Grp-D (treated with LC$^{+mcra}$ and BPEs) chickens at day 14, 21, and 28. Results are shown as mean ± SD, and '*' is used to denote the statistically significant ($p < 0.05$) differences in the CJ-Km count among control and treatment groups at a specific time point.

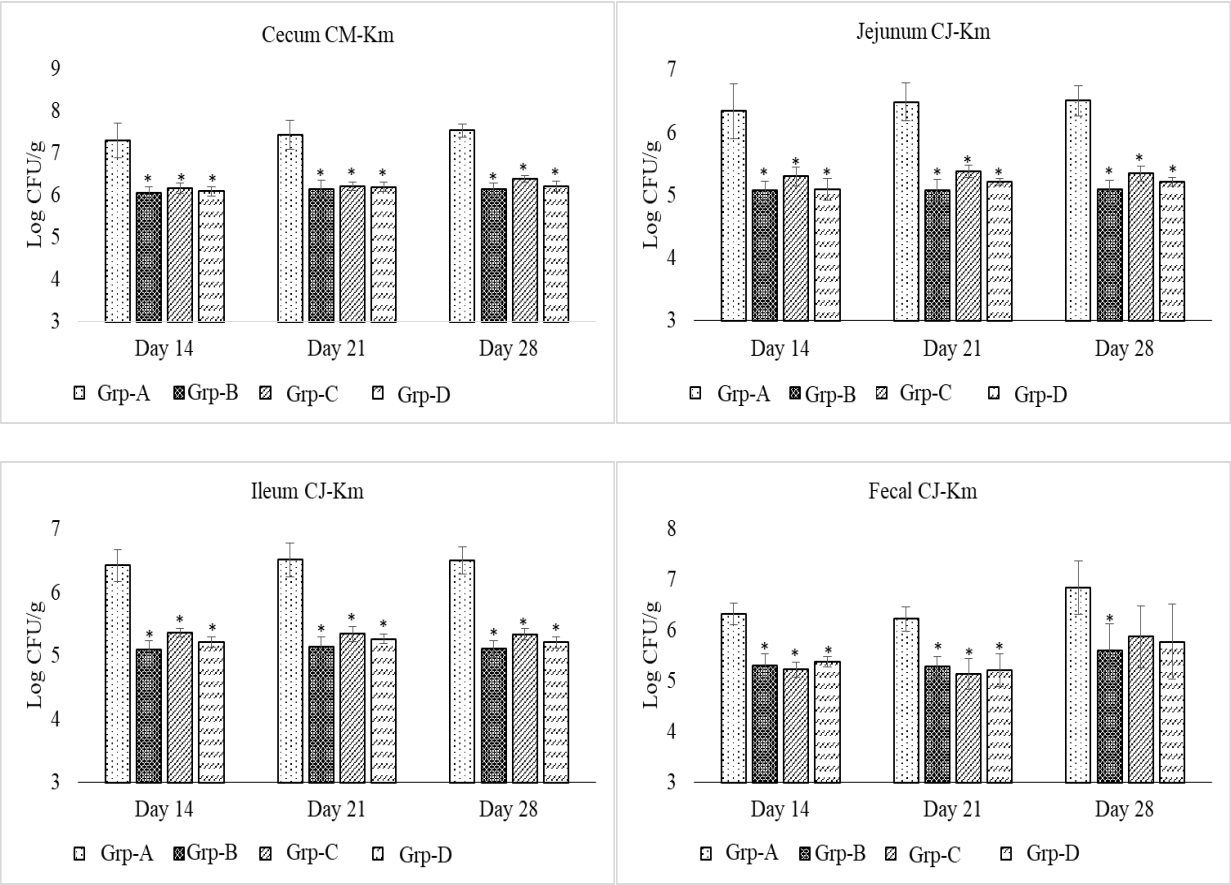


**Figure 4.3.** Comparison of reduction pattern in the colonization of overall *Campylobacter* (natural and challenged) in different intestinal contents including cecum, jejunum or ileum, and fecal shedding pattern of Grp-A (control), Grp-B (treated with AGPs), Grp-C (treated with $LC^{+mcra}$) or Grp-D (treated with $LC^{+mcra}$ and BPEs) chickens at day 14, 21, and 28. Results are shown as mean ± SD, and '*' is used to denote the statistically significant ($p < 0.05$) differences in the *Campylobacter* count among control and treatment groups at a specific time point.

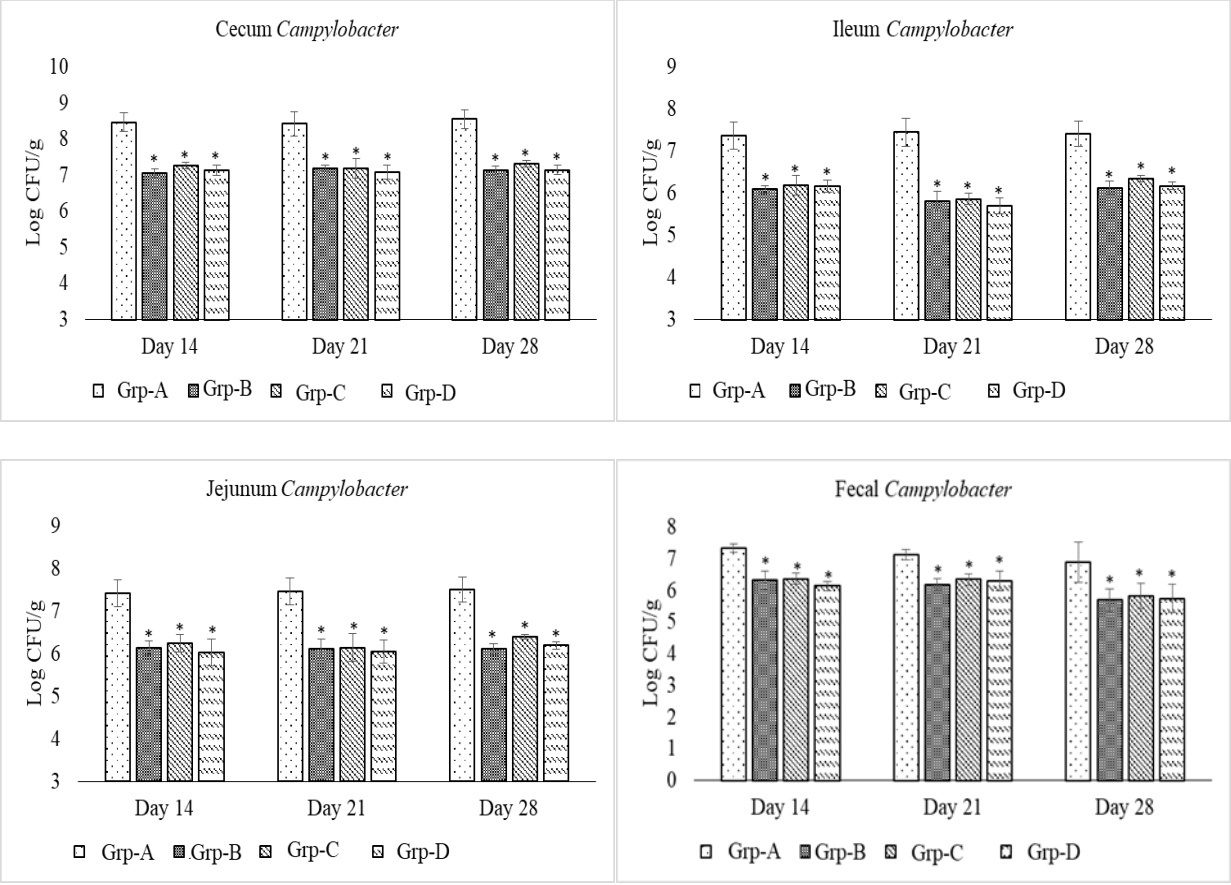


**Figure 4.4.** Comparison of taxonomic variation in the cecum contents of Grp-A (control), Grp-B (treated with AGPs), Grp-C (treated with LC$^{+mcra}$) or Grp-D (treated with LC$^{+mcra}$ and BPEs) chicken at phylum level in percentage (%) at day 28.

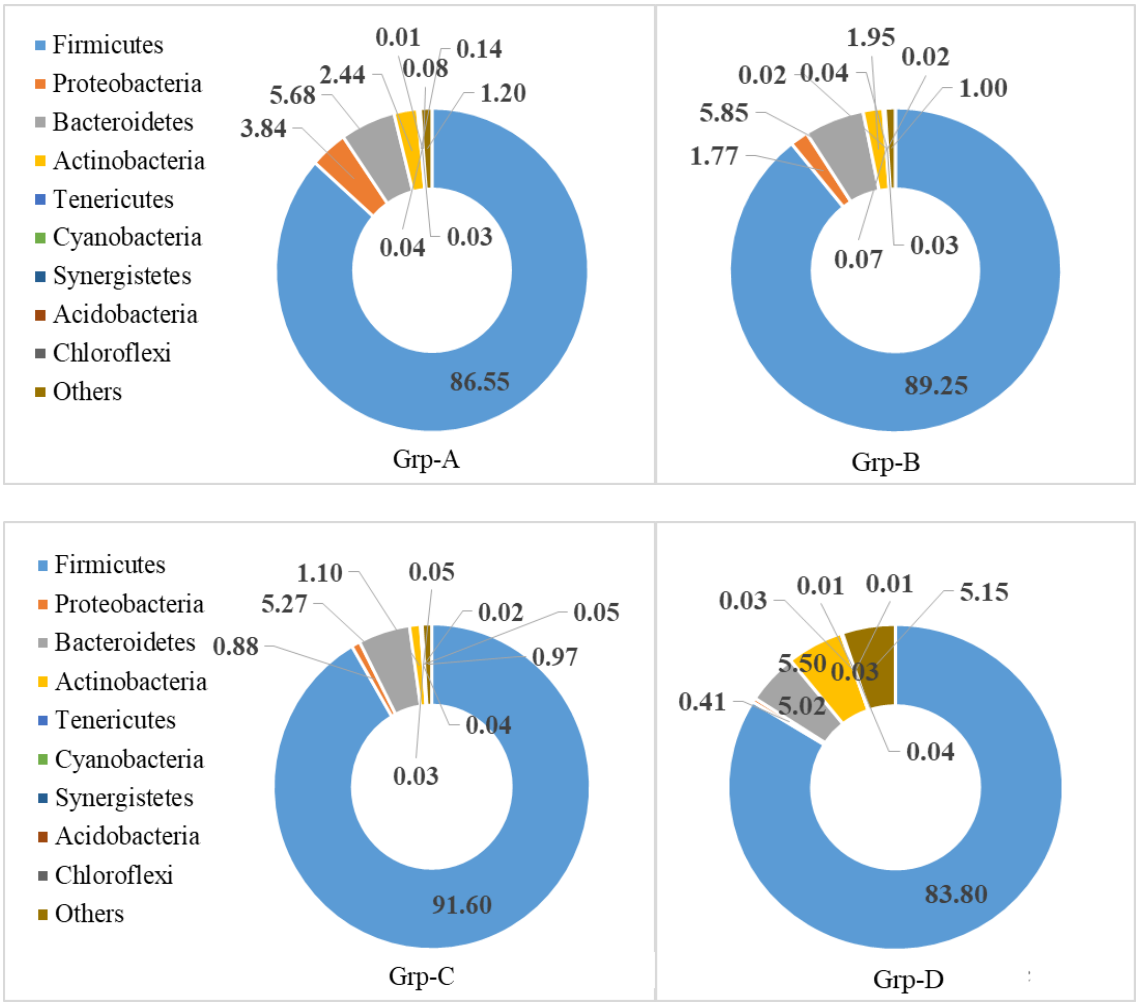


**Figure 4.5.** Comparison of taxonomic variation in the cecum contents of Grp-A (control), Grp-B (treated with AGPs), Grp-C (treated with $LC^{+mcra}$) or Grp-D (treated with $LC^{+mcra}$ and BPEs) chickens at genus level in percentage (%) at day 28.

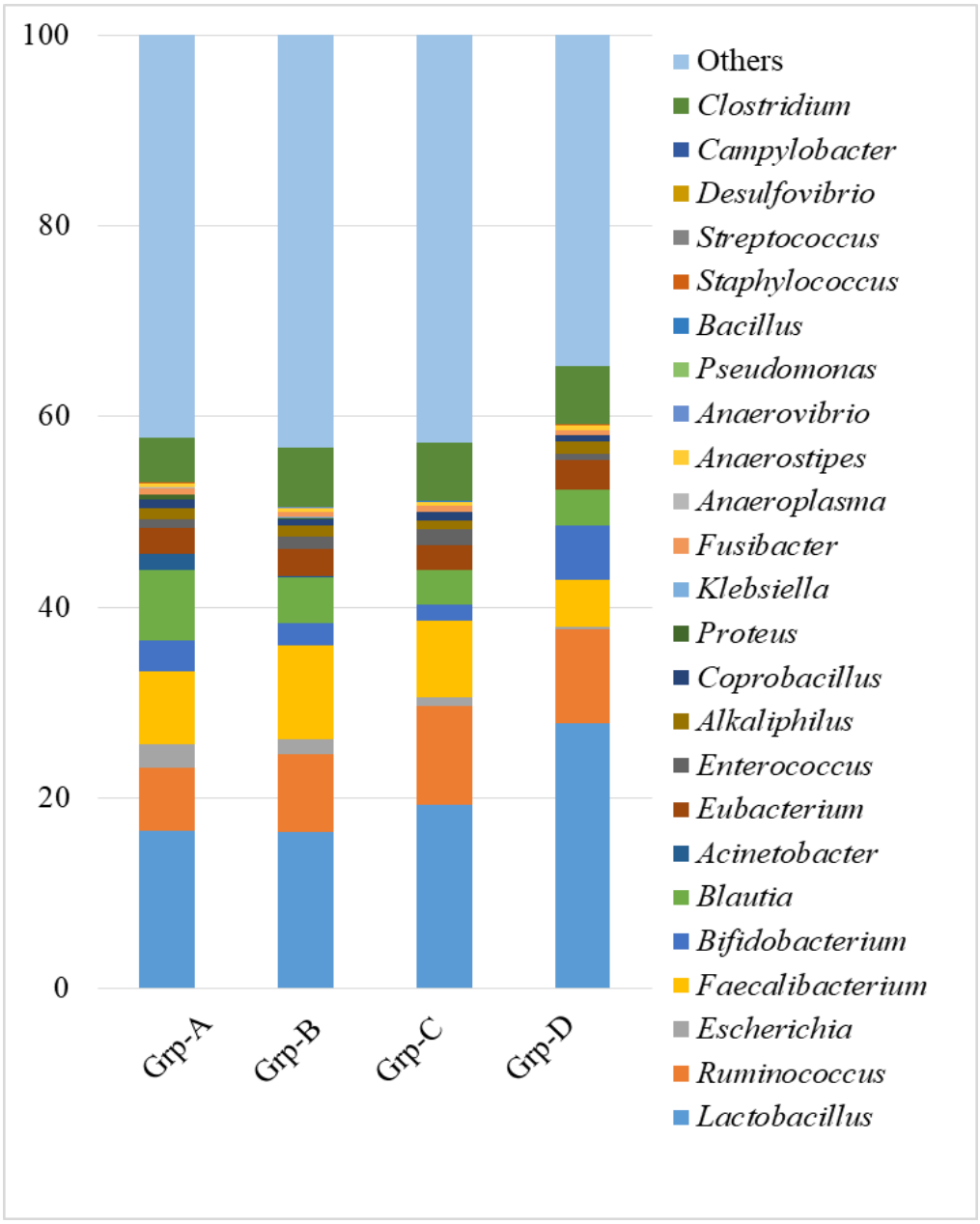


**Figure 4.6.** Comparison of diversity in the cecum contents of Grp-A (control), Grp-B (treated with AGPs), Grp-C (treated with $LC^{+mcra}$) or Grp-D (treated with $LC^{+mcra}$ and BPEs) chickens at species level in terms of number of species, Simpson index, Shannon index and Margalef's richness at day 28. Results are shown as mean ± SD, and '*' is used to denote the statistically significant ($p < 0.05$) differences in the control and treatment groups.

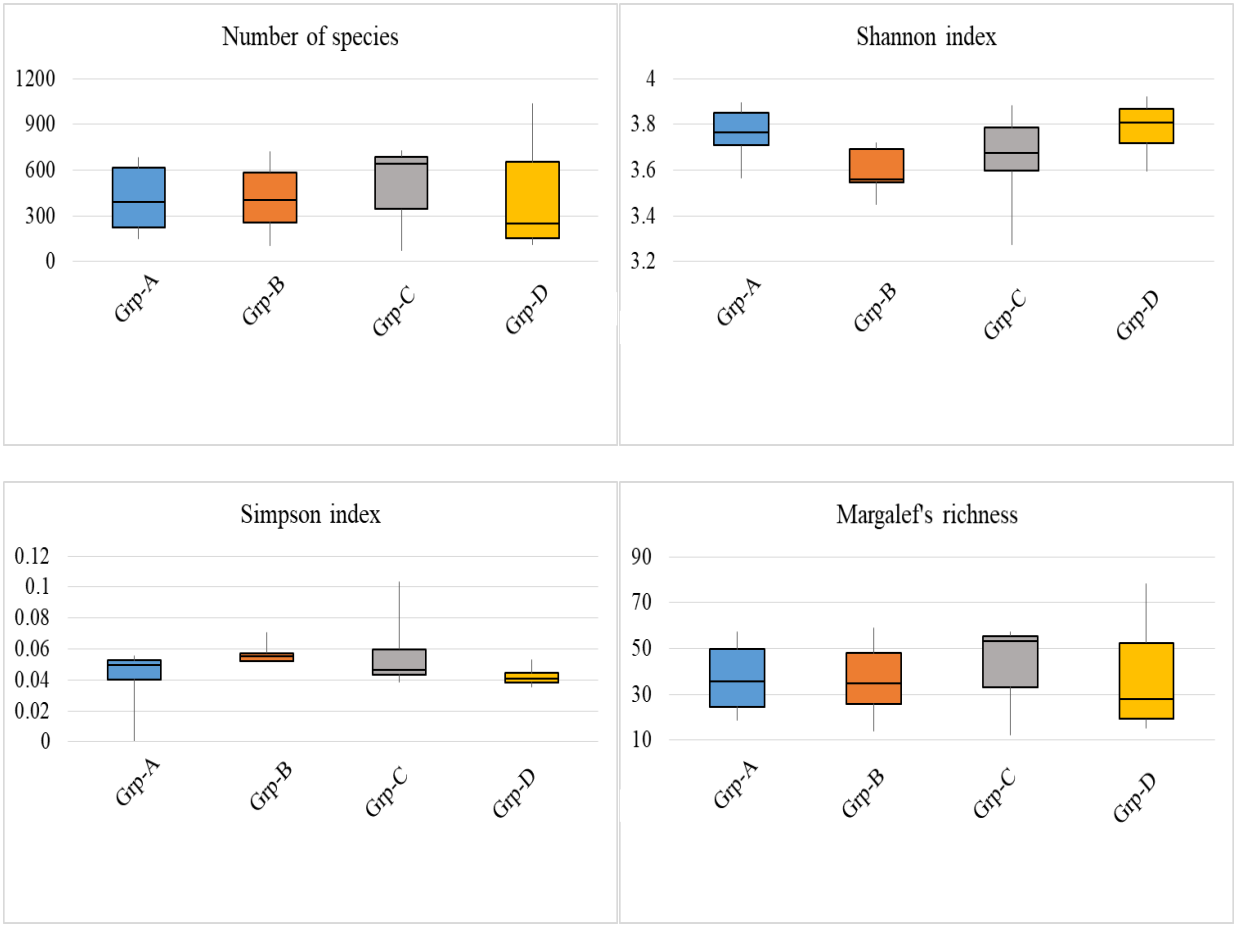


**Figure 4.7.** Weight gain pattern of Grp-A (control), Grp-B (treated with AGPs), Grp-C (treated with $LC^{+mcra}$) or Grp-D (treated with $LC^{+mcra}$ and BPEs) chickens at day 28. Mean of weight for each group is shown with the line marks.

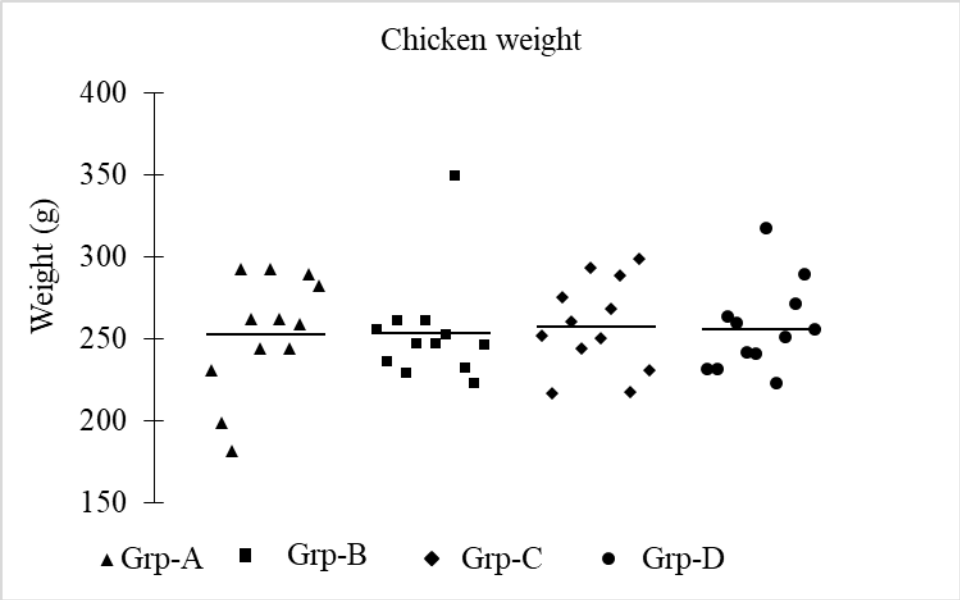


Table 4.1. Treatment groups and number of chickens for the study

| **Treatments** | **Trial 1** | **Trial 2** | **Total** |
|---|---|---|---|
| Phosphate buffered saline (PBS) [Grp-A] | 20 | 20 | 40 |
| Antibiotic growth promoters (AGPs) [Grp-B] | 20 | 20 | 40 |
| *mcra*-overexpressed *L. casei* ($LC^{+mcra}$) [Grp-C] | 20 | 20 | 40 |
| *mcra*-overexpressed *L. casei* ($LC^{+mcra}$) + berry phenolic extracts (BPEs) [Grp-D] | 20 | 20 | 40 |
| **Total** | **80** | **80** | **160** |

Table 4.2. Experimental design for individual trial

| Timeline | | Number of Chickens | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Daily PBS gavage** | **Daily Probiotic Colonization** | **Daily Prebiotic in water** | **Daily AGPs in water** | **Enteric Bacterial Challenge** | **Fecal Sample Collection** | **Euthanization** | **Sample Harvest** | **Remaining Live** |
| **Day 0** | | - | | | - | - | - | - | 80 |
| **Day 1-7** | 40 (A, B) | 40 (C, D) | 20 (D) | 20 (B) | - | - | - | - | 80 |
| **Day 8** | | - | | | 80 (A, B, C, D) | - | - | - | 80 |
| **Day 14** | | - | | | - | 48 (12/group) | 24 (6/group) | 24 (6/group) | 56 |
| **Day 21** | | - | | | - | 48 (12/group) | 32 (8/group) | 32 (8/group) | 24 |
| **Day 28** | | - | | | - | 24 (1/chick) | 24 (6/group) | 24 (6/group) | 0 |

# Chapter 5

## Overall conclusion and future directions

In previous study we have observed that LC$^{+mcra}$ have high potential for application in competitively inhibiting the growth of CJ, reducing the adhesive and invasive efficacy of CJ to different host cells, altering different physicochemical properties related to virulence and also altering the expression level of the virulence associated genes. The combination of bioactive probiotics, LC$^{+mcra}$ and anti-oxidative and anti-inflammatory, BPE complemented each other's ability to eliminate or alter pathogenic characteristics with elevated bidirectional activities in both accelerating growth of *Lactobacillus* strain and exclusion/inhibition of CJ. The promising functions of the combination of the genetically engineered probiotic, LC$^{+mcra}$ and bioactive BPE, in altering physicochemical properties, disruption of pathogen-cell interactions, and virulence genes suppression was also observed previously. In our present study, in simulated chicken gut condition, CFCS of LC$^{+mcra}$ and BPE has been shown that they can effectively reduce colonization of *Campylobacter*, as determined by cultural techniques. The results suggest that these antimicrobial alternatives can be implemented without any notable effect on the overall chicken gut microbiome composition as seen in the preservation of key bacterial phyla, genera, and species diversity indexes at the level of a simulated gut condition using cecum content. This serves as an intermediate step for validating novel procedures and microbial control protocols within a controlled system before implementing them in a live animal model. Then LC$^{+mcra}$, showed its effectiveness on the colonization of both marker-containing CJ-Km strain and naturally colonized *C. jejuni* in various sections of chicken intestine

along with the modulation of gut microbiota towards healthier gut. Though the study was conducted in controlled laboratory environment, the outstanding roles of $LC^{+mcra}$ provides a promising option for reducing the colonization of the most predominant zoonotic pathogens, specifically *C. jejuni* in chicken gut, which may eventually lead to lower incidence of cross-contamination in poultry products. Lastly, we can say that the colonization of *Campylobacter* in poultry gut can be controlled efficiently by combination of $LC^{+mcra}$ and BPE without interfering normal gut flora adversely. Therefore, $LC^{+mcra}$ with BPE could be an alternative to improve safety of poultry products and reduce campylobacteriosis in human in a sustainable way. For improving the poultry growth rate as an additional benefit of the combined effect of $LC^{+mcra}$ and BPE, and these effects may be amplified with extended period of application of this synbiotic compositions.

## Future directions

1. Evaluating the effect of $LC^{+mcra}$ and BPE on chicken physiology.
2. Assessing the mechanism of action of $LC^{+mcra}$ and BPE.
3. Determining the long-term effect of $LC^{+mcra}$ and BPE, i.e., resistance pattern.
4. Cost-benefit analysis and practical applicability of this synbiotic composition in animal production.
5. Application of this synbiotic composition at farm level.

## Glossary

| | |
|---|---|
| Microbiome | Collective microbial genomic content that can represent the total microbial community. |
| Probiotic | Live microorganisms provided into the host to improve host health. A probiotic must be a non-pathogen. |
| Prebiotic | Functional ingredients with specific traits of assisting host microflora for better health of the host. |
| Sustainable | Making balance between economic growth and environmental well-being while meeting the demand of current population with the scope of improvement for future. |
| Synbiotic | Mixtures of probiotic and prebiotic that effectively improve the host health and well-being. |

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