ABSTRACT

Title of Thesis: BIOACTIVE *LACTOBACILLUS CASEI* IN REDUCING GROWTH AND COLONIZATION OF *CAMPYLOBACTER JEJUNI*

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Master of Science
2018

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Campylobacter jejuni (CJ) is one of the pre-dominant causative agents of acute gastroenteritis in the US and occurs commonly through handling/consumption of contaminated poultry products. Probiotics with enhanced bioactive metabolites such as conjugated linoleic acids (CLAs) play crucial role in improving host health and act as antimicrobials. Further, prebiotic like components such as bioactive phenolics from berry pomace extract (BPE) can stimulate growth of beneficial microbes including *Lactobacillus casei* (LC) and inhibit bacterial pathogens *in vitro*. In this study, we aimed to assess efficiency of CLA overproducing LC (LC^{+mcra}) alone or in presence of BPE against CJ. LC^{+mcra} alone or LC^{+mcra} with BPE reduced CJ growth, adhesion and invasion efficiency to cultured cells and also altered physicochemical properties, gene expressions related to virulence. These findings suggest, BPE and LC^{+mcra} in combination may able to prevent CJ colonization in poultry and reduce cross-contamination, hence control foodborne infections with CJ in human.

BIOACTIVE *LACTOBACILLUS CASEI* IN REDUCING GROWTH AND COLONIZATION OF *CAMPYLOBACTER JEJUNI*

by

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Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science
2018

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Preface

This dissertation is original and independent work of the author, Zajeba Tabashsum under supervision of Dr. Debabrata Biswas

Acknowledgements

I would like to thank my mentor, Dr. Debabrata Biswas, for his support and guidance. I would also like to thank my M.S. thesis committee members for their constructive criticism to improve my work. Finally, I want to thank my friends and family.

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List of Abbreviations

AAD, Antibiotic Associated Diarrhea

BPE, Berry Pomace Extract

CFCS, Cell Free Cultural Supernatants

CFU, Colony Forming Unit

CLA, Conjugated Linoleic Acid

GAE, Gallic Acid Equivalent

PUFAs, Polyunsaturated Fatty Acids

SCFAs, Short Chain Fatty Acids

Chapter 1: Literature Review

Campylobacter, one of the most prominent causative agents of diarrheal illness in the US, affects more than a million people leading to thousand hospitalizations and hundred deaths each year (CDC, 2014). Campylobacteriosis can also cause post-infection complexity like Guillain-Barrie syndrome, irritable bowel syndrome, reactive arthritis and immune-proliferative small intestinal diseases (Chen et al., 2010; Garin et al., 2012). The most common sources of human *Campylobacter* gastroenteritis are handling of raw or consumption of partially cooked poultry meat or unpasteurized milk (Garin et al., 2012; Salaheen et al., 2016b; Wingstrand et al., 2006), as chickens and other warm blooded farm animals naturally harbor *Campylobacter* in their gastrointestinal (GI) tracts. Farm animals can also transmit the *Campylobacter* to other food products and environment through horizontal transfer specifically in organic mixed crop-livestock farms (Peng et al., 2016b; Salaheen et al., 2016b) where farmers are not permitted to use synthetic chemicals and antimicrobials. Further, in conventional farms antibiotics resistant *Campylobacter* are often survived and colonized in the gut of farm animals and associated with cross-contamination of food products and environment (DeWaal et al., 2013). As a result, alternative strategies to reduce the colonization of *Campylobacter* in farm animal gut and limit the cross-contamination of food products and environment are urgently required.

Campylobacter, as an enteric pathogen and its ecology. *Campylobacter* is a microaerophilic, spiral-shaped, Gram negative bacterium. *Campylobacter* genus includes 33 species and *C. jejuni* and *C. coli* are the most common and major

causative agents of infection. Optimum growth condition for *Campylobacter* is 42^{o}C temperature and low environmental oxygen concentration. These optimum conditions help *Campylobacter* in proper gene expression, regulation of energy/nutrient metabolism and propagation. As a result *Campylobacter* reside in the intestinal mucosa of worm blooded animals and there are possible risk factors involved in *Campylobacter* transmission from animal to human. Chicken is one of the major natural reservoirs for *Campylobacter* and possibility of transmission of *Campylobacter* from chicken to human increases as the number of interaction between the host increases. Generally shedding of *Campylobacter* starts about 2-3 weeks after hatching and over the time period all the birds can be contaminated. After colonization, bacterial load can reach up to 10^7 colony forming unit (CFU) per gram of cecum content (Stern et al., 1995). Genotypic similarity has been found among *Campylobacter* isolates from human and poultry (Nadeau et al., 2002). Again reports are also available stating colonization of *Campylobacter* is host specific which limit the occurrence of common serotype among human, poultry or other animal. *Campylobacter* can also reside in the intestinal tract of other animal like cattle, sheep, pig and goats.

Wild birds and animals can also be colonized with *Campylobacter* and serotypes from wild animal have been shown to be similar to the human clinical isolates. This paves the possibility of the wild animals as the possible vectors for *Campylobacter* transmission to farm animals (Hald et al., 2004; Meerburg et al., 2006). Farm animals can also transmit the *Campylobacter* to other food products and environment through horizontal transfer (Peng et al., 2016b; Salaheen et al., 2016b).

The Prevalence of *Campylobacter* in poultry meat samples were 87.5%, 71.43% and 33.33% in farmers markets, organic and retail supermarkets in Maryland and the DC metropolitan area respectively (Salaheen et al., 2016b). The prevalence of *Campylobacter* in the poultry meats from organic and conventional retail supermarkets ranges from 43% to 89% in the US (Smith et al., 1999; Zhao et al., 2001; Cui et al., 2005; Price et al., 2005; Luangtongkum et al., 2006; Price et al., 2007; Han et al., 2009). So, it is evident that post-harvest poultry products are highly contaminated with *Campylobacter*.

Infection and post infection sequelae by Campylobacter. The infection and virulence factors of *Campylobacter* are still to be explained entirely. The varied nature of the bacteria enables *Campylobacter* to survive in various environmental conditions and interacts with the mucus layer of the gut. From different studies it can be hypothesized that *Campylobacter* starts the infection by actively penetrating the intestinal mucosal layer followed by discharging toxin (cytolethal distending toxin, *cdt* A, B and C) or proteins (*Campylobacter* invasion antigen, *cia*) via flagellar apparatus which serve as Type III secretion system in the bacteria (Wallis, 1994) epithelial cell engulf *Campylobacter* which disrupts the integrity of the epithelial lining when modulated by proteins. Pro-inflammatory cytokines, chemokines and effector molecules of the innate immunity are highly induced when *Campylobacter* antigens are presented by the antigen presenting cells. Complications related to *Campylobacter* infections arise at this stage which include pancreatitis, cholecystitis, peritonitis and gastrointestinal hemorrhage. In immunocompetent patients, transient

bacteremia also arises occasionally. Rather than food poisoning and local Campylobacter enteritis, post infection sequelae of campylobacteriosis, such as Guillain-Barre syndrome (GBS), reactive arthritis, cardiac problem and uncreative colitis are more important clinically and economically (Chen et al., 2010; Garin et al., 2012) .

The symptoms of diarrheal infection caused by *Campylobacter* include presence of mucus and blood in stool, abdominal cramp, fever, nausea, vomiting and loss of appetite. GBS is considered as an acute demyelinating disease of the peripheral nervous system. Five percent of *Campylobacter*-associated reactive arthritis were found to be chronic or revert with muculoskeletal symptoms. Typical clinical symptoms of myocarditis and myopericarditis include transitory acute pain in chest with concomitant electrocardiogram variations and increased secretion of cardiac enzymes in association with antecedent and coincident enteritis. Ulcerative colitis is a chronic inflammatory condition of large intestine with bloody diarrhea, severe abdominal cramp and abnormal immune functions, defect in intestinal epithelial cell barrier function and gut microbiota (Pope et al., 2007; Uzoigwe, 2005; Gionchetti et al., 2001; Sasaki et al., 2012).

Use of antibiotic and development of antibiotic resistance. The morbidity and mortality in human were reduced at a dramatic level, when broad-spectrum antibiotics were first introduced in treatment of human bacterial diseases in the mid-twentieth century (Peng et al., 2014; Salaheen et al., 2014). The use of antibiotics is yet considered as the most vitally important medical event in human history, and the

application of antibiotic was boosted worldwide for both human medication and agricultural farm animal production (Wise, 2002; Anderson and Hughes, 2010). Broad-spectrum antibiotics are also used to control GI pathogens, i.e., erythromycin and ciprofloxacin are used for *Campylobacter* enterocolitis (Traa et al., 2010; Lund and O'Brien, 2011).On the downside, antibiotic treatment and therapy could disrupt the intestinal microbial ecosystem resulting in colonic microbiota imbalance and cause antibiotic-associated diarrhea (AAD), early aged antibiotic treatments could also result in permanent disruption on gut microbiome development and functions in infants and young children and cause disorders in adipose and hepatic cell metabolism leading to type-2 diabetes and obesity (Esteve et al., 2011; Kootte et al., 2012).

The improper use of antibiotics in human medicine and agriculture has resulted in widespread distribution of antibiotic-resistance genes and developed different resistant pattern among bacterial pathogens (Salyers and Shoemaker, 2006). Bacteria developed numerous complex mechanisms to resist antibiotics such as by reducing antibiotic uptake into bacterial cells, eliminating target receptors binding with antibiotics, enzymatic cleavage or modification of antibiotic molecule, overproduction of antibiotic targets, etc. (Todar, 2003), and these resistance could spread from animal to human through either direct contact with animals or even through food chain (Marshall and Levy, 2011). It has been observed that the antibiotic-resistant human pathogens evolved to be more virulent and aggressive in respect to disease occurrence (Guay, 2008; Lew et al., 2008; Woodford and Livermore, 2009). Over the years, the improper use of antibiotics designated bacterial

resistance and almost every known bacterial pathogens have developed at least single or multi-resistance to antibiotics (Todar, 2003). More than 70% of the infections-causing bacterial pathogens in hospitals have been reported single or multi drug-resistance and some bacteria are even resistant to all approved antibiotics (Marshall and Levy, 2011).

Again huge amount of antibiotics have been used in agricultural animal production in the US since 1950 as growth promoters because of their influence on animal growth promotion and ability to improve feed conversion efficiency. Flavophospholipol and virniamycin are two most commonly used antibiotics in the US for poultry production. Pre-harvest use of antibiotic is common in farm animal production but instead of specific targeted pathogens, antibiotics are used for diverse groups of pathogens. Recent studies suggest that some antibiotic treatment can disrupt the dynamics of gut flora and therefore impair animal health and productivity and even food safety. For these consequences, the European Union banned the use of antibiotics for farm animal production as growth promoter. In the US, certain use of cephalosporin in animal production were restricted by Food and Drug Administration (FDA) and now considering the option of banning non-therapeutic use of antibiotics in food animal production.

Alternatives to antibiotic. Due to the increased concern about antibiotic resistance, several non-antibiotic antimicrobials have been developed and introduced to prevent and inhibit foodborne bacterial pathogens. Development of alternatives to antibiotic growth promoters has been also advanced. The major potential agents include

bacteria and derivatives, animal- or plant-derived products, bacteriophages, and vaccines for both cases (Peng et al., 2014; Salaheen et al., 2014b).

***Bacteria and derivatives*.** Competitive exclusion products are one of the popular choices to exclude the pathogens. These competitive exclusion products are microbes comprising a variety of species of bacteria that are considered as being 'helpful'. The mechanism of action is believed to be that, colonization of the gastrointestinal tract by 'helpful' bacteria inhibit potential pathogens to colonize the gut and eventually causing infection (Salaheen et al., 2014b). This is the principle of competitive exclusion. These products are effective *in vitro* and can be administered to newborn animals to colonize the gastrointestinal tract and prevent *Salmonella* and *Campylobacter* infections (Peng et al., 2015a; Peng et al., 2015b; Shi et al., 2016). These products may also be used in animals those are treated therapeutically with antibiotics, to re-colonize a gut that may have been depopulated by the antimicrobial action of the antibiotics. Traits important to these 'helpful' bacteria include being non-pathogenic, resistance to stomach acids and bile, having the potential to colonize the host, production of nutrients, being free of antibiotic resistance genes or having reduced gene transfer ability and antagonistic to pathogens.

Derivatives of bacteria include different chemical products produced by them. Bacteriocins, one of the common derivatives are proteins or polypeptides with antimicrobial activities. These antimicrobial proteins are able to inhibit the growth of bacterial pathogens like *Salmonella*, *Listeria* and *Campylobacter* (Patton et al., 2007;

Stahl et al., 2004). Another class of derivatives are vitamins and most of the vitamins cannot be synthesized by animals but they can be produced by lactic acid bacterial fermentation (Patel et al., 2013). The SCFAs like acetate, propionate, and butyrate are the most important secondary metabolites of beneficial bacteria (Peng et al., 2016). Among various PUFAs, omega-3 and omega-6 fatty acids are of most importance and they are possibly produced from microbial sources including *Bifidobacterium*, *Lactobacillus*, *Lactococcus* etc (Peng et al., 2016; Stanton et al., 2005).

Plant derived products. Products derived from plants are natural, considered as less toxic than antibiotics and are generally residue free. Many of the plant derived products are certified as Generally Recognized As Safe (GRAS) by the Food and Drug Administration (FDA) and which makes them ultimate choice to use as antimicrobial and/or feed additives (Wang et al., 1998). One group of the plant derived products have antibacterial activities, while another group of plant derived products are considered as "antibiotic potentiators or adjuvants". Again, others are considered as "immuno-stimulants", those can promote the host immune system to effectively respond to the pathogen invasion. There are still another groups, those positively modulate the intestinal microbiota.

The term "phytobiotic" includes a wide range of substances differing with biological origin, chemical formulation and purity (Windisch et al., 2006). Some phytobiotics reduce microbial toxins by stabilizing microbiome (Periˇc et al., 2010; Steiner et al., 2006; Windisch et al., 2008) and can be beneficial in several growth and health promoting properties of animals. These positive effects of phytobiotics is mainly linked to the plant constituents including terpenoids (monoand 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 understanding of their modes of action and prospects of their application are still rudimentary (Windisch et al., 2008). Again, berries are considered as a generic source of bioactive compounds (Moussa et al., 2014). Proanthocyanidins from cranberry is getting the particular attention recently (Foo et al., 2000). Flavonoids of cranberry has been shown to reduce or prevent atherosclerosis by preventing oxidation of low density lipids (Reed et al., 2002). The plant tannins with proanthocyanidins, as potential alternatives to growth promoters in poultry has recently been reviewed by Redondo et al. (2014). Further studies have revealed that extracts from these sources can affect various bacterial functions including disruption of their cell envelope, which parallels that of some antibiotics widely used as growth promoters in the poultry industry (Salaheen et al., 2014b). Also, the spectrum of activity or the mode of action of purified components is often very narrow or non-specific and the use of berry extracts or pomace containing mixtures of bioactive compounds has become an attractive alternative to create an added value to animal feeds e.g., blueberry and blackberry pomace (Salaheen et al., 2016; Salaheen et al., 2014a; Salaheen et al., 2017b).

Animal derived products and others. Several animal-derived products have also been shown to be effective in foodborne-pathogen inhibition. Chitosan, isolated from the exoskeletons of crustaceans and arthropods has been shown to inhibit the growth of mold and several foodborne pathogens including *S.* Enteritidis, *E. coli*, and *L.*

monocytogenes (Leleu et al., 2011). A heat-stable and salt-tolerant peptide, pleurocidin has been isolated from myeloid cells and mucosal tissue of both vertebrates and invertebrates, which has shown inhibitory effect against different foodborne pathogens such 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, but their application in pre-harvest control of foodborne pathogens in farm animals needs to be studied further.

Bacteriophages can also be an alternative to antibiotic as they are active against specific bacterial strains. Specificity allows bacteriophages to be used against targeted pathogens in a mixed population without disturbing the composition of normal gut microflora (Inal et al., 2003). Vaccination is the method of inhibiting pathogens by inducing the defense mechanisms of host's immune systems, in turn decreasing infections and improving animal health. However, vaccines made from any one bacteria serovar cannot confer cross-protection against another serovar as a consequence, the super-high specificity as well as additional costs prevents vaccination from being commonly used (Singh et al., 2009).

Prebiotics and their effects on animal health. 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). Common prebiotics include inulin, lactulose, phenolics and oligosaccharides with characteristics of colonic microflora fermentable while

resistant to digestive enzymes in human gut (Kolida et al., 2002; Bielecka et al., 2002). Multiple preliminary studies have revealed that prebiotics can provide nutrients and energy for fermentation to native microflora, either by producing vitamins, SCFAs, and antioxidants for modulation of intestinal microflora composition or releasing antimicrobial byproducts as competitive advantages for exclusion of pathogenic bacteria (Videla et al., 2001; Cummings and Macfarlane, 2002; Fukuda et al., 2002). Some other beneficial traits include stimulating mineral absorption, enhancing effectiveness of immune system, and reduction of colorectal cancer risk (Geier et al., 2006; Scholz-Ahrens and Schrezenmeir, 2007; Lomax and Calder, 2009; Lohner et al., 2014).Prebiotics generally come from food sources. Beans, raw oats, Whole wheat, banana, berries etc are believed to be other traditional dietary sources of prebiotics.

The Food and Drug Administration (FDA) approved a health claim that suggest consuming 1.5 ounces (42 g) of most nuts, including peanuts (approximately 15 to 20 g of peanut), as part of a diet low in saturated fat and cholesterol may reduce the risk of heart disease. The benefits on reducing cardiovascular diseases (Isanga and Zhang 2007; Ozcan, 2010; Bao et al., 2013; Jones et al., 2013) with daily intake of peanut or other nut products has been suggested to be associated with their dietary fibers (prebiotics) as well as the phenolic compound resveratrol (3,4,5-trihydroxystilbene) (Bubonja-Sonje et al., 2011; Wang et al., 2011). Moreover, the prebiotic-like compounds from peanut and berries can promote the growth of selective beneficial bacteria, especially *Bifidobacteria* and *Lactobacillus* (Tzounis et al., 2011; Calatayud et al., 2013; Yaung et al.,2014).

Metabolites produced by probiotic and their biological functions. There are some dietary components from human food sources that cannot be digested, reach the large intestine and those can be fermented by gut microflora (Flint et al., 2012; Marcobal et al., 2013). During fermentation, beneficial bacteria (probiotics) especially lactic acid bacteria (LAB) produce a wide range of secondary metabolites (byproducts), most of which have been linked with health-promoting benefits. The major byproducts are lipid molecules differing in chemical structure from short chain fatty acids (SCFAs) such as acetate, propionate to polyunsaturated fatty acids (PUFAs) including conjugated linoleic acids (CLA) (Serini et al., 2009; Louis et al., 2014).

Prebiotics in combination with probiotics. The concept of 'synbiotic' was introduced by Gibson and Roberfroid (1995) as 'mixtures of probiotics and prebiotics that beneficially affect the host by improving the survival and implantation of live microbial dietary supplements in the GI tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, thus improving host welfare'. Several related studies have also revealed the combination of probiotics and prebiotics could possibly yield a synergistic effect in the limitation of foodborne pathogenic bacterial populations in the hosts. The symbiotic combination of inulin/oligofructose with *B. bifidum* and *L. plantarum* could promote the growth of bifidobacteria whereas reduce the growth of EHEC, *C. jejuni*, and *S.* Enteritidis *in vitro* (Fooks and Gibson, 2002). Likewise, *Bifidobacteria* in together with trans-galacto-oligosaccharides could protect mice from *S*. Typhimurium

and following lethal infections (Asahara et al., 2011). Moreover, synbiotics with *L. paracasei* and oligo-fructose significantly increased the amount of *Lactobacillus spp*., *Bifidobacterium spp*., total anaerobes, and total aerobes in weanling piglets' feces, while significantly reduced fecal concentrations of *Clostridium spp*. and *Enterobacterium spp.* (Bomba et al., 2002). In presence of peanut flour and cocoa, it has been observed that growth of probiotic like *Lactobacillus* spp is increased and growth of pathogens is inhibited (Peng et al., 2015a; Peng et al., 2014; Peng et al., 2015b). More recently, synbiotics have been suggested to be more effective than either probiotics or prebiotics alone in improving the gut health by modulation of gut microbiota (Saulnier et al., 2008; Adebola et al., 2014).

Bioactive metabolites produced by Lactobacillus. 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; Sharma et al., 2014; Peng et al., 2016a;). SCFAs particularly linoleic acid (LA) and conjugated linoleic acid (CLA) are known as the most beneficial due to their enormous benefits in human health and anti-pathogenic activities (O'Shea et al., 2012). 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 those are able to produce CLA during normal metabolic activities and in increased

concentration with additives/prebiotics, resulting in higher antimicrobial activities (Alonso et al., 2003; Lin et al., 2000; Ogawa et al., 2001).

Several factors like culture media, temperature, oxygen availability, period of fermentation, substrate concentration influence the CLA production (Pandit et al., 2012). For examples, *L. acidophilus* is able to form CLA in microaerophilic condition but not in aerophilic conditions (Ogawa et al., 2001); Lin et al. (2005) demonstrated that immobilized *L. bulgaricus* and *L. acidophilus* in chitosan and poly-acrylamide matrix exhibited higher CLA production ability than in normal cultural condition; Kishino et al. (2002) revealed that in nutrient medium supplemented with free linoleic acid inducer, the washed cell of *L. plantarum* was able to express higher level of CLA than obtained in growth cultures with extended incubation. In addition to these, the isomers formed are species-dependent (Van Nieuwenhove et al., 2011), indicating that several species like *L. paracasei* and *S. thermophilus* only form single isomer, whereas two or more CLA isomers could be produced by other species such as *L. acidophilus*, *L. casei*, and *L. plantarum*.

Linoleate isomerase (LAI) is the enzyme responsible for linoleic acid isomerization and CLA production in several bacteria including *Lactobacillus* and *Bifidobacteria* (Macouzet et al., 2010a). LAI has been reported to be found in *Lactobacillus* in two major forms: either as a membrane-bound protein or as a soluble enzyme which allows possible extraction from bacterial cell-free cultural supernatant (Gorissen et al., 2010; Macouzet et al., 2010b). It was observed in different studies that the optimal conditions for linoleic acid isomerization by LAI in washed cells of multiple *Lactobacillus* are pH 6.5 and 34-37 °C (Lee et al., 2003; Kishino et al.,

2010). Putative LAI proteins have been characterized and sequenced (Farmani, 2010), including LAI enzymes from *B. breve*, *B. dentium, Clostridium sporogenes*, *L. acidophilus*, *L. plantarum*, *L. lactis*, *L. reuteri*, *Propionibacterium acnes*, and *Rhodococcus erythropolis* strains (Rosson et al., 2001; Peng et al., 2007). The mechanism for the production of CLA by *Lactobacillus* has been demonstrated to involve hydroxyl fatty acids as intermediates (Kishino et al., 2011). Furthermore, the production/conversion of CLA in *Lactobacillus* was hypothesized to be a multiple-step, and was later revealed that the CLA formation is composed of three distinct steps- linoleic acid hydration into 10-hydroxy-octadecenoic acid (HOE), followed by isomerization and dehydration of 10-HOE into CLA (Yang et al., 2014).

The LAI is a myosin-cross-reactive antigen (MCRA) first found in *Streptococcus pyogenes* as a 67 kD protein (Kil et al., 1994). MCRAs contain a family of proteins present in a wide range of bacteria, especially LAB, and MCRAs from multiple LABs were confirmed as fatty acid hydratase (Yang et al., 2014). The BLAST search of MCRA protein revealed more than 148 conserved sequences across different bacterial genus including both Gram-positive and Gram-negative (Volkov et al., 2010). In a later study, the MCRA gene (*mcra*) encoding the multicomponent LAI catalyzing double bond migration (isomerization) in *L. plantarum* was transformed into *E. coli*, as a result of which, c9, t11-CLA and 10-HOE were produced at a significant level by the mutated *E. coli* strain (Kishino et al., 2011). Three years later, O'Connell et al. (2013) confirmed that the heterologous expression of *B. breve mcra* in *Lactococcus* and *Corynebacterium* induced larger amounts of hydroxyl-fatty acids production/conversion in the cultural condition. Moreover, these recombinant

bacterial cells turned out to possess higher resistance to solvent stress and heat when compared with wild type bacteria. Additionally, MCRAs found across a wide range of taxa including LAB have also been shown to contribute to *in vivo* survival and cell adherence (O'Flaherty and Klaenhammer, 2010; Volkov et al., 2010).

Beneficial effects of conjugated linoleic acid. Conjugated linoleic acid (CLA) is the common term used for the mixture of isomers of linoleic acid (C18:2, c9, c12). These isomers contain double bonds in either cis or trans configuration at different possible positions (Banni, 2002), among which c9, t11-CLA and t10, c12-CLA are the most common and mainly found isomers, and they are also observed to be associated with multiple health and nutritional benefits on human beings (Alonso et al., 2003). Dietary sources of CLA include dairy products, vegetable oils, animal meats, mixed nuts (including peanut), cocoa etc (Kris-Etherton, 2000; Sonwai et al., 2014). Because of numerous benefits, CLA has received great attention in recent years, and many researches have been conducted to evaluate the health effects of mixed isomers of CLA (Kelley et al., 2007). A number of biological functions and health benefits of CLA have been established, and certain CLA-producer probiotic strains including *Lactobacillus* have also been associated with a variety of systemic health promoting effects (O'Shea et al., 2012) and these benefits include anti-cancer, anti-inflammatory, and anti-pathogenic activities (Ewaschuk et al., 2006; Yang et al., 2015).

Both *in vitro* and *in vivo*, the anti-inflammatory properties of CLA have been reported. The overall anti-inflammatory mechanism of CLA is by COX-2 inhibition

which reduces PGE2 release (Li et al., 2005; Stachowska et al., 2007; Flowers and Thompson, 2009; Nakamura and Omaye, 2009). By activating peroxisome proliferator-activated receptors (PPARs), CLA can manage to inhibit the activation and translocation of NF-κB into nucleus (Kim et al., 2011) followed by reducing the level of pro-inflammatory cytokines formation such as TNF-α (Akahoshi et al., 2004), IL-1β (Albers et al., 2003; Tricon et al., 2004), and IL-8 (Jaudszus et al., 2005) which are generally activated by NF-κB (Clarke et al., 2010; Song et al., 2006). CLA has also been reported to stimulate the expression levels of anti-inflammatory cytokine TFG-β1 (Bassaganya-Riera et al., 2004; Bassaganya-Riera et al., 2012) and protective immunoglobulins IgA, IgG, and IgM, on the other hand suppressing IgE production (Martinez et al., 2010). c9, t11-CLA treatment on murine macrophage and dendritic cells suppressed the production of both IL-12 and IL-4 (Loscher et al., 2005), which indicates that CLA is able to attenuate both Th1 and Th2 polarization following an inflammatory challenge by bacterial pathogens, this is revealed in both *in vitro* and *in vivo* studies. The anti-inflammatory effects of CLA do not impair cellular immunity to intracellular pathogens, though the mechanism under it is still unknown (Turnock et al., 2001).

Many long chain saturated and unsaturated fatty acids have been revealed to own antimicrobial activity (Mbandi et al., 2004; Zheng et al., 2005; Shin et al., 2007). Among those, long chain unsaturated fatty acids especially linoleic acid has found to be bactericidal to *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Micrococcus Kristinae* (Zheng et al., 2005) with minimum antimicrobial effect against Gram-negative bacteria such as *E. coli* and *Salmonella* (Dilika et al., 2000; Sun et al., 2003).

On the other hand, CLA has also shown considerable promise as an antimicrobial bioactive agent against pathogenic bacteria. Meraz-Torres and Hernandez-Sanchez (2012) reported that lower concentration of potassium salt of CLA could slow down the growth rate of both Gram-positive bacteria like *Bacillus cereus*, *L. monocytogenes*, *S. aureus*, and *Streptococcus mutans* and Gram-negative bacteria including *P. aeruginosa*, *S.* Typhimurium, *Vibrio parahemolyticus*, *Klebsiella pneumoniae*, and *Proteus mirabilis*, while higher concentration of potassium salt of CLA could completely inhibit their growth. The possible antimicrobial mechanism is through CLA lipid peroxidation at bacterial membrane and cultural medium, since CLA had been found to be present in the membranes of all tested microorganisms, in the cultural medium and disrupted cell membrane surfaces were also observed (Byeon et al., 2009).

All of the above mentioned health benefits are probiotic species dependent but a significantly higher CLA producing bacterial colony forming units (CFU) is required for the optimal amount of CLA production (Mimura et al., 2004; Larsen et al., 2006; Gionchetti et al., 2007; Helwig et al., 2007). Therefore, increasing of CLA production by per bacterial cells might be a feasible plan to maintain the effectual dosage of CLA.

Overall hypothesis and Specific aims

The conjugated linoleic acid over-expressing *Lactobacillus casei* mutant can reduce the growth and colonization of *Campylobacter jejuni* and in conjugation with bioactive phenolics, the mutant has higher efficacy.

To evaluate the hypothesis, following aims need to be investigated-

Aim 1: *In vitro* evaluation of the effect of *Lactobacillus casei* mutant in growth and colonization of *Campylobacter jejuni*

Aim 2: *In vitro* evaluation of the cell free culture supernatants of *Lactobacillus casei* mutant in different virulence properties of *Campylobacter jejuni*

Aim 3: *In vitro* evaluation of the *Lactobacillus casei* mutant in presence of blackberry (*Rubus fruticosus*) and blueberry (*Vaccinium corymbosum*) pomace in controlling growth and colonization of *Campylobacter jejuni*

Chapter 2: *In vitro* evaluation of the effect of *Lactobacillus casei* mutant in growth, colonization and different virulence properties of *Campylobacter jejuni*

Introduction

Probiotics, especially lactic acid bacteria (LAB) are known to play important roles in reducing zoonotic bacterial pathogens including *Campylobacter jejuni* from farm animal gut by positively modulating the microbial ecosystem (Campana et al., 2012) and are getting much of the attention in recent years. *Lactobacillus casei* is being 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. Probiotics, prebiotics, or a combination of the two referred to as synbiotics, have emerged as a promising alternative approach in sustainable animal farming practices. Recently, it has been also identified their abilities to produce multiple antimicrobial derivatives (Peng et al., 2016a; Sharma et al., 2014) in the presence of prebiotic like components suggesting the possible usability of different *Lactobacillus* strains as favorable biological alternatives against control of foodborne bacterial pathogens in reservoirs. SCFAs particularly linoleic acid (LA) and conjugated linoleic acid (CLA) are known as the most beneficial bioactive metabolites due to their enormous benefits in human health and anti-pathogenic activities (O'Shea et al., 2012). Previous studies in our lab

also demonstrated antimicrobial properties of *L. casei* metabolites which were more effective in exclusion of *Salmonella*, *Listeria* and pathogenic *Escherichia coli* in presence of prebiotics, e.g., peanut fractions, cocoa, and various plant extracts (Peng et al., 2015a; Peng et al., 2014; Peng et al., 2015b; Peng et al., 2016b).

Given the emphasis on the potential beneficial properties of SCFAs, we aimed to stimulate the production of CLA of *L. casei* by over-expression of linoleate isomerase gene that will be capable of competitively inhibiting *C. jejuni* growth/survival and interacting with host cells. We also we aimed to investigate the expression levels of *C. jejuni* virulence mediatory genes and physicochemical properties to determine underlying mechanism of actions.

Material and methods

Bacterial strains and growth conditions. *Lactobacillus casei* (ATCC 334) (LC) was grown on de Man–Rogosa–Sharpe (MRS) agar (EMD Chemicals Inc., USA) for overnight at 37 °C under CO_2 enriched condition (5%) (Thermo Fisher Scientific Inc., USA). *Campylobacter jejuni* RM1221 (ATCC BAA-1062) (CJ) was grown on Karmali agar (EMD Chemicals Inc., USA) for overnight at 37 °C, under microaerophilic condition (10% CO_2, 5% O_2 and 85% N_2) (Thermo Fisher Scientific Inc., USA) (Salaheen et al., 2014b).

LC^{+mcra} mutant construction. Over-expression of linoleate isomerase gene in LC was performed following the method previously described by Rosberg-Cody et al. (2011) with slight modifications. Briefly, the 1720 bp myosin-cross-reactive antigen

(*mcra*) gene encoding linoleate isomerases from *L. rhamnosus* GG was amplified through PCR and blunt-end cloned into the pJET vector (Thermo Fisher Scientific Inc, USA). Transformation of pJET-*mcra* into *E. coli* DH5α (Thermo Fisher Scientific Inc, USA) was performed as following: 250 µL bacterial suspensions in cold 50 mM $CaCl_2$ was mixed with 10 µL ligated product and incubated with 10 min on ice followed by 50 s at 42 °C. After 2 min incubation on ice, 250 µL Luria-Bertani (LB) broth (EMD Chemicals Inc., USA) was added for 10 min at room temperature, followed by selection on LB agar (EMD Chemicals Inc., USA) with 100 µg/mL ampicillin (IBI Scientific Inc., USA). The *mcra* gene was then double-excised from pJET-*mcra* with BamHI and XbaI (BioLabs Inc., USA) and ligated into the same sites of pMSP3535 vector (AddGene, USA) at 16 °C overnight. The constructed pMSP3535-*mcra* was transformed into *E. coli* DH5α following the same way described above and mixed with LC at ratio of 1:1, 1:5, and 1:10 (donor cells: recipient cells) for bacterial mating. The final LC mutant (LC^{+mcra}) was selected on MRS agar containing 300 µg/mL erythromycin (IBI Scientific Inc., USA) at 37 °C under micro-aerophilic condition.

Cell lines and culture conditions. Chicken macrophage, HD-11 (provided by Dr. Uma S. Babu, Immunobiology Branch, Food and Drug Administration, Laurel, MD, USA) and human epithelial, HeLa (ATCC® CCL2™) cells were cultured at 37 °C, standard condition (5% CO_2) in Dulbecco's Modified Eagle Medium (DMEM) (Corning cellgro, USA) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (Corning cellgro, USA) and 50 µg/mL of gentamycin (Lonza, USA). Both HD-

11and HeLa cells were seeded in 24-well culture plate (Greiner bio-one Inc., USA) at 2×10^5 cells/mL and then they were cultured at the standard condition as above up to 90% confluence. The semi-confluent cultures were washed with phosphate-buffered saline (PBS) three times and immersed in antibiotic free DMEM supplemented with 5% heat-inactivated FBS for cell adhesion and invasion assay (Peng et al., 2014).

Peanut flour preparation. In shell Jumbo Virginia raw peanut was purchased from local market and shelled by hand to separate the kernel fractions. Peanut skin was removed and the white kernel was grounded to peanut flour. Peanut flour was defatted by 2 extractions with n-hexane (10 mL of n-hexane for per gram of peanut flour) for 12 h at 25 °C. Peanut flour fraction (defatted) suspensions was prepared in sterilized distilled water (pH adjusted to 8.0 with 1 N NaOH), mixed well and sterilized with UV irradiation for 2 h (Peng et al., 2014).

Mixed-culture of LC with CJ in broth. LC, LC^{+mcra} and CJ were grown on selective respective agar plates following the method described above. LC bacterial suspension with or without 0.5% peanut flour and LC^{+mcra} containing 10^7 colony forming unit (CFU) /mL of 400 µL was co-cultured with 400 µL of CJ bacterial suspension containing 10^6 CFU/mL in 3.2 mL of DMEM supplemented with 5% heat-inactivated FBS at 37 °C under microaerophilic conditions. Serial dilutions were performed in PBS, followed by plating on Karmali agar for CJ at 0, 24, 36, and 48 h time points.

Cell free culture supernatant on the growth of CJ. Overnight liquid cultures of LC with or without 0.5% peanut flour, LC^{+mcra} in DMEM containing 5% FBS were centrifuged at 4000 × g for 20 min (Thermo Fisher Scientific Inc., USA). Cell free culture supernatants (CFCSs) were then filtered by sterile syringe 0.2 μm filter (VWR Inc., USA) (Peng et al., 2017). Filtered CFCS from LC (CFCS-LC), peanut flour supplemented LC (CFCS-LC+PF) and LC^{+mcra} (CFCS-LC^{+MCRA}) were collected and stored at 4 °C. The pH of CFCS-LC, CFCS- LC+PF and CFCS-LC^{+MCRA} were adjusted to the pH of DMEM+5% FBS (with 1 N NaOH) resulting CFCS-ALC, CFCS-ALC+PF and CFCS-ALC^{+MCRA} respectively. CJ bacterial cell suspension containing 10^6 CFU/mL of 400 µL was inoculated in separate culture tubes with 1.6 mL DMEM+5% FBS and 2 mL of CFCS-LC, CFCS- LC+PF, CFCS-LC^{+MCRA}, CFCS-ALC, CFCS- ALC+PF and CFCS-ALC^{+MCRA} respectively and incubated at 37 °C under microaerophilic conditions. Serial dilutions were performed in PBS, followed by plating on Karmali agar at 0, 24, 48 h and 72 h time points.

Cell adhesion and invasion assay. The HD-11 and HeLa cell monolayers grown in the wells of 24-well plate with 800 µL DMEM containing 10% FBS were pre-treated with 100 µL DMEM (control), 2 × 10^6 CFU/mL LC with and without 0.5% peanut flour, LC^{+mcra} and CFCS-LC, CFCS-LC+PF, CFCS-LC^{+MCRA}, CFCS-ALC, CFCS-ALC+PF, or CFCS-ALC^{+MCRA} for 1 h with each treatment in triplicates. After pre-treatment, 100 µL of CJ bacterial suspension with multiplicity of infection (MOI) of 10 (2 × 10^6 CFU/mL) were inoculated into each wells. Infected cell monolayers were incubated at 37°C in standard condition for 2 h, and after incubation, the cell

monolayers were washed three times with DMEM with 10% FBS. The cells were then lysed by treating with 0.1%Triton X-100 for 15 min, serially diluted and plated on specific agar plates for counting the adhesive bacterial cells. Cell invasive activity was measured by further 1 h incubation of the washed monolayers in DMEM containing 10% FBS and 100 µg/mL gentamicin followed by three times washing, Triton X-100 lysis, serial dilution and plating on specific agar plates (Peng et al., 2015b; Salaheen et al., 2017a).

Physiological properties of CJ treated with CFCSs. Physicochemical properties, e.g., cell surface hydrophobicity, auto-aggregation and injured cell ratio, were evaluated following the methodologies previously described by Ahn et al. (2014) and Salaheen et al. (2016a) with slight modifications. In brief, bacterial cells were grown in DMEM in absence (control) or presence of CFCS-LC, CFCS-LC+PF, CFCS-LC^{+MCRA}, CFCS-ALC, CFCS-ALC+PF, CFCS-ALC^{+MCRA} at 37 °C for 18 h. The CJ cells were harvested by centrifuging at 3000 ×g for 20 min followed by hydrophobicity, auto-aggregation assays and serially diluted and plated on Karmali agar and LB agar containing 5% FBS at 37 °C, under microaerophilic conditions for injured cell rate assay.

RNA extraction and cDNA synthesis. The cells were grown in the absence or presence of CFCSs and RNA was extracted according to the protocol of ZR Bacterial RNA MiniPrep kit (Zymo Research Corp.,USA). The quantification of RNA was carried out using NanoDrop spectrophotometer (Thermo Scientific Inc.,USA). The

synthesis of cDNA was performed according to the protocol of qScript cDNA SuperMix (Quanta Biosciences,USA). The eluted RNA (1 µg) was mixed with 4 µL of 5X qScript cDNA SuperMix containing optimized concentration of $MgCl_2$, dNTPs, RNase inhibitor protein, qScript reverse transcriptase, random primers, oligo(dT) primer, and stabilizers and then incubated at 25 °C for 5 min, 42 °C for 30 min, and 85 °C for 5 min. (Salaheen et al., 2014a).

Quantitative RT-PCR assay. The PCR reaction mixture containing 10 µL of PerfeCTa SYBR Green Fast Mix, 2 µL of each primer (100 nM), 2 µL of cDNA (10 ng), and 4 µL of RNase-free water were amplified using an Eco Real-Time PCR system (Illumine, USA) with 30 s denaturation at 95 °C, followed by 40 cycles of 95 °C for 5 s, 55 °C for 15 s, and 72 °C for 10 s. The custom-synthesized oligonucleotide primers (Erofins MWG Operon, USA) for *cadF* (*Campylobacter* adhesion fibronectin-binding protein), *cdtB* (Cytolethal distending toxin), *ciaB* (*Campyloabacter* invasion antigen), *flaA* (Flagellin A subunit synthesis), *flaB* (Flagellin B subunit synthesis) of CJ were used following methodology previously described by Salaheen et al. (2014a). These genes were chosen as *cadF, ciaB, flaA* and *flaB* genes commonly known for their critical role in CJ colonization to host cells and *cdtB* is imperious in CJ toxin production in host cells (Fouts et al., 2005). The relative transcription levels of target genes were estimated by the comparative log fold change. The CT (Cycle threshold) values of target genes in treated bacterial cells were compared to those in untreated bacterial cells and normalized to the housekeeping gene.

Statistical analysis. Data were analyzed by the Statistical Analysis System software (SAS Institute Inc., USA). The one-way analysis of variance (ANOVA) for each single time point followed by Tukey's test was used to evaluate the treatments and determine the significant differences among control and treatments based on significant level of 0.05.

Results

***Competitive inhibition of CJ with LC in co-culture condition*.** To determine the effect of probiotic and its byproducts, and synbiotics on the growth inhibition of CJ, we co-cultured CJ with LC with or without peanut flour and LC^{+mcra} in DMEM to provide best possible cellular nutrients to support the growth of both bacterial strains *in-vitro* culture condition. In the presence of only peanut flour, growth of CJ was observed unchanged compared to the control whereas in the presence of LC regardless with or without peanut flour (0.5%) the growth of CJ was reduced (Fig 1A). It was observed that LC with peanut flour and LC^{+mcra} alone successfully killed all viable CJ cells in time dependent manner and both mixed-culture condition completely inhibited the growth of CJ (undetected level) within 48 h compared to LC without peanut flour (>6.3 logs CFU/mL reduction) (Figure 2.1A).

***Effect of CFCSs on growth inhibition of CJ*.** Bacterial cell free culture supernatants from LC [CFCS-LC (pH = 4.60±0.16)], LC with peanut flour [CFCS-LC+PF (pH = 4.47±0.07)] and LC^{+mcra} [CFCS-LC^{+MCRA} (pH = 4.38±0.05)] were collected from

overnight culture started with the initial inoculums of 10^6 CFU/mL and ended with approximately 2×10^9 CFU/mL (CFCS-LC), 10^{10} CFU/mL (CFCS-LC+PF) and 4×10^9 CFU/mL (CFCS-LC^{+MCRA}) respectively. Then the pH of all the CFCSs were also adjusted at 7.4 (pH of DMEM+5% FBS) to get CFCS-ALC, CFCS-ALC+PF and CFCS-ALC^{+MCRA}, respectively and all of these CFCSs were tested to inhibit the growth of CJ.

All the CFCSs collected from overnight culture of LC in the presence or absence of peanut flour or LC^{+mcra} showed inhibitory effects on the growth of CJ (Figure 2.1B). After 24 hour, no noteworthy reduction in growth of CJ was observed (< 1.0 log CFU/mL) in the DMEM culture media supplemented with 50% CFCSs. After 48 h the growth of CJ was reduced more than 4.5 logs CFU/mL by CFCS-LC^{+MCRA} and more than 4.8 logs CFU/mL by CFCS-ALC^{+MCRA} compared to control group (no CFCS added to the growth medium). After 72 h of incubation, the most effective growth reduction of CJ was observed by CFCS-LC^{+MCRA} (>7.1 logs CFU/mL reduction) and CFCS-ALC^{+MCRA} (>7.0 logs CFU/mL reduction) followed by CFCS-LC, CFCS-ALC+PF and the rests (Figure 2.1B).

***Alteration of adherence and invasion abilities of CJ to cultured chicken and mammalian cells*.** In the presence of LC, LC with 0.5% peanut flour or linoleic acid over-expressed mutant, LC^{+mcra}, and their CFCSs, the adherence and invasion abilities of CJ to both HD-11 and HeLa cells were reduced (Figure 2.2). It was observed that, the adhesion ability of CJ to HD-11 cells in the presence LC with peanut flour (0.5%) or LC^{+mcra} was significantly reduced >0.12 logs CFU/mL and >0.07 logs CFU/mL,

respectively. In the same assay into HD-11 cells, invasion efficacy of CJ was also significantly reduced >0.14 logs CFU/mL and >0.11 log CFU/mL in the presence of LC with peanut flour and LC^{+mcra} alone, respectively (Figure 2.2A).

Only numerical but no significant reduction of adhesion efficacy of CJ to HD-11 cells was observed in the presence of CFCSs collected from LC with or without peanut flour or LC^{+mcra} but the invasion abilities of CJ into HD-11 cells were reduced significantly by > 0.09 logs CFU/mL in the presence of CFCSs collected from LC^{+mcra}, for both pH neutralized and non-neutralized CFCSs (Figure 2.2B).

It was also observed that in co-culture condition LC^{+mcra} reduced the adherence of CJ to HeLa cells most effectively (> 0.12 log CFU/ml) followed by LC with peanut flour (> 0.09 log CFU/ml) and LC alone (> 0.05 log CFU/ml) (Figure 2.2C). In the same assay, LC^{+mcra} also showed to be the most effective in reducing invasive ability of CJ to HeLa cells(>0.28 log CFU/ml). LC with peanut flour and LC alone were observed to reduce the invasive efficacy of CJ into HeLa cells by >0.24 log CFU/ml and >0.15 log CFU/ml, respectively (Figure 2.2C).

It was also found that only CFCS collected from LC with peanut flour significantly reduced the adhesion efficacy of CJ to HeLa cells by > 0.22 log CFU/mL. On the other hand, the pH neutralized CFCSs collected from LC with peanut flour and LC^{+mcra} alone reduced the adhesion efficacy of CJ to HeLa cells significantly by > 0.18 log CFU/mL and > 0.13 log CFU/mL, respectively. Similarly, the CFCSs collected from LC alone, LC with peanut flour (0.5%) or LC^{+mcra} alone reduced the invasion into HeLa cells by CJ significantly by >0.22 log CFU/mL, > 0.09 log CFU/mL, >0.13 log CFU/mL, respectively. Whereas when we neutralized

the pH of collected CFCSs from LC alone, LC with peanut flour or LC^{+mcra} alone, there was significant reduction of invasion ability of CJ into HeLa cells by >0.2 log CFU/mL, >0.13 log CFU/mL, >0.22 log CFU/mL, respectively (Figure 2.2D).

Alteration of physicochemical properties of CJ treated with CFCSs. The physicochemical properties of CJ specifically ratio of injured cells, auto-aggregation ability and hydrophobicity, were altered due to the pre-treatment with CFCSs collected from the overnight cultures of various conditions of LC with or without peanut flour or linoleic acid over-expressed mutant, LC^{+mcra} (Table 2.1).

We observed that pre-treatments of CJ with various cultural supernatants including only probiotic (CFCS-LC), both probiotic and prebiotic (CFCS- LC+PF) and genetically modified probiotc (CFCS-LC^{+MCRA}) significantly increased the percentage of injured bacterial cells by 44.25%, 38.62% and 46.21% respectively. Whereas, pH neutralized CFCSs displayed attenuated effects on the ratio of injured CJ bacterial cells; treatments with the pH adjusted supernatants CFCS-ALC, CFCS-ALC+PF and CFCS-ALC^{+MCRA} increased the injured cell percentage only numerically by 17.35%, 13.18% and 25.3% respectively when compared to control (without any CFCSs in growth media) (Table 2.1).

The auto-aggregation capacity of CJ decreased significantly by the CFCSs pre-treatments, CFCS-ALC^{+MCRA} (42.27% decrease) being the most effective followed by CFCS-LC^{+MCRA} (41.65% decrease), CFCS- ALC+PF (29.62% decrease), CFCS- LC+PF (27.33% decrease) and CFCS-ALC (25.43% decrease) when

compared to control group. The auto-aggregation capacity of CJ was not changed in the presence of CFCS-LC though (Table 2.1).

We also found that cell surface hydrophobicity of CJ was reduced significantly 59.2% and 55.38% with the pre-treatment by CFCS-LC+PF and CFCS-LC^{+MCRA}, respectively when compared to control group. CFCS-LC, CFCS-LC^{+MCRA}, CFCS-ALC and CFCS-ALC+PF could also reduce the cell surface hydrophobicity of CJ by 41.26%, 50%, 33.3% and 43.17% respectively when compared to control group but only numerically.

Effects of CFCSs on CJ virulent gene expression. We assessed the expression level of several virulence genes of CJ including *cadF*, *cdtB*, *ciaB*, *flaA* and *flaB* by pre-treatment of different CFCSs (Figure 2.3). The *cadF* gene expression level was significantly down-regulated >4.3 folds, >5.5 folds and >5.1 folds by CFCS-ALC, CFCS-ALC+PF and CFCS-ALC^{+MCRA}, respectively. The expression level of *cdtB* gene was down regulated significantly by CFCS-LC^{+MCRA} (>5.7 folds), CFCS-ALC (>5.8 folds), CFCS-ALC+PF (>7.4 folds) and CFCS-ALC^{+MCRA} (>5 folds) pre-treatments. The expression level of *ciaB* was also down regulated significantly around 1 fold, more than 3.0 folds, more than 2.2 folds and more than 2.8 folds by CFCS-LC^{+MCRA}, CFCS-ALC, CFCS-ALC+PF and CFCS-ALC^{+MCRA}, respectively. The expression level of *flaB* was upregulated significantly by CFCS-LC^{+MCRA} (>2 folds), CFCS-ALC (>3.1 folds) and CFCS-ALC^{+MCRA} (>2.3 folds). All other up-regulation and down-regulation of the above mentioned gene expression levels were not significant.

Discussion

In this study, we investigated role of CLA, one of the bioactive SCFAs produced by LC during their growth and metabolism in the presence of appropriate growth conditions, in the growth, survival and metabolic activities of CJ and its interactions with host (HD-11 and HeLa) cells. In our previous study, we observed that in the presence of prebiotic like components, LC can stimulate the production of CLA and LC in the presence of prebiotic like component or its supernatants (CFCSs) inhibit the growth and survival of various enteric bacterial pathogens (Peng et al., 2015b; Peng et al., 2014; Salaheen et al., 2014b). In this study, we confirmed that CLA over-producing LC mutant, LC^{+mcra} alone can perform the same activities in the absence of prebiotic like components. Here we determined the effects of LC^{+mcra} to inhibit the growth and alter different pathogenic traits of CJ and compared genetically engineered strain's efficacy with LC in presence or absence of 0.5% peanut flour. To compare the effect of only metabolites including CLA and metabolites with active bacterial cells, LC or LC^{+mcra}, we also investigated the effect of CFCSs in the inhibition of growth, survival of CJ and its interaction with cultured cells.

To create an equal growth and survival condition for both LC and CJ, in this study we used the cell culture media (DMEM) for evaluating the interactions between LC and CJ in the co-culture condition. Previously in our laboratory, we also found that only peanut flour had no direct effect on the growth of CJ in DMEM (Salaheen et al., 2014b). However, in this study, we found LC in presence of peanut flour (0.5%) and LC^{+mcra} alone were able to effectively inhibit the growth of CJ in co-culture

condition but LC without peanut flour failed to inhibit significantly the growth of CJ in the similar pattern in co-culture condition. These findings satisfy the previous reports in which we have shown that in the presence of peanut flour (0.5%), the growth of LC was stimulated and ultimately inhibited the growth of several enteric bacterial pathogens such as, *E.coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* (Peng et al., 2015b; Peng, 2014; Salaheen et al., 2014b). Possible reasons behind this inhibitory effect of LC with peanut flour against CJ growth in co-couture condition are induction of LC with peanut flour to produce higher amount of anti-pathogenic metabolites particularly LA/CLA and other SCFAs, which ultimately cause the growth inhibition, and/or stimulated higher number of LC may out-compete CJ through nutritional competition (Salaheen et al., 2014b) and LC^{+mcra} was genetically engineered to produce increased amount of anti-pathogenic metabolite CLA.

Previously our laboratory also reported that attenuated antimicrobial property of CFCSs at high pH growth condition was crucial in the ionic state of lactic acids and other metabolites production by LC and by which growth of foodborne bacterial pathogens were reduced *in-vitro* culture condition (Peng et al., 2015b). Findings from this study suggest that the inhibitory activity of CFCSs may not always depend only on medium acidification, since pH neutralized CFCSs (7.4) were also effective in CJ growth reduction. Furthermore, as there was no complete inhibition of CJ growth with CFCSs after 72 h compared to the total inhibition of CJ growth by the LC with peanut flour (0.5%) and LC^{+mcra} alone within 48 h, so it can be hypothesized that both anti-pathogenic metabolites and competition for nutrient uptake were involved in CJ growth inhibition.

Jean-Richard et al. (2000) found that similar carbohydrate-binding specific proteins are displayed on *Lactobacillus* spp. surface and involved in decreasing the adhesive and invasiveness of enteric bacterial pathogens like *E.coli, Salmonella* Typhimurium by pre-occupying the surface receptors on host cells. Further, researchers also reported that CFCSs, collected from various condition of *Lactobacillus* spp. also restricted the cell adhesion to and invasion into host epithelial cells of different enteric bacterial pathogens (Bendali et al., 2014; Campana et al., 2012).

In this study we found that LC with peanut flour or LC^{+mcra} alone reduced the adhesion and invasion efficacy of CJ significantly in both host cells, HD-11 and HeLa cells. However, in this study, adhesion efficacy of CJ was not reduced significantly in HD-11 cultured model by the CFCSs obtained from overnight culture of LC supplemented with or without peanut flour or LC^{+mcra} alone. Though CFCSs obtained from LC with 0.5% peanut flour reduced the adhesion efficacy to HeLa cells significantly. Again invasion efficacy of CJ was reduced by CFCSs obtained from LC^{+mcra} in HD-11 and the CFCSs collected from LC with or without peanut supplement or LC^{+mcra} reduced CJ invasion into HeLa cells significantly when compared to control group (growth media without any CFCSs). On the other hand, pH neutralized CFCSs could not significantly reduce the adhesion efficacy of CJ to HD-11 cells but pH neutralized CFCSs of LC with 0.5% peanut flour and LC^{+mcra} reduced the adhesion efficacy of CJ to HeLa cells significantly. Invasiveness of CJ was reduced in both HD-11 and Hela cells by pH neutralized CFCSs collected from overnight culture of LC supplemented with or without peanut flour or LC^{+mcra} alone.

These findings revealed, reduced adhesion and invasion ability of CJ in human epithelial cells than in chicken macrophage cells by the parallel co-culture or CFCSs treatments, which are similar to the outcomes of previous studies from our laboratory though the mechanism is not clear (Salaheen et al., 2016a; Salaheen et al., 2014a).

Injured CJ cell (viable) percentage was increased significantly by CFCSs obtained from LC with or without peanut supplement or LC^{+mcra} alone but only numerically when pre-treated with pH neutralized CFCSs in comparison to control group (growth media without any CFCSs). So it can be inferred that the ratio of injured CJ cells depends on the pH of the growth media along with other anti-pathogenic metabolites present in the CFCSs of LC with or without peanut supplement or LC^{+mcra} alone. It was also observed that there was no significant intra CFCSs treatments variation in the ration of injured CJ cells.

Previously several research groups found that auto-aggregation capacity serves as virulence marker of many Gram-negative enteric bacterial pathogens (Chiang et al., 1995; Menozzi et al., 1994). In this study, we also found auto-aggregation capacity of CJ decreased significantly by the CFCSs from LC with peanut flour supplements or LC^{+mcra} alone when compared to control group (without any CFCS) or to CFCSs of LC without peanut flour. The pH neutralized CFCSs also decreased auto-aggregation capacity of CJ significantly when compared to control group. Though role of auto-aggregation in CJ pathogenesis is not well documented, it has been suggested that auto-aggregation may play roles in CJ invasiveness into intestinal epithelial cells (Golden et al., 2002), which is one of the possible

explanation of the reduced invasiveness of CJ in this study when treated with different CFCSs.

In this study there was noticeable reduction in cell surface hydrophobicity of CJ when pre-treated with CFCSs collected from LC with or without peanut supplement and LC^{+mcra} alone, in agreement with Peng et al. (2015b) showing reduced cell surface hydrophobicity of other Gram negative intestinal bacterial pathogens. The reduction of cell surface hydrophobicity is possibly the result from specific alterations of the distribution and proportions of cell surface-associated proteins and polysaccharides which act as the mediators in the aggregation process (Schachtsiek et al., 2004). It was previously demonstrated by Saran et al. (2012) and Lorite et al. (2013) that, there is positive correlation between bacterial cell surface hydrophobicity and cell attachment activities. Similarly in this study, cell surface hydrophobicity reduction of CJ may be connected with the attenuated adhesion abilities of CJ to chicken macrophage and mammalian epithelial cells.

We further investigated the relative expression levels of several virulence associated genes of CJ when cultured with different CFCSs obtained from overnight cultures of LC with or without peanut or LC^{+mcra}. The expression of virulence genes *cadF, ciaB, flaA, flaB* and *cdtB,* were altered particularly down-regulated in the presence of CFCSs. In our previous study un-altered relative expression level of *cadF, cdtB, ciaB, flaB* genes and up-regulation of *flaA* gene expression levels were also observed during the growth of CJ cells in the presence of bioactive phenolic components obtained from blueberry and blackberry pomaces which is antibacterial to several enteric pathogens (Salaheen et al., 2014a). Studies have also demonostrated

that cell-free spent media/extracts of pro-biotics can down-regulate the expression levels of *ciaB* and *flaA* gene (Mundi et al., 2013; Ding et al., 2005). In this study, CFCSs down-regulated the expression of *cadF, cdtB, ciaB* genes and up-regulated the expression of *flaB* gene significantly. The mechanism behind the decreased and increased expression levels of the genes are still not fully understood but as the genes play important role in colonization and toxin production of CJ, these down regulated relative expressions of the genes are vital in control of CJ infection and the metabolites particularly CLA showed their strong antimicrobial effects.

Conclusion

In conclusion, it can be suggested that LC with 0.5% peanut flour or LC^{+mcra} alone 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 use of pre-biotic like compounds, i.e. peanut flour is not always a feasible option with probiotic LC, hence CLA over-producing LC mutant, LC^{+mcra} without peanut flour can be a hands-on alternative to the use of peanut flour in improving feed quality and safety along with furthered aptitude to combat CJ infection and gut health improvements.

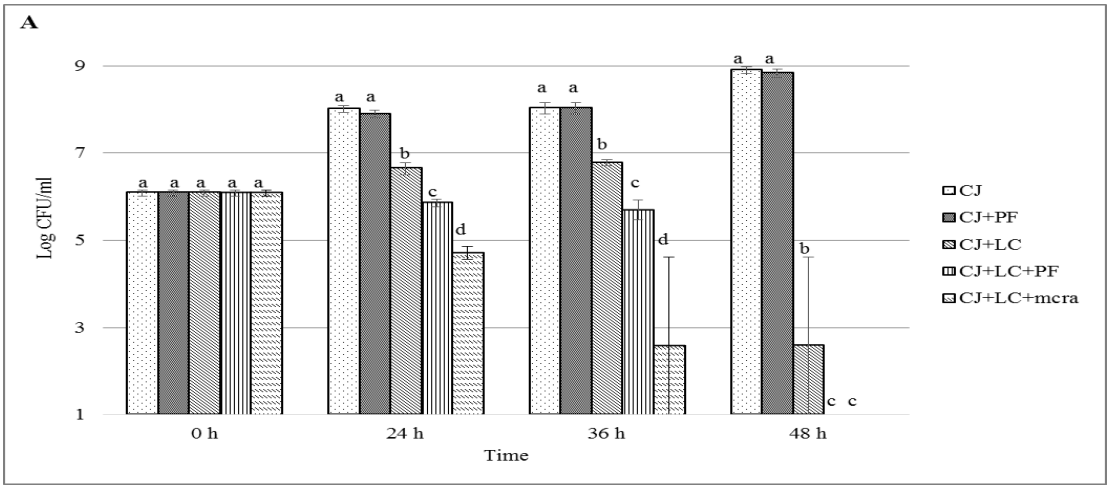
List of Tables and Figures

Table 2.1: Physicochemical properties of CJ treated with CFCS treatments

Treatment	Injured cell (%)	Auto-aggregation (%)	Hydrophobicity (%)
Control	40.0±5.16*,a	53.2±3.53a	15.0±5.05a
CFCS-LC	57.7±1.59b	43.5±8.44a	8.8±5.23a
CFCS-LC+PF	55.4±3.72b	38.7±10.67b	6.1±4.28b
CFCS-LC+MCRA	58.5±3.548b	31.1±6.23b	6.7±3.56b
CFCS-ALC	46.9±5.16ab	39.7±3.43b	10.0±3.41a
CFCS-ALC+PF	45.3±6.27ab	37.5±7.25b	8.52±2.09a
CFCS-ALC+MCRA	50.1±2.61ab	30.7±7.59b	7.5±5.03aa

*Values indicate Mean ± Standard deviation and means with different letters (a-c) within the same column are different when compared with control and among the treatments at $p<0.05$.

Figure 2.1: Growth pattern of CJ in co-culture at 0, 24, 36, and 48 h (A) and Inhibitory effects of CFCSs of LC with or without peanut four, LC^{+mcra} (with and without pH adjustment) on growth of CJ at 0, 24, 48 and 72 h (B). Error bars indicate standard deviation from 6 parallel trails. Different letters (a-f) at each time point indicate the significant growth reduction when compared with single culture or control and among the treatments at $p < 0.05$.



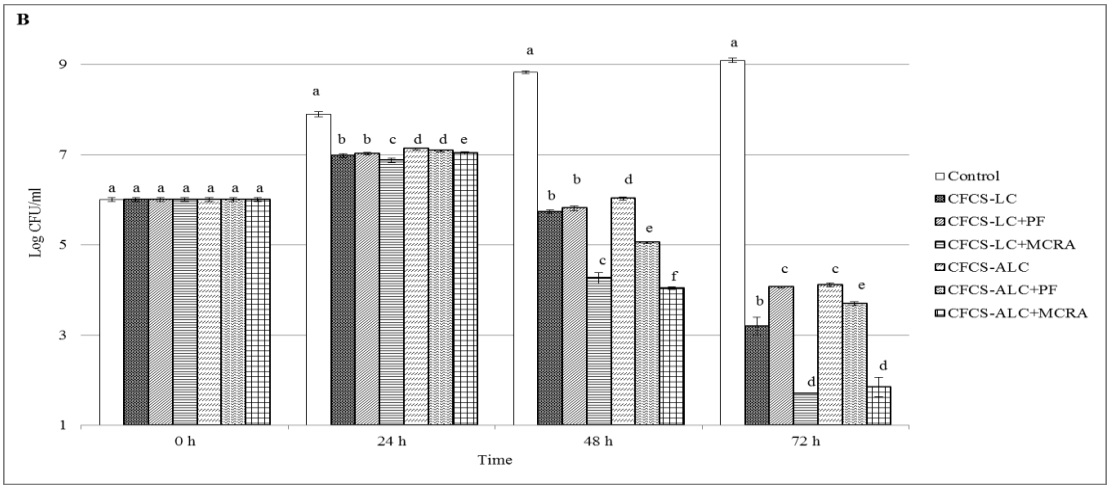
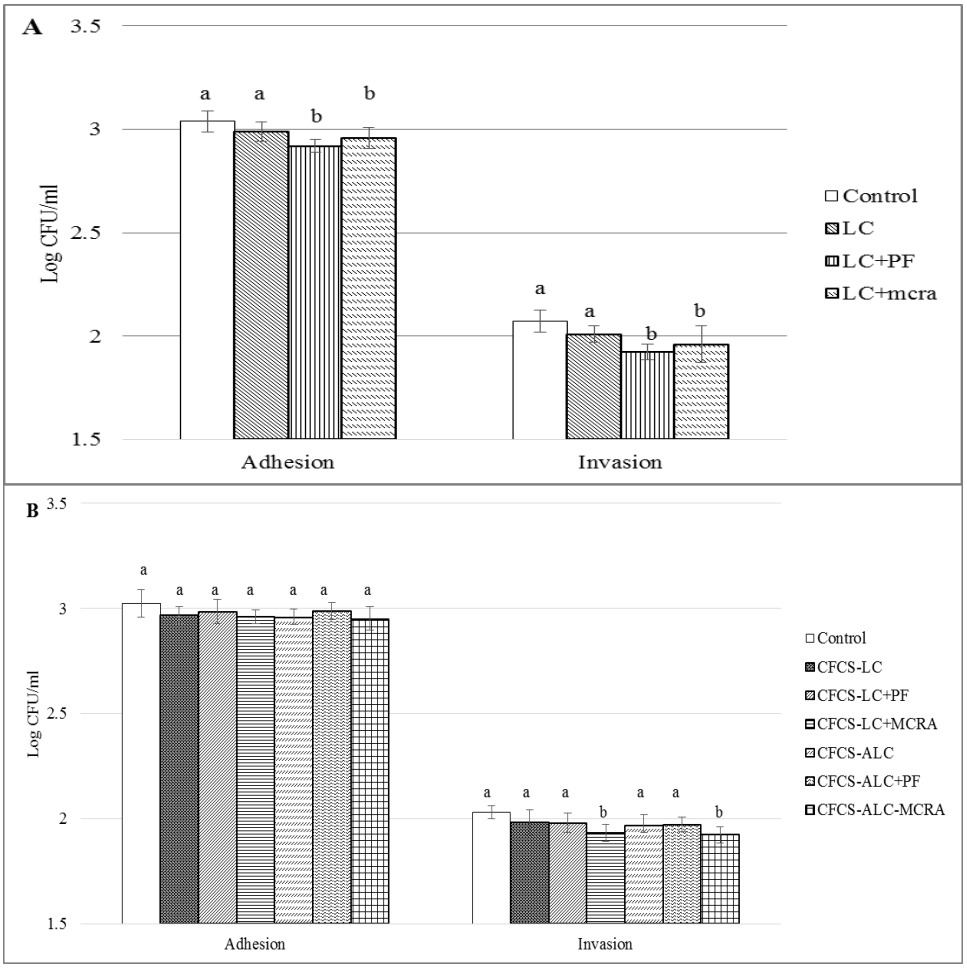
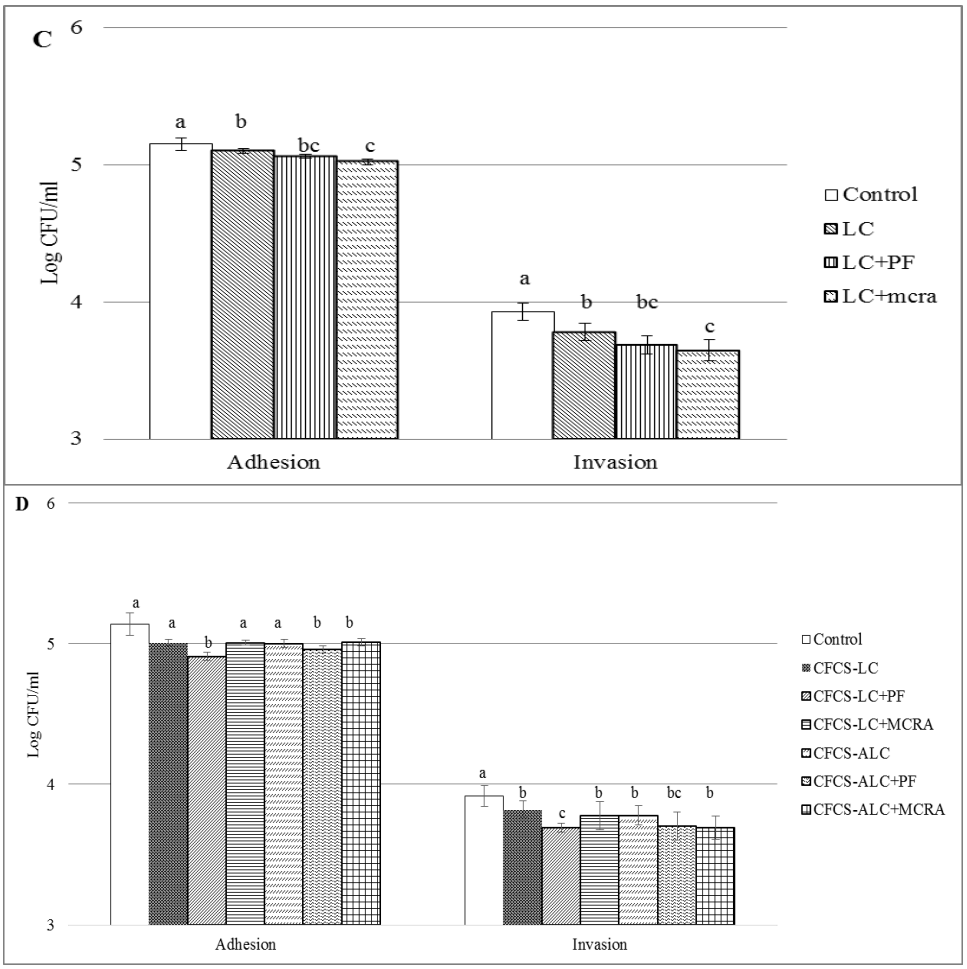


Figure 2.2: Cell adhesion and invasion levels of CJ to HD-11 cells with pre-treatment of LC, LC with 0.5% peanut flour, LC^{+mcra} (A) and with pre-treatment of CFCSs (B), to HeLa cells with pre-treatment of LC, LC with 0.5% peanut flour, LC^{+mcra} (C) and with pre-treatment of CFCSs (D). Error bars indicate standard deviation from 6 parallel trails. Bars with different letters (a through c) are significantly different when compared with control and among the treatments at $p < 0.05$.

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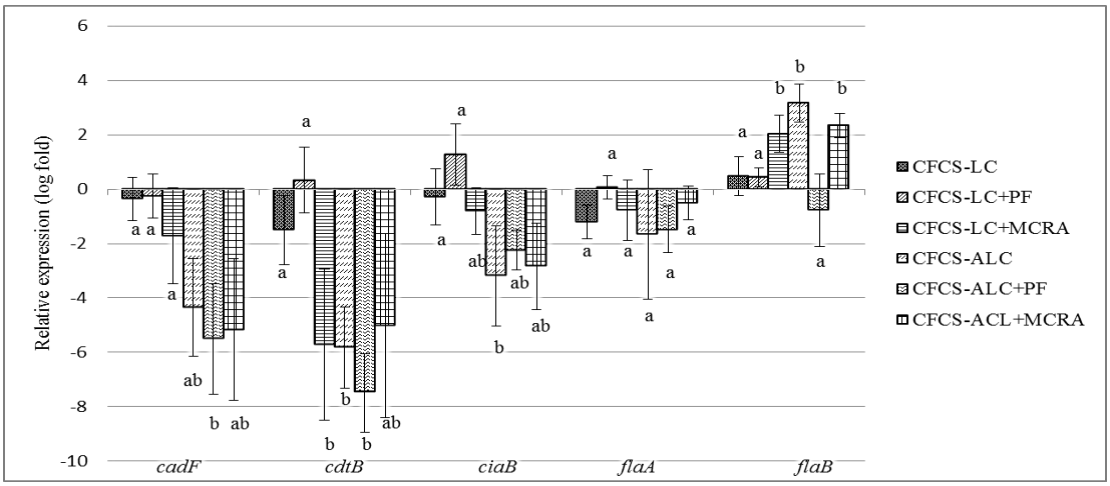
Adhesion
Invasion
Control
CFCS-LC
CFCS-LC+PF
CFCS-LC+MCRA
CFCS-ALC
CFCS-ALC+PF
CFCS-ALC+MCRA

Figure 2.3: Relative expression of different virulence genes of CJ treated with CFCSs. Error bars indicate standard deviation from 6 parallel trails. Bars with different letters (a,b) are significantly different at $p < 0.05$.



Chapter 3: *In vitro* evaluation of the *Lactobacillus casei* mutant in presence of blackberry (*Rubus fruticosus*) and blueberry (*Vaccinium corymbosum*) in controlling growth and colonization of *Campylobacter jejuni*

Introduction

According to the data from the most recent outbreaks caused by multi-drug resistant bacterial isolates in the US, *Campylobacter* becomes one of the major concerns for health professional and food producing industries (CDC, 2018). From 2010 to 2015, there were 209 foodborne *Campylobacter* outbreaks that lead to 2,234 illnesses in the US (CDC, 2017). Further this huge number of campylobacteriosis increases the risk of post infection complexity specifically Guillain Barrie Syndrome, irritable bowel syndrome, reactive arthritis and immune proliferative small intestinal diseases (Chen et al., 2010; Garin et al., 2012). The most commonly identified causative agent of human campylobacteriosis is *Campylobacter jejuni* (CJ) and commonly occurred due to handling or consumption of raw or partially cooked poultry products, unpasteurized milk or milk products (Garin et al., 2012; Salaheen et al., 2016; Wingstrand et al., 2006), as chickens and other warm blooded farm animals naturally harbor *Campylobacter* in their gastrointestinal (GI) tracts (Salaheen et al., 2016). In addition, organic or pasture practicing farmers cannot use any antibiotics or chemicals, so with the increased chance of the cross-contamination level with zoonotic pathogens especially CJ in organic animal food products (Peng et al., 2016;

Salaheen et al., 2016), alternative strategies in reduction of the colonization of *Campylobacter* in farm animal gut to limit the cross-contamination of animal food products and environment are urgently required.

Probiotics are known to play important roles in modulating gut microbial ecosystem particularly reducing the colonization of bacterial pathogens in the gut and improving host gut health (Campana et al., 2012; Maldonado et al., 2006; Servin et al., 2003). Although the molecular basis of this modulation has not been fully explored, but various mechanisms including the ability of probiotics to use quorum sensing to control the virulent genes of pathogens (Medellin-Peña et al., 2007), disruption of the specific protein synthesis and secretion, limit motility, flagellar assembly and other pathogenicity associated traits (Sperandio et al., 2002) are proposed. The effectiveness of probiotics against different enteric bacterial pathogens generally depend on the number of probiotics and the amount of bioactive products specifically short chain fatty acids i.e., conjugated linoleic acid (CLA) produced by them (Peng et al., 2016). *Lactobacillus casei* (LC) is already accepted as an effective probiotic in relieving gastrointestinal pathogenic bacterial infections both *ex vivo* and *in vivo* with no pathogenic traits in human and animals (Peng et al., 2015; Shi et a., 2016; Tabashsum et al., 2017). Further, the antimicrobial properties of LC and its metabolites showed effectiveness in presence of prebiotics or prebiotic like components, e.g., peanut fractions, cocoa, olive, and various plant extracts (Peng et al., 2015; Peng et al., 2015b; Peng., 2017; Salaheen et al., 2014).

Various plant byproducts, especially blackberry (*Rubus fruticosus*) and blueberry (*Vaccinium corymbosum*) byproducts/pomace contain bioactive phenolics

along with various other compounds, which have shown antimicrobial effects against different enteric pathogens including *Campylobacter* (Puupponen-Pimiä et al., 2005; Salaheen et al., 2014; Salaheen et al., 2016; Salaheen et al., 2014). Further, previous studies in our laboratory have provided evidence that berry juice also stimulate the growth of probiotics (Yang et al., 2014).

In this study, we aim to evaluate the combined effect of genetically modified LC with over-expression of linoleate isomerase gene and berry pomace extract (BPE) against the survival ability of one of the major zoonotic pathogens, CJ and its interaction with host cells. In addition, physicochemical properties, and expression levels of pathogenic virulence mediatory genes were also investigated to determine underlying mechanism of actions.

Material and methods

Bacterial strains and growth conditions. *L. casei* strains including wild-type *L. casei* (ATCC 334) (LC), and over-expression of linoleate isomerase gene in *L. casei* (LC^{+mcra}) were used in this study. Both LC and LC^{+mcra} were grown on de Man–Rogosa–Sharpe (MRS) agar (EMD Chemicals Inc., USA) for overnight at 37°C under CO_2 (5%) enriched environment (Thermo Fisher Scientific Inc., USA). *C. jejuni* RM1221 (ATCC BAA-1062) (CJ) was grown on Karmali agar (EMD Chemicals Inc., USA) for overnight at 37°C, under microaerophilic condition (10% CO_2, 5% O_2 and 85% N_2) (Thermo Fisher Scientific Inc., USA) (Salaheen et al., 2014).

Preparation of pomace extracts. Commercial blackberry and blueberry pomaces (donated by Milne Fruit Products Inc., USA) were stored at -20°C and was used to extract phenolics according to the protocol previously described by Salaheen et al. (2014a) and Salaheen et al. (2014b). Spectrophotometric method 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 blackberry and blueberry pomace extracts at 1:1 v/v ratio.

Cell lines and culture conditions. Chicken macrophage, HD-11 (kindly provided by Dr. Uma S. Babu, Immunobiology Branch, Food and Drug Administration, Laurel, MD, USA) and human epithelial, HeLa (ATCC® CCL2™) cells were cultured at 37°C, standard condition (5% CO_2) in Dulbecco's Modified Eagle Medium (DMEM) (Corning cellgro, USA) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (Corning cellgro, USA) and 50 µg/mL of gentamycin (Lonza, USA). HD-11 and HeLa cells were seeded in 24-well culture plate (Greiner bio-one Inc., USA) at 2×10^5 cells/mL and then they were cultured at the standard condition as above up to 90% confluence. The semi-confluent cultures were washed with phosphate-buffered saline (PBS) three times and immersed in antibiotic free DMEM supplemented with 10% heat-inactivated FBS for cell adhesion and invasion assay (Salaheen et al., 2016a).

Growth inhibition assay. LC, LC^{+mcra} and CJ were grown on selective respective agar plates following the method described above. Different concentrations of BPEs (0.1 mg/ml GAE or 0.3 mg/ml GAE), LC, LC^{+mcra} bacterial suspension containing 10^7 colony forming unit (CFU) /mL in presence of BPEs (total 400 µL) was co-cultured with 400 µL of CJ bacterial suspension containing 10^6 CFU/mL in 3.2 mL of Bolton broth (Himedia, India) supplemented with 10% heat-inactivated FBS at 37°C under microaerophilic conditions. Serial dilutions were performed in PBS, followed by plating on Karmali agar for CJ at 24, 48, and 72 h time points.

Overnight liquid cultures of LC or LC^{+mcra} were centrifuged at 4000 × g for 20 min (Thermo Fisher Scientific Inc., USA) and cell free culture supernatants (CFCSs) were then filtered by sterile syringe 0.2 µm filter (VWR Inc., USA) (Tabashsum et al. 2017). Filtered CFCS from LC (CFCS-LC), and LC^{+mcra} (CFCS-LC^{+MCRA}) were collected and stored at 4°C. CJ bacterial cell suspension containing 10^6 CFU/mL of 400 µL was inoculated in separate culture tubes with different concentrations of BPEs, 10% of CFCS-LC or CFCS-LC^{+MCRA} with BPEs (0.1 mg/ml GAE or 0.3 mg/ml GAE) in Bolton broth with10% FBS respectively and incubated at 37°C under microaerophilic conditions. Serial dilutions were performed in PBS, followed by plating on Karmali agar at 24, 48 h and 72 h time points.

Cell adhesion and invasion assay. The HD-11 and HeLa cell monolayers grown in the wells of 24-well plate with 800 µL DMEM containing 10% FBS were pre-treated with 100 µL DMEM (control), different concentrations of BPEs (0.1 mg/mL GAE or 0.3 mg/mL GAE) or LC, LC^{+mcra}, CFCS-LC, CFCS-LC^{+MCRA} in presence of different

concentrations of BPEs (0.1 mg/mL GAE or 0.3 mg/mL GAE) for 1 h with each treatment in triplicates. After pre-treatment, 100 µL of CJ bacterial suspension with multiplicity of infection (MOI) of 10 (2×10^6 CFU/mL) were inoculated into each wells, incubated at 37°C in standard condition for 2 h, and after incubation, the cell monolayers were washed three times with DMEM with 10% FBS. The cells were then lysed by treating with 0.1% Triton X-100 for 15 min, then serially diluted and plated on specific agar plates for counting the adhesive bacterial cells. Cell invasive activity was measured by further 1 h incubation of the washed monolayers in DMEM containing 10% FBS and 100 µg/mL gentamicin followed by three times washing, Triton X-100 lysis, serial dilution and plating on specific agar plates (Peng et al., 2017).

Physiological properties of CJ treated with CFCSs. Physicochemical properties, e.g., injured cell ratio, auto-aggregation and, cell surface hydrophobicity were evaluated following the methodologies previously described by Ahn et al. (2014) with slight modification. In brief, bacterial cells were grown in absence (control) or presence of different concentrations of BPEs (0.1 mg/mL GAE or 0.3 mg/mL GAE) or CFCS-LC, CFCS-LC^{+MCRA} in presence of different concentrations of BPEs (0.1 mg/mL GAE or 0.3 mg/mL GAE) at 37°C for 18 h. The CJ cells were then harvested by centrifuging at 3000 ×g for 20 min followed by serially diluted and plated on Karmali agar and LB agar containing 5% FBS at 37°C, under microaerophilic conditions for injured cell rate assay, auto-aggregation and, hydrophobicity assays.

Quantitative RT-PCR assay. The cells were grown in the absence or presence of BPEs (0.1 mg/mL GAE or 0.3 mg/mL GAE) or/and CFCSs and RNA was extracted with ZR Bacterial RNA MiniPrep kit (Zymo Research Corp., USA). The synthesis of cDNA was performed according to the protocol of qScript cDNA SuperMix (Quanta Biosciences, USA).The custom-synthesized oligonucleotide primers (Erofins MWG Operon, USA) for *ciaB* (*Campyloabacter* invasion antigen), *cdtB* (Cytolethal distending toxin), *cadF* (*Campylobacter* adhesion fibronectin-binding protein), *flaA* (Flagellin A subunit synthesis), *flaB* (Flagellin B subunit synthesis) of CJ were used following methodology previously described by Tabashsum et al. (2017). The relative transcription levels of target genes were estimated by the comparative log fold change. The CT (Cycle threshold) values of target genes in treated bacterial cells were compared to those in untreated bacterial cells and normalized to the housekeeping gene (16s).

Statistical analysis. Data were analyzed by the Statistical Analysis System software (SAS Institute Inc., USA). The one-way analysis of variance (ANOVA) for each single time point followed by Tukey's test was used to evaluate the treatments and determine the significant differences among control and treatments based on significant level of 0.05.

Results

Effect of probiotic strains and/or BPE on the growth and survival of CJ. Zoonotic bacterial pathogen (CJ) and probiotic strains (LC or LC^{+mcra}) in presence of BPEs (0.1

mg/ml GAE or 0.3 mg/ml GAE) were co-cultured to determine the combined effect of these probiotic strains in combination with prebiotic like component, BPE on the growth and survival of CJ at various time points. The growth inhibition pattern of CJ is shown in Figure 3.1. The growth of CJ was reduced >1.8 logs during first 24 h in presence of both concentrations (0.1 mg/ml GAE or 0.3 mg/ml GAE) of BPEs. We also observed that both the probiotic strains, LC and LC^{+mcra} in presence of the BPEs (0.1 mg/ml GAE or 0.3 mg/ml GAE) reduced the growth of CJ in a similar pattern at a time dependent manner (Figure 3.1). Overall, LC^{+mcra} in presence of BPEs showed higher inhibitory effect than the wild-type probiotic strain, LC with BPEs. It was also observed that LC^{+mcra} in presence of high concentration of BPE (0.3 mg/ml GAE) was the most efficient in inhibiting the growth and survival of CJ.

In the similar experiment, both CFCS-LC and CFCS-LC^{+MCRA}, collected from overnight culture of LC and LC^{+mcra}, respectively, in presence of BPEs showed strong inhibitory effects on the growth of CJ, however CFCS-LC^{+MCRA} with high concentration of BPE was the most intensive throughout 72 h (Figure 3.1). During first 24 h, the growth of CJ was reduced 2.23 log CFU/ml in presence of BPE at a concentration of 0.1 mg/ml GAE with CFCS-LC and 4.0 log CFU/ml in presence of BPE at a concentration of 0.3 mg/ml GAE with CFCS-LC^{+MCRA}. After 48 h of incubation, growth of CJ was reduced >3.6 logs in the presence of BPE at low concentration (0.1 mg/ml GAE of BPE) with CFCS-LC, and >4.5 logs in presence of BPE at same concentration with CFCS-LC^{+MCRA}, respectively. At the same time point, CJ growth was inhibited >5.0 log CFU/ml in presence of BPE at high concentration (0.3 mg/ml GAE) with both CFCS-LC, CFCS-LC^{+MCRA} compared to

control group (only the growth medium). The most effective growth inhibition of CJ was observed by the treatment of BPE at a concentration of 0.3 mg/ml GAE with CFCS-LC^{+MCRA} (collected from LC+^{mcra}), after 72 h of incubation (Figure 3.1).

Alteration of adherence and invasion abilities of CJ to cultured cells. Adhesion to and invasion into pre-treated cultured cells in presence of BPE and/or probiotic strains or their CFCSs were assessed to evaluate their role in alteration of interaction of CJ with host cells. In presence of BPEs alone, both probiotic strains LC and LC^{+mcra} or their CFCSs, reduced the adhesion and invasion abilities of CJ to HD-11 and HeLa cells significantly (Figure 3.2).

It was observed that, the adhesion ability of CJ to HD-11 cells was also significantly reduced from a range of 0.46 log CFU/ml to 2.0 log CFU/ml when pre-treated with both concentrations of BPEs (0.1 mg/ml GAE or 0.3 mg/ml GAE) or either probiotic strains/their cell free culture supernatants with or without BPEs (Figure 3.2A). In the same assay, invasion efficacy of CJ into HD-11 cells was significantly reduced by 1.0 log CFU/ml in presence of both concentrations of BPEs (0.1 mg/ml GAE or 0.3 mg/ml GAE) or low concentration of BPE (0.1 mg/ml GAE) with either of the probiotic cells or CFCS collected from respective overnight culture of the probiotic strains. Further, invasion efficacy of CJ into HD-11was significantly reduced more than 1.0 log CFU/ml with the pre-treatment of high concentration of BPE (0.3 mg/ml GAE) in combination with either LC, or LC^{+mcra} or CFCS-LC or CFCS-LC^{+MCRA} (Figure 3.2B).

When the adhesion ability of CJ to HeLa cells was assessed, low concentration of BPE (0.1mg/ml GAE) reduced the adhesiveness by 0.18 log

CFU/ml. High concentration of BPE (0.3 mg/ml GAE) or the probiotic strains, LC/LC^{+mcra} or their cell free culture supernatants in presence of both concentrations of BPEs (0.1 mg/ml GAE or 0.3 mg/ml GAE) significantly reduced the adhesion ability of CJ by 1.0 log CFU/ml or more (Figure 3.2C). The invasion ability of CJ into HeLa cells was reduced by 0.41 log CFU/ml and 0.92 log CFU/ml, in presence of low or high concentrations of BPEs, respectively. Further probiotic strain, LC or its CFCS in presence of low concentration of BPE (0.1 mg/ml GAE) reduced the invasion ability by 0.94 log CFU/ml and 0.68 log CFU/ml, respectively. Other pre-treatments (LC or its CFCS in presence of high concentration of BPE or LC^{+mcra} or its CFCS in presence of both concentrations of BPEs) reduced the CJ invasion ability by more than 1.0 log CFU/ml into cultured HeLa cells (Figure 3.2D).

Alteration of physicochemical properties of CJ in the presence of CFCS and/or BPE. The physicochemical properties of CJ, specifically ratio of injured cells or auto-aggregation and hydrophobicity ability, were altered when pre-treated with BPEs or/and CFCSs obtained from overnight cultures of LC or LC^{+mcra} (Table 3.1). For example, we observed significantly increased percentage of injured CJ cells, when compared to control (only growth media) and CFCS-LC^{+MCRA} with high concentration of BPE (0.3 mg/ml GAE) was the most effective pre-treatment resulting in 37.07% injured CJ cell (Table 3.1). The auto-aggregation capacity of CJ was significantly decreased by the pre-treatments, while BPE (0.3 mg/ml GAE) in presence of CFCS-LC^{+MCRA} was the most effective (63.04% decrease) and BPE of 0.1 mg/ml GAE was the least effective (22.13% decrease) (Table 3.1). We also found that cell surface hydrophobicity of CJ was reduced significantly by high concentration of

BPE (0.3 mg/ml GAE) or CFCSs in presence of both concentrations of BPE (0.1 mg/ml GAE or 0.3 mg/ml GAE) but low concentration of BPE (0.1 mg/ml GAE) could reduce the cell surface hydrophobicity of CJ only numerically when compared to control group.

Effects of BPEs or/and CFCSs of probiotics on CJ virulent gene expression. In presence of different concentrations of BPEs with or without CFCSs, we evaluated the expression level of several virulence genes of CJ including *ciaB*, *cdtB*, *cadF*, *flaA* and *flaB* based on qPCR analysis (Figure 3.3). These genes were chosen as *ciaB*, *cadF*, *flaA* and *flaB*, are commonly known for their critical role in CJ colonization to host cells and *cdtB* is imperious in CJ toxin production in host cells (Fouts et al., 2005). The relative expression level of *ciaB* gene was significantly down-regulated >2 folds, >1.5 folds and >1.5 folds by BPE of 0.3 mg/ml GAE, BPE of 0.1 mg/ml GAE in presence of CFCS-LC^{+MCRA} and BPE of 0.3 mg/ml GAE in presence of CFCS-LC^{+MCRA}, respectively. The relative expression level of *cdtB* gene was also significantly down regulated more than 1.7 folds by BPE of 0.1 mg/ml GAE in presence of CFCS-LC^{+MCRA} and more than 1.3 folds by BPE of 0.3 mg/ml GAE in presence of CFCS-LC^{+MCRA}. The expression levels of *cadF* gene were down-regulated significantly by BPE of 0.3 mg/ml GAE and CFCS-LC^{+MCRA}. The relative expression level of *flaA* and *flaB* genes were up-regulated significantly in presence of different concentrations of BPEs with or without CFCSs from 3.4 folds to 8.35 folds. All other relative expression levels of the above mentioned genes were insignificant (Figure 3.3).

Discussions

In this study, we investigated the combined role of genetically modified *Lactobacillus* strain, LC+MCRA containing *mcra* gene, and bioactive phenolic compounds, BPEs extracted from blackberry and blueberry pomaces/byproducts, in growth, survival and metabolic activities of CJ as well as their effect on the interactions between host cells (HD-11 and HeLa) and CJ. The effectiveness of metabolites alone, in absence of live LC or LC^{+mcra}, the cultural supernatants produced by both *L. casei* strains, wild-type (LC) or mutant (LC^{+mcra}) was tested for their role in inhibition of CJ growth and survival as well as in alteration of CJ pathogenesis in presence of sub-lethal concentration of BPE.

Previously, we reported that CLA overproducing *L. casei* strain, LC^{+mcra} or its supernatant (CFCS-LC^{+MCRA}) or BPE alone can inhibit the growth and survival of CJ (Salaheen et al., 2014; Tabashsum et al., 2017). In this study, we aimed to combine the effects of conjugated linoleic over-producing strain, $LC+^{mcra}$ and berry phenolic extracts, BPEs for intensifying their role in inhibiting the growth of CJ in co-culture conditions with LC or LC^{+mcra} in presence of BPEs. Our finding indicates that BPE and LC^{+mcra} in combination are able to inhibit growth; reduce the adherence and invasion abilities of CJ in chicken and human origin cultured cells more aggressively.

Possible reasons behind this inhibitory effect of LC or LC^{+mcra} against CJ growth in co-culture condition may include, production or over-production of anti-pathogenic metabolites particularly CLA with other SCFAs and nutritional competition (Peng et al., 2016; Peng et al., 2015). The mechanism behind growth inhibition by the BPEs possibly include, phenolics damaging bacterial cell membrane,

and its role in inhibiting extracellular microbial enzyme secretion, obstruction on microbial metabolism and also increased growth of probiotic strain in presence of phenolic compounds may out-compete CJ in the growth media (Salaheen et al., 2014; Salaheen et al., 2014; Yang et al., 2014). The BPEs in presence of CFCSs from overnight culture of the probiotic strains were also able to reduce the growth of CJ and the explanation behind this inhibition may include acidic conditions generated by the metabolites from the probiotics (Adams et al., 1988; Gyawali et al., 2012), hydrogen peroxide and antimicrobial polypeptides produced by the probiotics (Atassi et al., 2010; Wannun et al., 2014; Xu et al., 2008). Anti-pathogenic traits of phenolic compounds and CLA along with other metabolites produced by the probiotic strains had enhanced efficacy when combined, as combined effect shows better performance than previous individual effect studies in our laboratory (Peng et al., 2015; Peng et al., 2015a; Salaheen et al., 2014; Salaheen et al., 2014a; Salaheen et al., 2014b; Salaheen et al., 2016; Tabashsum et al., 2017; Yang et al., 2014).

Attachment is one of the prerequisites for bacterial colonization to host cells followed by invasiveness and considered to be an important virulence property (Adegbola, 1988; Edwards et al., 1998). In this study, adhesion efficacy of CJ was reduced significantly in cultured chicken fibroblast cells, chicken macrophage cells, and mammalian epithelial cells in co-culture conditions or by the metabolites obtained from overnight culture with BPEs or only BPEs without probiotic strains/ their metabolites when compared to control group (growth media without any supplements). This finding indicates that in the presence of this genetically modified probiotic strain with feed or water supplement with BPE may able to reduce the

colonization of CJ in poultry. It was previously reported that similar carbohydrate-binding specific proteins are displayed on *Lactobacillus* spp. surface which may involve in decreasing the adhesive and invasiveness of enteric bacterial pathogens by pre-occupying the surface binding receptors on host cells (Neeser et al., 2000; Salaheen et al., 2018). Further, researchers also reported that CFCSs, collected from various strains of *Lactobacillus* spp. also restricted the adhesion to and invasion into host cells of different enteric bacterial pathogens in agreement to our findings (Bendali et al., 2014; Campana et al., 2012).

According to the injured cell ratio assay, higher percentage of injured CJ cells (viable) were observed in the presence of BPEs and CFCS-LC^{+MCRA}, collected from LC^{+mcra}. This amplification of effect from high concentration of BPEs or higher concentrations of CLA in CFCS-LC^{+MCRA} produced by the LC^{+mcra} strain also supported their synergistic effect. This finding was also supported by several previous reports (Salaheen et al., 2014; Salaheen et al., 2014a; Salaheen et al., 2016; Tabashsum et al., 2017). Bacterial cell surface hydrophobicity and auto-aggregation capability are believed to be positively correlated with its host cell association activities (Lorite et al., 2013; Oliveira et al., 2007; Saran et al., 2012) and auto-aggregation capacity serves as virulence marker of many Gram-negative enteric bacterial pathogens (Chiang et al., 1995; Menozzi et al., 1994). Treatments like CFCSs from LC/LC^{+mcra} with BPEs result in alteration of mechanical and physicochemical properties (decreased hydrophobicity and auto-aggregation) may have impact on the reduction of invasiveness in CJ, also supported by previous studies (Salaheen et al., 2014; Yang et al., 2014).

We further investigated the relative expression levels of several virulence associated genes of CJ when cultured with different concentrations of BPEs with or without the presence of CFCSs/metabolites obtained from both the probiotic strains. The expression of several virulence genes were altered particularly *ciaB, cdtB, cadF* were down-regulated and *flaA*, *flaB* were upregulated in the presence of the treatments in agreement with the findings of different previous studies (Ding et al., 2005; Mundi et al., 2013; Salaheen et al., 2014; Tabashsum et al., 2017). The mechanism behind the decreased or increased expression levels of the genes are still partially understood but as the genes play important role in colonization and toxin production of CJ, these down regulated relative expressions of the genes are considered to be vital in control of CJ infection.

Conclusion

In conclusion, as probiotic strains specifically LC^{+mcra} in presence of sub-lethal concentration of BPE exhibited distinctive effects on CJ pathogenesis, the combination of bioactive probiotics and anti-oxidative and anti-inflammatory natural component complemented each other's ability to eliminate or alter pathogenic characteristics with elevated bidirectional activities in both accelerating growth of Lactobacillus strains and exclusion/inhibition of CJ. This study also confirms the promising functions of the combination of the genetically engineered probiotic, LC+MCRA and bioactive BPEs, in altering physicochemical properties, disruption of pathogen-cell interactions, and virulence genes suppression. These traits might be

applied in farm animals to reduce cross contamination of zoonotic pathogens and to improve quality of poultry products.

List of Tables and Figures

Table 3.1: Physicochemical properties of CJ treated with CFCSs/BPEs

Treatment	Injured Cell (%)	Auto-aggregation (%)	Hydrophobicity (%)
Control	19.71±1.07*,[a]	49.28±2.36[a]	14.50±2.98[a]
0.1 mg/ml GAE	24.04±0.87[b]	38.37±6.7[b]	10.90±1.36[ab]
0.3 mg/ml GAE	27.54±1.12[c]	30.80±1.43[b]	8.2±1.45[b]
0.1 mg/ml GAE + CFCS-LC	25.99±0.77[c]	25.41±1.31[c]	9.0±0.88[b]
0.3 mg/ml GAE + CFCS-LC	29.80±0.66[d]	22.06±2.0[d]	7.88±1.83[b]
0.1 mg/ml GAE + CFCS-LC^{+MCRA}	32.32±1.73[e]	21.62±1.41[d]	6.92±1.41[b]
0.3 mg/ml GAE + CFCS-LC^{+MCRA}	37.08±3.55[e]	18.21±1.19[e]	5.96±1.35[b]

*Values indicate Mean ± Standard deviation and means with different letters (a through e) within the same column are different when compared with control and among the treatments at $p<0.05$.

Figure 3.1: Growth pattern of CJ at 24, 48, and 72 h. The sign '+' indicate the combination of the treatments. Error bars indicate standard deviation from 3 parallel trails. Different letters (a through i) at each time point indicate the significant growth reduction when compared with single culture as a control and among the treatments at $p < 0.05$.

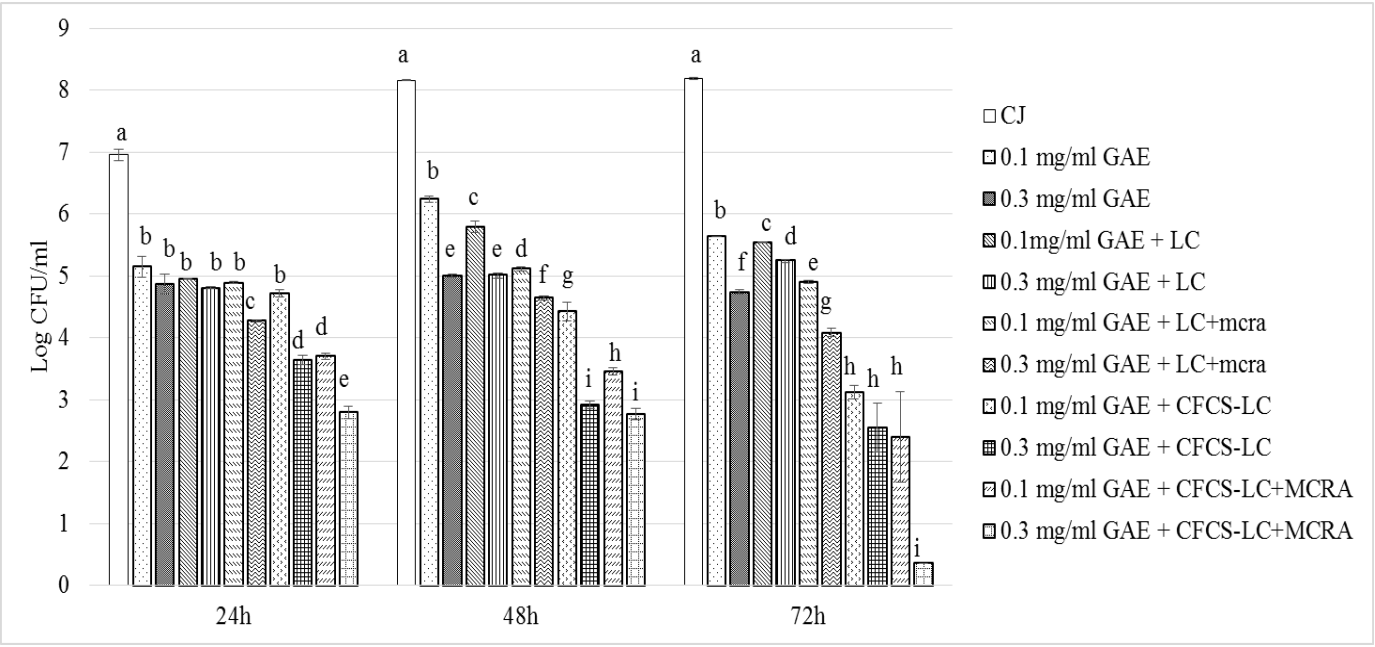
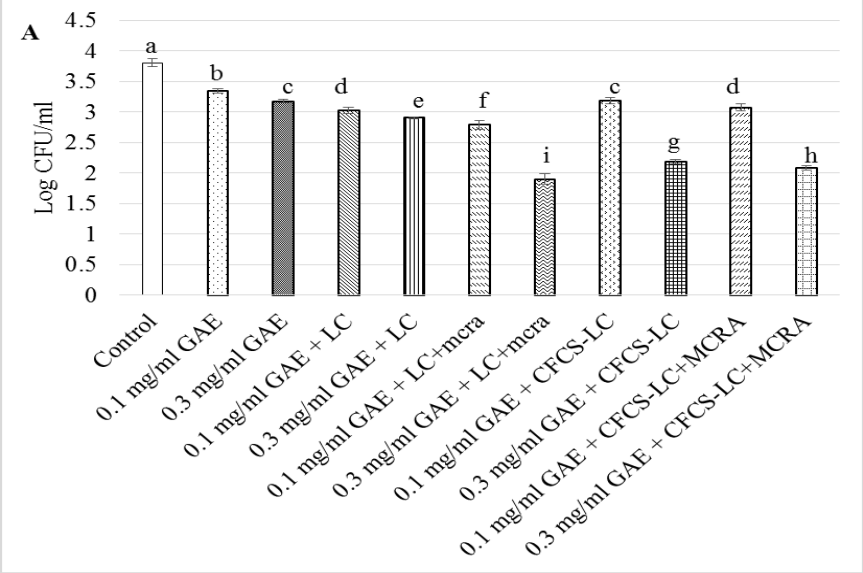
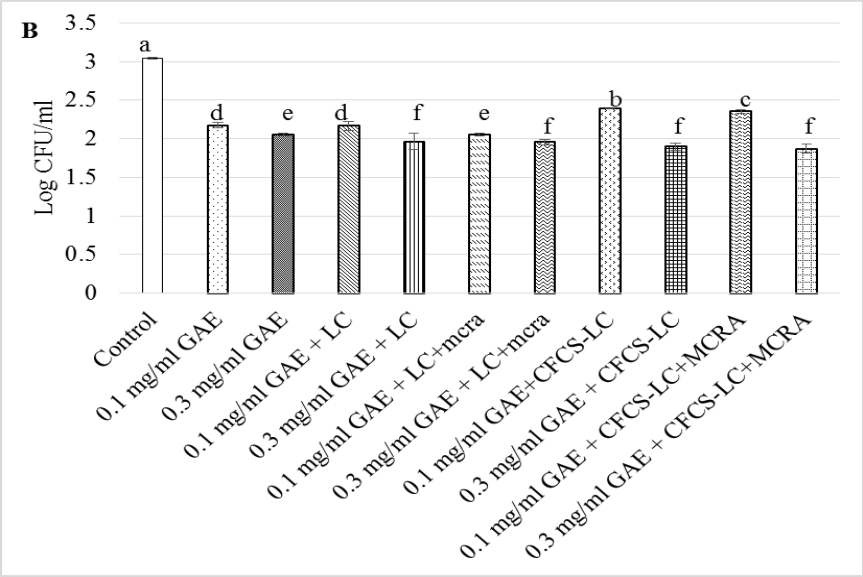


Figure 3.2: Cell adhesion levels of CJ to HD-11 cells with pre-treatments of BPEs (0.1 mg/ml GAE or 0.3 mg/ml GAE) or live probiotic strains LC, LC^{+mcra} or CFCSs (CFCS-LC or CFCS-LC^{+MCRA}) collected from overnight culture of LC or LC^{+mcra} (A), cell invasion levels of CJ into HD-11 cells with pre-treatments of BPEs (0.1 mg/ml GAE or 0.3 mg/ml GAE) or live probiotic strains LC, LC^{+mcra} or CFCSs (CFCS-LC or CFCS-LC^{+MCRA}) collected from overnight culture of LC or LC^{+mcra} (B), cell adhesion levels of CJ to HeLa cells with pre-treatments of BPEs (0.1 mg/ml GAE or 0.3 mg/ml GAE) or live probiotic strains LC, LC^{+mcra} or CFCSs (CFCS-LC or CFCS-LC^{+MCRA}) collected from overnight culture of LC or LC^{+mcra} (C), cell invasion levels of CJ into HeLa cells with pre-treatments of BPEs (0.1 mg/ml GAE or 0.3 mg/ml GAE) or live probiotic strains LC, LC^{+mcra} or CFCSs (CFCS-LC or CFCS-LC^{+MCRA}) collected from overnight culture of LC or LC^{+mcra} (D). The sign '+' indicate the combination of the treatments. Error bars indicate standard deviation from 3 parallel trails. Bars with different letters (a through i) are significantly different when compared with control and among the treatments at $p < 0.05$.


A
Log CFU/ml
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
a
b
c
d
e
f
i
c
g
d
h
Control
0.1 mg/ml GAE
0.3 mg/ml GAE
0.1 mg/ml GAE + LC
0.3 mg/ml GAE + LC
0.1 mg/ml GAE + LC+mcra
0.3 mg/ml GAE + LC+mcra
0.1 mg/ml GAE + CFCS-LC
0.3 mg/ml GAE + CFCS-LC
0.1 mg/ml GAE + CFCS-LC+MCRA
0.3 mg/ml GAE + CFCS-LC+MCRA


B
Log CFU/ml
3.5
3
2.5
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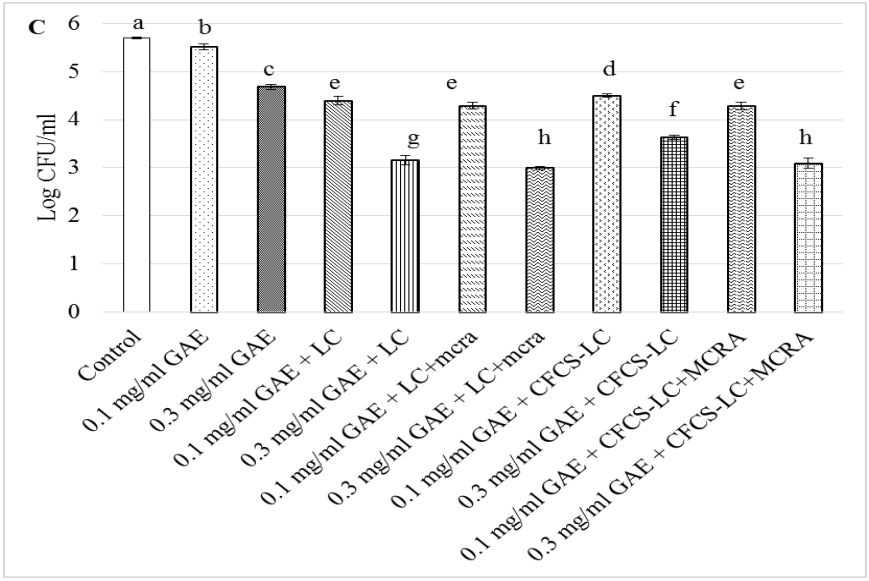
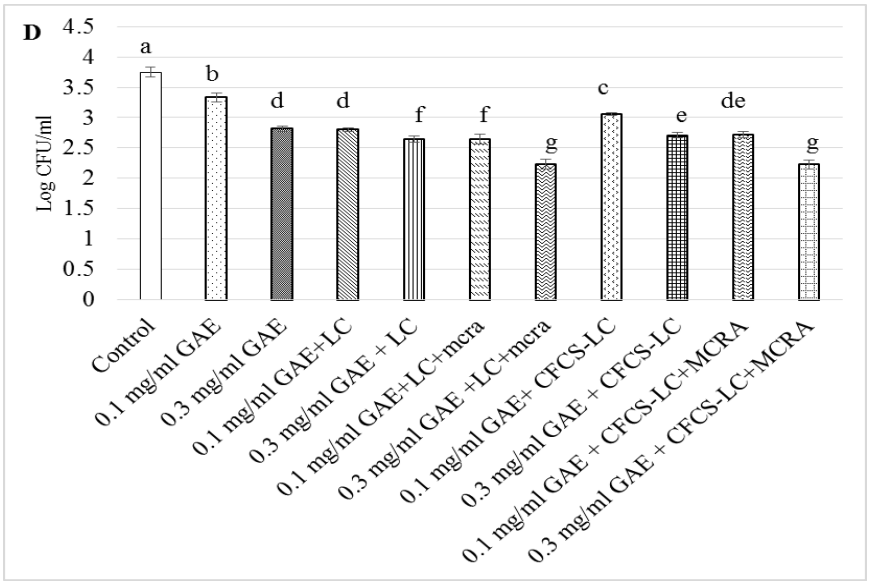
1.5
1
0.5
0
a
d
e
d
f
e
f
b
f
c
f
Control
0.1 mg/ml GAE
0.3 mg/ml GAE
0.1 mg/ml GAE + LC
0.3 mg/ml GAE + LC
0.1 mg/ml GAE + LC+mcra
0.3 mg/ml GAE + LC+mcra
0.1 mg/ml GAE+CFCS-LC
0.3 mg/ml GAE + CFCS-LC
0.1 mg/ml GAE + CFCS-LC+MCRA
0.3 mg/ml GAE + CFCS-LC+MCRA


C
Log CFU/ml
0
1
2
3
4
5
6
a
b
c
e
g
e
h
d
f
e
h
Control
0.1 mg/ml GAE
0.3 mg/ml GAE
0.1 mg/ml GAE + LC
0.3 mg/ml GAE + LC
0.1 mg/ml GAE + LC+mcra
0.3 mg/ml GAE + LC+mcra
0.1 mg/ml GAE + CFCS-LC
0.3 mg/ml GAE + CFCS-LC
0.1 mg/ml GAE + CFCS-LC+MCRA
0.3 mg/ml GAE + CFCS-LC+MCRA


D
Log CFU/ml
0
0.5
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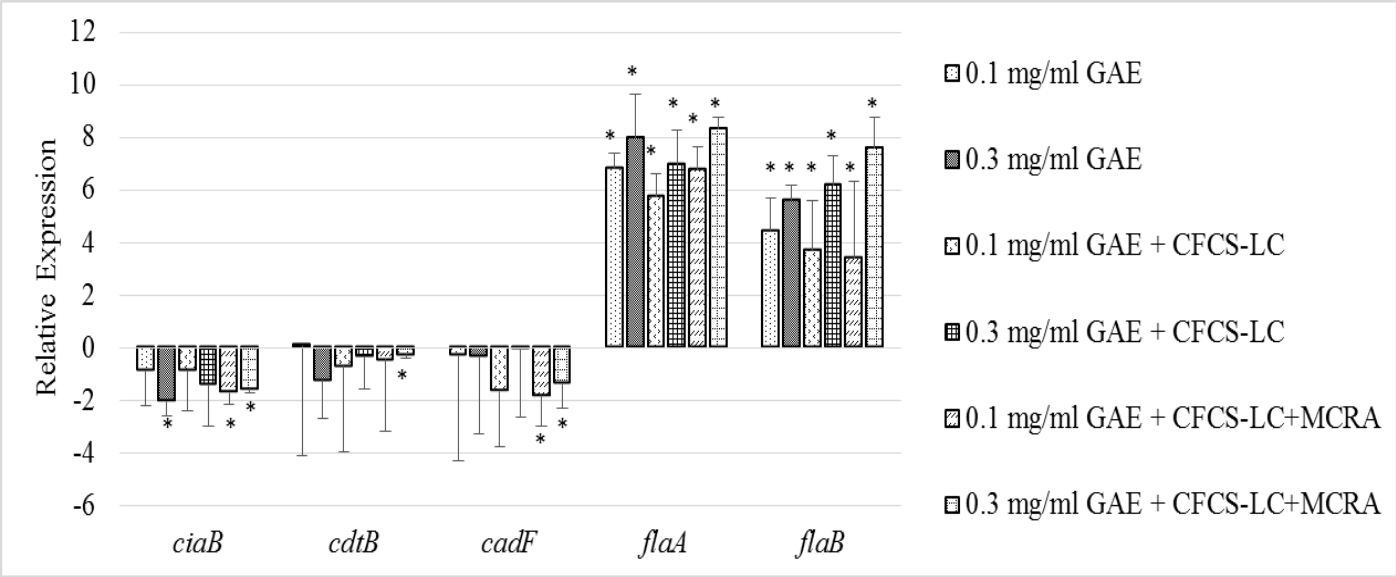
1.5
2
2.5
3
3.5
4
4.5
a
b
d
d
f
f
g
c
e
de
g
Control
0.1 mg/ml GAE
0.3 mg/ml GAE
0.1 mg/ml GAE+LC
0.3 mg/ml GAE + LC
0.1 mg/ml GAE+LC+mcra
0.3 mg/ml GAE +LC+mcra
0.1 mg/ml GAE+ CFCS-LC
0.3 mg/ml GAE + CFCS-LC
0.1 mg/ml GAE + CFCS-LC+MCRA
0.3 mg/ml GAE + CFCS-LC+MCRA

Figure 3.3: Relative expression of different virulence genes of CJ treated with BPEs alone or CFCSs from LC/LC^{+mcra} in presence of BPEs (0.1 mg/mL GAE or 0.3 mg/mL GAE). The sign '+' indicate the combination of the treatments. Error bars indicate standard deviation from 3 parallel trails. Bars with asterisk (*) are significantly different at $p < 0.05$.



Overall conclusions

LC^{+mcra} inhibits the growth and survival of CJ *in vitro* and alter the interaction of CJ to cultured human intestinal and chicken cells. The metabolic produced by LC^{+mcra} in cell-cultured media modulates the physicochemical properties and gene expression of CJ associated to virulence properties including infectious activities and host damages. In combination of BPE with LC^{+mcra}, growth, and survival ability was reduced sharply and alternation of virulence properties of CJ was amplified. These findings suggest that CLA overproducing LC^{+mcra} has the potential to be an alternative in control of CJ colonization in different reservoir and infection along with other beneficial attributes of LC and BPEs as an water or feed supplement might enhance effect of LC^{+mcra}. Therefore, BPEs and LC^{+mcra} in combination may able to stimulate the growth of beneficial microbiota and its metabolics, and prevent CJ colonization in poultry and reduce cross-contamination in poultry products and control poultry-borne campylobacteriosis in human.

Future directions

1. *In vivo* examination of the effectiveness of LC^{+mcra} and BPE in animal model.
2. Analyze the mode of action of LC^{+mcra} and BPE.
3. Metagenomics and metabolomics study to reveal the effect on host.
4. Delivery method in chickens at farm level.
5. Large scale application at farm level and extensive cost-benefit analysis.

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