

ABSTRACT

Title of Dissertation:

STIMULATION OF GROWTH AND
METABOLITES PRODUCTION OF
LACTOBACILLUS IN CONTROL OF
ENTERIC BACTERIAL PATHOGEN
INFECTION AND IMPROVING GUT
HEALTH

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Doctor of Philosophy
2018

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Foodborne enteric diseases cause millions of illness and thousands of deaths annually in the United States. Major enteric bacterial pathogens include *Salmonella*, enterohemorrhagic *Escherichia coli* O157:H7 (EHEC), *Campylobacter*, *Listeria*, *Shigella*, *Vibrio*, and *Yersinia* which account for more than 90% cases of culture-confirmed infections. Among these causative agents, *Salmonella enterica* is responsible for the highest rate of hospitalization and EHEC has the lowest infectious dose. Their pathogenesis involves numerous virulent factors whereas their colonization and invasion on host gut intestine mainly depend on the type III secretion system. The prevention of foodborne enteric diseases is of great concern to public health

professionals, farmers, and food producers. Due to the increased public health concern about antibiotic-resistance dissemination, alternative strategies such as pro-commensal approach by applying probiotics, prebiotics, and combination of both (synbiotics) are of interests for prevention and therapy of foodborne enteric diseases. In this study, we both *in vitro* and *in vivo* evaluated the preventive capabilities of *Lactobacillus* against enteric pathogenic bacterial colonization and infection. Functional food cocoa and peanut containing prebiotic-like ingredients selectively promoted the growth of beneficial bacteria and stimulated the production of bio-active metabolites especially conjugated linoleic acids in *Lactobacillus*. We also detected the synergistic effects of *Lactobacillus* and cocoa/peanut on competitive exclusion of *S. Typhimurium* and EHEC, alteration on physicochemical properties, disruption of host-pathogen interactions, and down-regulation on virulence gene expressions. Furthermore, with homologous recombination, we overexpressed myosin cross-reactive antigen gene encoding linoleate isomerase in *L. casei* and improved the efficiency in their linoleic acids production as well as the gut intestinal adherence and colonization. By applying genetically engineered LC-CLA, *S. Typhimurium* and EHEC were much effectively controlled and restricted from all aspects *in vitro* mentioned before. Additionally, the *in vivo* pre-administration of LC-CLA reduced *S. Typhimurium* gut intestinal colonization/infection in a significant level and induced anti-inflammatory effects, which benefitted the overall mice gut health. Our findings established a baseline upon which self-promoting probiotic independent from prebiotic in prevention or treatment against enteric diseases can be explored.

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by

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Dissertation 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
Doctor of Philosophy
2018

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Preface

This dissertation is original and independent work by the author Mengfei Peng under the supervision of Dr. Debabrata Biswas.

Dedication

To my parents, who taught me the value of honesty and integrity

To my teachers, who advised me on hard-working and persistence

To my special, who always support me, from the beginning to the end

Acknowledgements

I would like to express my sincere gratitude to my advisor and mentor, Dr. Debabrata Biswas, for his patient guidance and constant support. His motivation and immense knowledge in research helped me the most in my Ph.D. study.

Besides my advisor, I would also like to thank the rest of my Ph.D. dissertation committee members for their constructive criticism and precious instructions, plus for the challenging questions incited me to future research from a variety of perspectives.

Finally, I thank all the supports from my dear family and friends.

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Scholarly Articles

Findings and reviews from this dissertation have been published in the following peer-reviewed articles:

1. **Peng, M.**, Aryal, U., Cooper, B., and Biswas, D. (2015). Metabolites produced during the growth of probiotics in cocoa supplementation and the limited role of cocoa in host-enteric bacterial pathogen interactions. *Food control*, 53, 124-133.
2. **Peng, M.**, Bitsko, E., and Biswas, D. (2015). Functional properties of peanut fractions on the growth of probiotics and foodborne bacterial pathogens. *Food Science*, 80(3), M635-M641.
3. **Peng, M.**, Reichmann, G., and Biswas, D. (2015). *Lactobacillus casei* and its byproducts alter the virulence factors of foodborne bacterial pathogens. *Functional Foods*, 15, 418-428.
4. **Peng, M.** and Biswas, D. (2016). Short chain and polyunsaturated fatty acids in host gut health and foodborne bacterial pathogen inhibition. *Critical Review of Food Science and Nutrition*, 57(18): 3987-4002.
5. **Peng, M.**, Zhao, X., and Biswas, D. (2017). Polyphenols and tri-terpenoids from *Olea europaea L.* in alleviation of enteric pathogen infections through limiting bacterial virulence and attenuating inflammation. *Functional Foods*, 36: 132-143.

List of Abbreviations

5-FU, 5-fluorouracil
AAD, antibiotic-associated diarrhea
A/E, attaching and effacing
CFCS, cell free culture supernatant
CFU, colony forming unit
CLA, conjugated linoleic acid
EHEC, enterohemorrhagic *Escherichia coli* O157:H7
FBS, fetal bovine serum
GAE, gallic acid equivalent
GI, gastro-intestinal
HUS, hemolytic uremic syndrome
IBS, irritable bowel syndrome
LAB, lactic acid bacteria
LEE, locus for enterocyte effacement
Ler, locus for enterocyte effacement-encoded regulator protein
LI, linoleate isomerase
MCRA, myosin cross-reactive antigen
MIC, minimum inhibitory concentration
MOI, multiplicity of infection
OD, optical density
PBS, phosphate buffer saline
PUFA, polyunsaturated fatty acid
SCFA, short chain fatty acid
T3SS, Type III secretion system
T_h, T, helper
Tir, translocated intimin receptor
UC, ulcerative colitis

Chapter 1: Literature review

Foodborne enteric bacterial pathogens and diseases

Foodborne enteric diseases are estimated to be responsible for 47.8 million people cases of illness, 127,839 hospitalizations, 3,037 deaths, and \$77.7 billion economic burden each year (Hoffmann, Batz, & Morris, 2012) in the United States (US). Prevention of foodborne diseases is of great concern to public health professionals, farmers, as well as food producers. However, to further deteriorate the current situation, the commonly used antibiotics for control and treatment of zoonotic bacterial pathogen infections have been recorded to worsen the niche of beneficial gut microflora (Andersson & Hughes, 2010), and the efficacy of these antibiotics is compromised since numerous bacterial pathogens are evolving into multi-drug-resistant strains (DeWaal, Roberts, & Plunkett, 2013).

Major foodborne bacterial pathogens in the US include *Salmonella*, enterohemorrhagic *Escherichia coli* O157:H7 (EHEC), *Campylobacter*, *Listeria monocytogenes*, *Shigella*, *Vibrio*, and *Yersinia* (D. Adams et al., 2015, 2017; D. A. Adams et al., 2016). Common diseases caused by these foodborne bacterial pathogens are summarized in Table 1-1. Most of the foodborne pathogens inhabit at gastrointestinal (GI) tract of food-producing animals as normal microflora and in the farm

and processing environment, which allows them contaminate meat and milk products during slaughter and milking or contaminate produce vegetables if the soil was fertilized with improperly composted animal manure (McEwen and Fedorka-Cray, 2002). From both the pre-harvest (farm level) food safety and transmission through food products in the US, *Salmonella* especially *Salmonella enterica serovars* Typhimurium and Enteritidis, EHEC, *Campylobacter jejuni*, and *L. monocytogenes* are the most important foodborne pathogens with severe public health concerns (Bryan, 2002; Oliver, Patel, Callaway, & Torrence, 2009; Mengfei Peng et al., 2016; S. Salaheen, Peng, & Biswas, 2016). Farm animals including chickens, turkeys, swine, cattle, and sheep as well as wild birds around farm environments are the principle reservoirs and sources of these foodborne bacterial pathogens (Oliver et al., 2009). Further specifically, EHEC usually shed and harbor in dairy and beef cattle (Meng, LeJeune, Zhao, & Doyle, 2013) which animals can remain asymptotically due to the lack of vascular expression of Gb3 (Pruimboom-Brees et al., 2000). *S. Typhimurium*, *C. jejuni*, and *L. monocytogenes*, are normally carried by poultry and swine as well as found in the associated wild environments (D'Aoust & Maurer, 2007; Nachamkin, 2002).

Salmonella

Salmonella (Gram-negative) is a genus of rod-shaped bacteria belongs to Enterobacteriaceae family which is responsible for nearly half of all foodborne infections annually in the US (K. J. Cummings et al., 2012). *Salmonella* has two species - *Salmonella bongori* and *Salmonella enterica*, but over 2500 serovars have been isolated worldwide, in which *S. Typhimurium* and *S. Enteritidis* are the most frequently isolated strains associated with human diseases. According to the report from Scallan *et al.* (2011), *Salmonella enterica* alone is responsible for approximate 1.2 million illnesses, 20 thousand hospitalizations, and 400 deaths each year in the US. Moreover, it was estimated that annual *Salmonella* infections cause \$4.4 billion economic lose including medical costs and productivity lost (Scharff, 2012). Factors including virulence plasmids, flagella, fimbriae, endotoxins (lipopolysaccharide), and exotoxins all contribute to *Salmonella* infections (Van Asten & Van Dijk, 2005) which induce salmonellosis with manifestations including diarrhea, abdominal cramps, vomiting, dehydration, fever, anorexia, and malaise after 12 to 72 hours of infection, while its invasive infections might induce meningitis, osteitis, osteomyelitis, hypovolemic shock, septic shock, and even death for infants, elderly, and immunocompromised individuals (Arshad et al., 2008; T. F. Jones et al., 2008). Post-infectious irritable bowel syndrome (IBS) is one of the chronic disorders coming with salmonellosis in the form of

constipation and diarrhea alteration and accompanied by abdominal pain (Mearin et al., 2005). IBS could further induce chronic mucosal immunological dysregulation accompanied with intestinal permeability and motility alterations leading to persistent intestinal symptoms (DuPont, 2008). Reiter's syndrome is another post-infection sequelae of *Salmonella*, in which, a certain percentage of infected individuals could develop reactive and even chronic arthritis as a result of attacking on cartilaginous tissues in the joints by immune system in response to *Salmonella* bacteria in short or long term period (Townes, 2010).

Enterohemorrhagic Escherichia coli O157:H7

EHEC (Gram-negative) is an emerging foodborne pathogen associated with severe form of human diseases including enterohemorrhagic diarrhea and hemolytic uremic syndrome (HUS) (Nguyen & Sperandio, 2012). According to Teunis *et al.* (2008), EHEC has an extremely low infectious dose of fewer than 100 colony forming units (CFUs), some cases ingesting as few as 10 of this pathogen could cause individual illness. As a result of infection by a prophage containing the structural coding for shiga toxin, the non-producing strain evolved and obtained the ability to express shiga-like toxins which induce antibiotic treatment in EHEC-induced diarrheal illness has been shown to increase the risk of severe HUS by 17-fold due to the induced bacterial

membrane injury which favors the acute release of shiga-like toxins (Safdar, Said, Gangnon, & Maki, 2002). Other factors such as acid resistance gene systems, lipopolysaccharides, enterotoxins, fimbriae, and flagella also play important roles in EHEC infection (Nguyen & Sperandio, 2012). Except for symptoms such as abdominal pain, diarrhea, vomiting, and fever, in some cases particularly young children and elderly, EHEC infection can lead to life-threatening HUS. Characterized by acute kidney failure, hemolytic anemia, and thrombocytopenia, HUS was estimated to have a fatality rate ranging from 3 to 5%, and neurological complications like seizure, stroke, and coma caused by HUS can also happen in 25% of the HUS patients (Nguyen & Sperandio, 2012). Even though recovering from HUS, long-term renal complications including hypertension, renal insufficiency, and end-stage renal failure can occur in approximately 50% of the previous patients (Oliver et al., 2009). Furthermore, EHEC is also the predominant causative agent for ulcerative colitis (UC), which is the chronic inflammation in human large intestine (Jess et al., 2011; Ternhag, Törner, Svensson, Ekdahl, & Giesecke, 2008). UC is characterized by severe abdominal cramps, bloody diarrhea, immune abnormality, and intestinal barrier functional defection (Sasaki & Klapproth, 2012), and is also considered as the pre-condition of colorectal cancer (Rhodes & Campbell, 2002; J. Yang et al., 2006).

Major virulence factors and pathogenesis of foodborne pathogen infections

Foodborne bacterial pathogens once ingested with food or water manages to survive and pass through the acidic stomach environment and move to intestine. They usually colonize the gut intestine especially large intestine and cross the mucosal barrier through active invasion process or translocation with phagocytic M cells (A. J. Müller et al., 2012). Eventually, some of them randomly taken up by different phagocytes (macrophages, dendritic cells, and poly-morphonuclear cells) could disseminate to extra-intestinal sites such as lymph nodes, liver, kidney, spleen, and brain, causing inflammation and local tissue damage (Bhunia, 2008). The host cell invasion pathway for invasive bacterial pathogen acts as the essential role in foodborne infections and the design of therapeutic agents for preventing/blocking invasion ability is one of major considerations. Type III secretion system (T3SS) is the most common system play important role in several invasive bacterial pathogens especially *Salmonella* and EHEC for bacterial invasion of host cells.

Pathogenicity islands, conferring a virulence phenotype, are genetic elements within the core bacterial genome (Shames, Auweter, & Finlay, 2009) involve in invasiveness. The *Salmonella* pathogenicity islands 1 carries critical genes required for the biosynthesis of functional T3SS apparatus, including multiple regulatory proteins, effector proteins, and their chaperones (Galán & Collmer, 1999). Once locating the

lumen of small intestine and sensing the gut environment, T3SS-1 (encoded by SPI-1) genes could be expressed and subsequent secretion apparatus could be assembled on the surface of bacterial membrane (Lara-Tejero & Galán, 2009). The host cell invasion is initiated by pathogen binding which activates the assembly and insertion of translocons (molecular syringe) into the host cell membrane through its affinity for cholesterol (Hayward et al., 2005). The needle complex is regulated by more than 20 virulence genes including *prgI* (needle), *invG* (outer rings), *prgK* (neck), *prgH* (inner rings), and *invA/invC/spaP/spaQ/spaR/spaS* (inner membrane components). The molecular syringe complex is precisely coordinated to ensure the secretion is in a coherent order with translocases (SipB and SipD) secreted earlier to assist in translocation of other effector proteins (Lara-Tejero, Kato, Wagner, Liu, & Galán, 2011). At least 15 effector proteins were found in *Salmonella* to be translocated into host cells by T3SS-1 and result in followed bacterial entry (McGhie, Brawn, Hume, Humphreys, & Koronakis, 2009). Among these effector proteins, 5 major effectors (SopB, SopE, SopE2, SipA, and SipC) in *Salmonella*, each with the capability in manipulation of the cytoskeletal machinery within the host cells, are known to drive bacterial engulfment. SopB (inositol phosphatase), SopE (guanine exchange factors), and SopE2 (guanine exchange factors) target the RhoGTPase and activate Rho family members Cdc42 and Rac, inducing the activation of N-WASP- and Scar/WAVE-

Arp2/3 complexes whose function is triggering the actin remodeling/polymerization (J. C. Patel & Galán, 2006; Zhou, Chen, Hernandez, Shears, & Galán, 2001). SipA and SipC engage in actin and control/localize actin polymerization at the bacterial attachment site. SipC with distinct N- and C- domains is able to nucleate F-actin and promote its bundling (Myeni & Zhou, 2010). SipA on the other hand, could induce invasion-competent ruffles either indirectly by stimulating SipC activities or directly by antagonizing depolymerizing factors and stabilizing F-actin (McGhie, Hayward, & Koronakis, 2004; Zhou, Mooseker, & Galán, 1999). It is noteworthy that these effectors mentioned above functioning in T3SS-1-dependent *Salmonella* entry also have profound effects on later infection processes, which include membrane trafficking, disruption of tight junctions and intestinal barrier integrity, host cell apoptosis, antigen presentation cytokine, and chemokine production (Boyle, Brown, & Finlay, 2006; Santos et al., 2009). After bacterial entry, effector protein SopB also plays a role in *Salmonella*-containing vacuole biogenesis and packaging, which prevents *Salmonella* from directly fusing with lysosomes (Dove et al., 2002; Knodler, Winfree, Drecktrah, Ireland, & Steele-Mortimer, 2009; J. C. Patel, Hueffer, Lam, & Galán, 2009). As the infection progresses, *Salmonella* also takes use of T3SS-2 encoded by the SPI-2 in secreting and delivering of additional effector proteins (such as SseF, SseG, and SseJ)

through the *Salmonella*-containing vacuole membrane to ensure intracellular bacterial survival and replication (Figueira & Holden, 2012; Malik-Kale et al., 2011).

T3SS also assist EHEC in forming attaching and effacing (A/E) lesions on the mucosal epithelium once passing through the acidic barrier and allows its colonization on gut intestine. The virulence genes required for T3SS, regulators, chaperones, and effector proteins related to A/E lesions formation are encoded within the chromosomal pathogenicity island known as the locus for enterocyte effacement (LEE) (Elliott et al., 1998). The LEE-encoded regulator protein (Ler) acts as the master transcription factor of the entire pathogenicity island and regulates the expression of the LEE (D. Müller et al., 2009). Following the initial attachment of EHEC to intestinal cell, several LEE encoded proteins including EspA, EspB, and EspD are secreted into the host cells by T3SS. EspA forms filamentous appendages between EHEC and host cell which acts as the translocation machinery. EspB and EspD once translocated into host cell rapidly alter the host cell's signal transduction pathways (Nguyen & Sperandio, 2012). In order to move on to the next stage of attachment and effacement, intimate attachment between EHEC and host cell is required. To accomplish the intimate attachment, EHEC manage to translocate its own receptor protein translocated intimin receptor (Tir), encoded by *tir* gene, to the host cell and express on the host cell membrane. While simultaneously, EHEC also expresses intimin, encoded by *eae* gene, which is an outer

membrane protein for tightly binding with the expressed Tir on host cell surface (Deibel, Krämer, Chakraborty, & Ebel, 1998). Following this tight binding, *E. coli* secreted protein F-like protein from prophage U, a non-LEE coded effector protein, is secreted and cooperates with Tir in recruiting actin nucleation/cytoskeleton reorganization-promoting factors and regulators, and eventually forms actin pedestals in host cell beneath the attached EHEC for disruption the overall mucosal barrier and intestinal cell functions (Campellone, Robbins, & Leong, 2004; Weiss et al., 2009).

Antibiotic treatment and the development of antibiotic resistance in bacterial pathogens

Since the mid-twentieth century when broad-spectrum antibiotics were first introduced in treatment of human bacterial and fungal diseases, they have reduced the morbidity and mortality in human by a dramatic level (M Peng, Salaheen, Biswas, & Park, 2014; Serajus Salaheen, Peng, & Biswas, 2015). The application of antibiotics have been considered as the most vitally important medical event in human history, and the use of them was boosted worldwide for both human medication and agricultural farm animal production (Andersson & Hughes, 2010; Wise, 2002). Broad-spectrum antibiotics are also used to control GI pathogens specifically foodborne pathogens in severe situations. For example, erythromycin and ciprofloxacin are used for *Campylobacter* enterocolitis, metronidazole and vancomycin are used for colitis caused

by *Clostridium difficile*, ciprofloxacin and co-trimoxazole are used for enterocolitis caused by *Salmonella* (Lund & O'Brien, 2011; Traa, Fischer Walker, Munos, & Black, 2010). However, antibiotic treatment and therapy could disrupt the intestinal microbial ecosystem resulting in colonic microbiota imbalance and cause antibiotic-associated diarrhea (AAD), which is usually induced by opportunistic pathogenic microorganisms especially *C. difficile*. In addition, early aged antibiotic treatments could also induce permanent disruption on gut microbiome development and functions in infants and young children and even cause disorders in adipose and hepatic cell metabolism which ultimately develops into type-2 diabetes and obesity (Esteve, Ricart, & Fernández-Real, 2011; Kootte et al., 2012).

Furthermore, the intensive and improper use of antibiotics in both human medicine and agriculture has induced the widespread dissemination of antibiotic-resistance genes and altered the resistant frequency among human bacterial and fungal pathogens (Salyers & 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, 2008), and these resistance could spread from animal to human through either direct contact with animals or transmission in food chain (Marshall & Levy, 2011). It turned

out that the antibiotic-resistant human pathogens evolved to be more virulent and aggressive in respect to disease occurrence (Guay, 2008; Lew, Pai, Oxlade, Martin, & Menzies, 2008; Woodford & Livermore, 2009), which complicate the original situations by raising fatality as well as economic burden on health care (Depuydt et al., 2008; Roberts et al., 2009; Sipahi, 2008). According to Saga and Yamaguchi (2009), Penicillin resistance in several strains of *staphylococcus* was recognized immediately after the introduction of the drug in the late 1940s. Likewise, the resistance to chloramphenicol, streptomycin, and tetracycline was also noted soon. Then the strain of *Shigella dysenteriae* also exhibited resistance to chloramphenicol, streptomycin, tetracycline, and sulfonamides. Over the years, the use of all antibiotics selected bacterial resistance, and almost every known bacterial pathogens have developed at least single or multi-resistance to antibiotics for clinical use (Todar, 2008). Some drug-resistant foodborne bacterial pathogens include EHEC, *S. aureus*, *Salmonella enterica*, *Campylobacter*, *L. monocytogenes*, *Streptococcus pneumonia*, *Vibrio*, *Shigella* etc. Nowadays, more than 70% of the infections-causing bacterial pathogens in hospitals are single or multi drug-resistance and some bacteria are even resistant to all approved antibiotics (Marshall & Levy, 2011).

Alternative strategies in prevention and therapy of foodborne illness

Because of the increased concern about antibiotic resistance, several non-antibiotic antimicrobials have been developed and introduced to prevent and inhibit foodborne bacterial pathogens. The major potential agents include plant- or animal-derived products, bacteriophages, and vaccines (M Peng et al., 2014).

Various products with plant origin can be served as antimicrobial agents and the antimicrobial properties of different plant products like spices/herbs and vegetable/fruit extracts are well documented. For example, a significant number of scientific research have been conducted on the antimicrobial properties of different spices and herbs like mustard, garlic, cinnamon, cumin, bay, clove, thyme, pepper, rosemary, basil, and turmeric (Lai & Roy, 2004; Skrinjar & Nemet, 2009; Vallverdú-Queralt et al., 2014; Yanishlieva, Marinova, & Pokorný, 2006). These spices, herbs, and their extracted products have been recommended to be used in food to reduce microbial contamination and increase overall shelf life of food products at a more natural and healthy way (Skrinjar & Nemet, 2009). Antimicrobials from spices and herbs are collected at different ways, from volatile or oily liquids, seeds, leaves, barks, and sometimes roots of plants (Tajkarimi, Ibrahim, & Cliver, 2010). It has been concluded that the presence of alkaloids, glycosides, steroids, phenols, coumarins, and tannins are important antimicrobial elements but oily substances, especially essential

oils, are the main factors responsible for their antimicrobial properties (Ebana, Madunagu, Ekpe, & Otung, 1991). Additionally, different fruits and vegetables such as pomegranate, raspberry, cranberry, grapes, olive (*Olea europaea*), and their extracts are rich in bio-active compounds, which have antimicrobial activity. These compounds are broadly grouped into phenolic compounds, essential oils, terpenoids, alkaloids, polypeptides, lectins, etc. Different compounds have different roles and different modes of action on pathogenic and spoiling microorganisms (Serajus Salaheen et al., 2015).

Multiple animal-derived products have also been documented as being effective in foodborne-pathogen inhibition. Chitosan, isolated from the exoskeletons of crustaceans and arthropods (insects, spiders, millipedes, and centipedes), has been shown to inhibit the growth and reduce trans-shell penetration 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, could be isolated from myeloid cells and mucosal tissue of both vertebrates and invertebrates, whose inhibitory effect against different foodborne pathogens such as *L. monocytogenes* and EHEC has already been documented (H. J. Jung et al., 2007). Other products such as defensin, lactoferrin, lactoperoxidase, lysozyme, and ovotransferrin have all shown their potential in meat or the preservation of milk products 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 be active against specific bacterial strains because of their high specificity in recognizing and injecting 'disrupting DNA' into a host bacterium. Specificity allows bacteriophages to be used against targeted foodborne pathogens in a mixed population without disturbing the composition of normal gut microflora. Owing to their rapid replication and high level of specificity, bacteriophages can serve as a potential treatment against foodborne pathogens. However, the efficacy of bacteriophages against infecting bacteria needs to be tested in the lab before application. The specificity of bacteriophages is also a disadvantage when a need to target multiple pathogens or causative agents of disease is not confirmed (Inal, 2003). In addition, compared to antibiotics, bacteriophages are more complex organisms that are able to transfer genes between bacteria and induce pathogenic mutation. Only by careful selection of strictly lytic bacteriophages and sequencing their hereditary materials can cross-gene transfer be prevented (Inal, 2003).

Vaccination is the method of inhibiting pathogens by inducing the defense mechanisms of host's immune systems. Some live-attenuated specific vaccination has already shown great efficacy in reducing the levels of foodborne pathogens in mice. Since 2007, millions of funds have been provided by both government and

organizations for the purpose of developing vaccines against the most well-known foodborne bacteria *Shigella* and EHEC. However, vaccines made from any one bacteria serovar cannot confer cross-protection against another serovar, no matter how much antigenic similarity there is between them, whereas more than 2500 serovars of *Salmonella* and various *Campylobacter* species are found in animals and humans (Singh, 2009). As a consequence, the super-high specificity as well as additional costs prevents vaccination from being commonly used in prevention and control of foodborne illness.

Competitive exclusion is a strategy with the presentation of non-pathogenic single or mixed bacterial cultures for colonizing the GI tract and providing pathogen-exclusion effects (Steer, Carpenter, Tuohy, & Gibson, 2000). Depending on the purpose and diseases, competitive exclusion can be the exclusion of pathogens from the native gut intestine or the displacement of invading pathogenic populations (Kamada, Chen, Inohara, & Núñez, 2013; Woo & Ahn, 2013). Several potential proposed modes of action for competitive exclusion in eliminating pathogenic bacteria include direct and indirect competition for limiting nutrients, competition for attachment sites on the host intestinal cells, and the production of antimicrobial compounds such as bacteriocins and other antimicrobial polypeptides (Kamada et al., 2013; Woo & Ahn, 2013). Recently, much attention is paid on new called

‘procommensal strategies’ by establishing non-pathogenic native or introduced microbial intestinal ecosystem to reduce and exclude foodborne bacterial pathogens. Procommensal strategies at one hand promote the growth of target groups of beneficial bacteria that are competitive with or antagonistic to the pathogens of interest, and on the other hand prevent the opportunistic pathogens from remaining in the gut intestine. Current procommensal strategies applied in both human and farm animals include probiotics, prebiotics (as well as foods with prebiotic-like effects), and synbiotics (functional foods containing both probiotics and nutrients with prebiotic-like effects).

The active role of human gut microbes and their metabolites production in gut intestine

Human gut epithelial cells and mucus-containing surfaces are colonized by enormous microorganisms commonly known as normal gut microflora, and they comprise a crucial and complex ecosystem with considerable microbial diversity (Tlaskalová-Hogenová et al., 2011). These commensal microorganisms protect the human intestinal tract from pathogenic bacterial colonization and infection, either directly, through competitive exclusion, or indirectly by producing antimicrobial byproducts/metabolites or modulating gut immunity (Mengfei Peng, Reichmann, & Biswas, 2015). Several genera, in particular *Bifidobacteria* and *Lactobacillus*, produce essential secondary metabolites during intestinal fermentation (Louis, Hold, & Flint,

2014; Marcobal et al., 2013); however, the quantity and composition of bio-active metabolites in the gut are mediated by bacterial spatial distribution as well as host dietary intake (Pédron et al., 2012; Walter & Ley, 2011).

The importance of human gut microbiome homeostasis

The human gut microbiome is composed of bacteria, viruses (mainly phages), fungi, and protozoans. Together, these microorganisms entail a microbial genetic repertoire that is approximately 100 times greater than that of their human host (Fujimura, Slusher, & Cabana, 2010). The human distal GI microbiome possesses 1000 distinct bacterial species and the number was estimated to be at least 10^{14} CFU/g, ten times more than all the human cells in the body. Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria are the dominant phyla in the gut microbiome, among which *Bacteroides*, which alone constitutes approximately 30% of all bacteria therein, along with *Bifidobacterium*, *Clostridium*, *Eubacterium*, *Escherichia*, *Fusobacterium*, *Lactobacillus*, *Peptococcus*, *Peptostreptococcus*, and *Ruminococcus* form the majority genera (Guarner & Malagelada, 2003; Khanna & Tosh, 2014; Vedantam & Hecht, 2003).

The human gut microbiome is integral to the natural defense mechanisms of the gut mucosal surfaces, playing an important role in safeguarding the host against

incoming pathogenic attackers (Mengfei Peng, Reichmann, et al., 2015). By either directly interfering with pathogenic bacteria through competition for niches and nutrients, or by modulating/enhancing host immune defense mechanisms, the microbiota prevent the attachment and proliferation of pathogenic microorganisms on intestinal surfaces as well as their invasion and circulation into intestinal cells (Tlaskalová-Hogenová et al., 2011; Turner, 2009). Indirect evidence points to the complex microbiome-host interactions and the microflora's role in controlling the outgrowth and infections of pathogenic bacteria; e.g., alteration in the composition of the gut microbiome following antibiotic treatment was associated with an over-shedding of *Clostridium difficile* in the stool of infected mice (Lawley et al., 2008; Rupnik, M.; Wilcox, M.H.; Gerding, 2009). Moreover, germ-free and antibiotic-treated mice are more susceptible to infection by numerous enteric pathogenic bacteria, including *S. Typhimurium* (S. Fukuda et al., 2011; Lawley et al., 2009).

As the first line of defense, the intestinal mucosal barrier has evolved complex protective mechanisms in order to resist xenobiotic pathogen adherence and invasion, and more than 80% of immune cells act through the mucosal-associated lymphatic tissue (Tlaskalová-Hogenová et al., 2004). The healthy gut microbiome is the key of protection against colonization and infection of enteric bacterial pathogens through regulation of host intestinal epithelial cell function by both maintaining the epithelial

barrier and associated immune tissue functions (Peterson & Artis, 2014; Round & Mazmanian, 2009). It has been reported that germ-free mice have impaired Paneth cells which is responsible for producing antimicrobial peptides in small intestine, as well as deficient intestinal mucosal barriers and fragile in pathogenic bacterial translocation (K. S. Kobayashi et al., 2005; Vaishnava, Behrendt, Ismail, Eckmann, & Hooper, 2008). Moreover, commensal gut bacterial signaling mechanism also promotes the mucosal barrier functions by stimulating IgA production; these antibodies bind with and neutralize pathogenic antigens, thus protecting against bacterial infection (Fagarasan, Kawamoto, Kanagawa, & Suzuki, 2010; Frantz et al., 2012; Strugnell & Wijkburg, 2010; Suzuki et al., 2010). Recent studies have also revealed that commensal gut anaerobes can induce the production of T helper (T_h) 17 cytokines (IL-17 and IL-22) by CD4⁺ T_h cells (Ivanov et al., 2009), whereas aberrant T_h17 populations are associated with certain GI-dysbiosis-associated chronic disorders such as rheumatoid arthritis, lupus, and inflammatory bowel disease (Chow & Mazmanian, 2009; T. Kobayashi et al., 2008). In addition, another protective mechanism is through stimulation of the host innate immunity (Kamada et al., 2013). This important microbial immune-modulation is amplified by IL-1 β , the cytokine critical for neutrophil recruitment in response to infection. The gut microbiome facilitates pro-IL-1 β production via resident intestinal

macrophages which are able to quickly respond to enteric infections by rapid conversion of pro-IL-1 β to active IL-1 β (Franchi et al., 2012).

Both commensal and pathogenic bacteria require similar ecological niches to colonize host epithelial cells and proliferate throughout the intestine. A healthy gut microbiota is able to competitively exclude incoming pathogens (Hammami, Fernandez, Lacroix, & Fliss, 2013). Commensal bacteria produce certain antimicrobials which inhibit the adhesion, multiplication, survival and colonization of neighbor bacterial species, including pathogens. For example, intestinal bacteriocins produced by *E. coli* and enterotoxins secreted by *Bacillus cereus* can directly block the colonization space of pathogenic *E. coli* and foodborne pathogenic *Bacillus* (Ceuppens et al., 2012; Schamberger & Diez-Gonzalez, 2002). Moreover, *Bifidobacterium* and *Lactobacillus* inhibit the growth of several intestinal pathogens including EHEC by generating and secreting bio-active fatty acids and acidifying the local gut environment (Cherrington, Hinton, Pearson, & Chopra, 1991; R. Shin, Suzuki, & Morishita, 2002). Furthermore, commensal gut bacterial flora and enteric bacterial pathogens share certain nutritional requirements, and as such, the indigenous microbial community plays a critical role in limiting pathogen colonization and infection through competing nutrients. For example, commensal *E. coli* consumes the lion's share of necessary amino acids and organic acids, out-competing any pathogenic *E. coli* invaders for these

resources, which leads to starvation and death of the foreign pathogen (Fabich et al., 2008; Leatham et al., 2009; Momose, Hirayama, & Itoh, 2008a, 2008b). In addition, interactions between gut microbiota and their host cells can suppress and compromise the virulent properties of pathogens. Production of certain metabolites such as butyrate by commensal bacteria can down-regulate the expression of virulent genes involved in T3SS of *S. Enteritidis* and *S. Typhimurium* (Gantois et al., 2006). Interactions between *Bacteroides thetaiotaomicron* and host-produced mucins induce the production of fucose by the fucosidase-bearing commensal bacteria; the secreted fucose then down-regulates expression of the master regulator *ler* for EHEC LEE genes (Pacheco, Munera, Waldor, Sperandio, & Ritchie, 2012). Finally, disruption of the ambient conditions essential for virulence activity can also inhibit pathogenesis. *Enterobacteriaceae* can consume residual oxygen, restricting the virulence of bacterial pathogens like *Shigella flexneri*, which requires an oxygen-rich environment for growth in the intestine (Marteyn et al., 2010).

Probiotics and their beneficial effects

Probiotics are live non-pathogenic microorganisms that are administered in order to improve gut intestinal microbial balance as well as protect the host from infective agents (Callaway et al., 2003). Live probiotic cultures are usually available in

fermented dairy products and probiotic strains fortified foods, whereas freeze dried probiotics are also commercial available in the form of tablets, capsules, powders, or sachets. Several documented probiotic strains include *Bifidobacterium*, *Bacillus coagulans*, *Streptococcus thermophilus*, *Saccharomyces cerevisiae*, and most importantly multiple *Lactobacillus spp.* (*L. acidophilus*, *L. boulardii*, *L. bulgaricus*, *L. casei*, *L. johnsonii*, *L. paracasei*, *L. plantarum*, *L. rhamnosus*, *L. reuteri*) (Hutkins et al., 2016; M E Sanders, Gibson, Gill, & Guarner, 2007; Mary Ellen Sanders, 2008). However, presence of bacteriophage of these probiotic strains play an important role in production of fermented common in dairy and other products. They are responsible for most of the incomplete and slow fermentation (Marcó, Moineau, & Quiberoni, 2012). As a consequence, minimizing the contamination by bacteriophage and developing phage-resistant starter strains are of major concern for both researchers and industrial technologists. General characteristics of probiotic strains especially *Lactobacillus* include the abilities to modulate gut innate and acquired immunity, maintain the epithelial gut barrier, and the antimicrobial properties against enteric pathogens by secreting hydrogen peroxide, organic acids, antimicrobial polypeptides, etc. (Monachese et al., 2011). These special characteristics are all associated with their functions in prevention and therapy of (chronic) enteric infections and diarrhea (Monachese et al., 2011). The goal of this bacterial-strategy is to fill up all available

gut niches with beneficial bacteria so as to block and exclude the establishment of pathogenic bacteria (Doyle & Erickson, 2006; Gaggia, Mattarelli, & Biavati, 2010).

Various beneficial attributes associated with combatting foodborne illness by probiotics (Table 1-2) include production of antimicrobial agents such as lactic acids, hydrogen peroxide, and bacteriocins, production of short chain fatty acid (SCFA), immunologic enhancement, colonization/adhesion resistance, and modulation of mucosal barrier functions. Besides these, other benefits on human beings are connected with their properties in improving digestion, anti-inflammation, anti-carcinogenesis, and assimilation and lowering cholesterol (Amalaradjou & Bhunia, 2012; Dicks & Botes, 2010; Nagpal et al., 2012; Salminen et al., 2010; Thomas & Ockhuizen, 2012).

Evidences for using wild type probiotics in prevention and control of enteric infections have been recorded recently. Both *Lactobacillus* and *Bifidobacterium* are found to enhance colonization resistance which contributes in their competition with enteric bacterial pathogens (Wagner, Johnson, & Rubin, 2009). Probiotic *bifidobacteria*, *Enterococcus faecium* and non-pathogenic probiotic *E. coli* strains inhibited EHEC growth, protected mice against EHEC infection, and caused reduction in Shiga toxin production (Takashi Asahara et al., 2004; Lema, Williams, & Rao, 2001; Reissbrodt et al., 2009). Multiple *Lactobacillus* strains including *L. acidophilus*, *L. casei*, *L. fermentum*, *L. plantarum*, *L. reutri*, and *L. rhamnosus* could attach efficiently

to host intestinal cells and diminish the intestinal and extra-intestinal dissemination of EHEC, *Salmonella*, *C. jejuni*, and *V. parahemolyticus* by competitive exclusion and displacement (Lema et al., 2001; Satish Kumar et al., 2011). Administration of several combined probiotic strains could synergistically reduce *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella*, *S. aureus*, and *Shigella sonnei* (Higgins et al., 2008; Zschüttig et al., 2012). In addition, Probiotics like *L. paracasei* were also shown to up-regulate dendritic cells, activate T_h cells and antibody production, as well as down-regulate pro-inflammatory cytokines, which resulted in a reversed/enhanced gut intestinal integrity and protection against enteric bacterial pathogens especially *Salmonella* (Tsai, Cheng, & Pan, 2010; Wagner et al., 2009).

Over the last decade, scientists have been working on development of novel and innovative genetically-recombinant probiotics with enhanced advantages such as higher ability in mucosal and intestinal colonization, higher tolerance to bacteriophage, gastric acid, and bile salts for survival in GI tract, prolonged colonization as well as competition with enteric pathogens, and higher expression of target antimicrobial molecules against pathogens (Sleator & Hill, 2008; J. Wells, 2011; J. M. Wells & Mercenier, 2008). To achieve these purposes, various bioengineering actions could be applied. First, toxin-specific host cell receptors could be expressed on probiotics strain surface for competitively binding with lethal toxins secreted by enteric pathogens

(Paton, Morona, & Paton, 2006). Adhesins and other key factors in secretory systems of enteric pathogens could be cloned and expressed on probiotic strains to create competitive environment for specific pathogen colonization (Jagadeesan et al., 2011). Furthermore, by mimicking expression of virulence genes from enteric bacteria, probiotic strains could interrupt pathogenic quorum sensing pathway and suppress the associated virulence gene expression in the target pathogen (Waters & Bassler, 2005). Finally, the development and administration of probiotic strains with heterologous antigen expression could stimulate immune responses (cytokine production) and assist in suppressing intestinal inflammation and providing cyto-protection (J. M. Wells & Mercenier, 2008).

Prebiotics and prebiotic-like foods in gut microbiome modulation

Gibson and Roberfroid (1995) first defined the terminology ‘prebiotics’ as ‘non-digestible food ingredients which improve the host health by selectively stimulating the growth and activity of limited numbers of bacteria in the colon’. Nine years after at 2004, they redefined the term 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’ (Glenn R. Gibson, Probert, Loo, Rastall, & Roberfroid, 2004a). Common prebiotics include inulin, lactulose, and

oligosaccharides, and their major characteristics are colonic microflora fermentable while resistant to digestive enzymes in human gut (Bielecka, Biedrzycka, & Majkowska, 2002a; Kolida, Tuohy, & Gibson, 2002a). Moreover, multiple preliminary studies have revealed that several bifidogenic prebiotics could provide native microflora limiting nutrients and energy for fermentation, either producing vitamins, SCFAs, and antioxidants for modulation of intestinal microflora composition or releasing antimicrobial byproducts as competitive advantages for exclusion of pathogenic bacteria (J. H. Cummings & Macfarlane, 2002; M. Fukuda et al., 2002; Videla et al., 2001). Besides all of these mentioned above, research has also demonstrated the potential effects of prebiotics on stimulating mineral absorption (such as calcium), enhancing immune system effectiveness, and reduction of colorectal cancer risk (Geier, Butler, & Howarth, 2006; Lohner, Küllenberg, Antes, Decsi, & Meerpohl, 2014; Lomax & Calder, 2009; Scholz-Ahrens & Schrezenmeir, 2007).

Prebiotics are generally coming from food sources. The top-10 prebiotic-containing foods, their prebiotic fiber content, and recommended daily serving are summarized in Table 1-3. Chicory root is considered the richest natural source of inulin and oligosaccharides. Beans, raw oats, Whole wheat, banana, et al. are believed to be other traditional dietary sources of prebiotics. Certain oligosaccharides naturally exist in breast milk are found to play an active and vital role in the development of gut

immune system in infants (Seifert & Watzl, 2007). Except for inulin, lactulose, and oligosaccharides, several other compounds such as flavanols and polyunsaturated fatty acid (PUFA) especially conjugated linoleic acids (CLA) are also found to possess similar prebiotic-like effects (Andoh, Tsujikawa, & Fujiyama, 2003; Tzounis et al., 2011).

In 2003, the Food and Drug Administration (FDA) approved a qualified 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. Per 20 g peanut contains 5 g dietary fibers, 10 g proteins, 5 g total fat, and multiple other bio-active compounds. The benefits on reducing cardiovascular diseases (Bao et al., 2013; Isanga & Zhang, 2007; J. B. Jones et al., 2014; Ozcan, 2010) with daily intake of peanut or other nut products has been suggested to associate with their dietary fibers (prebiotics) as well as the phenolic compound Resveratrol (3,4,5-trihydroxystilbene) (Bubonja-Sonje, Giacometti, & Abram, 2011; Wang, Yan, Li, Jiang, & Liu, 2011). Additionally, the prebiotic-like benefits of multiple compounds from peanut in large intestine can promote the growth of selective beneficial gut microflora, especially *Bifidobacteria* and *Lactobacillus* (Calatayud et al., 2013; Tzounis et al., 2011).

Cocoa is also a rich source of dietary fibers and various polyphenols including flavanols and procyanidins, which are free radical cleaning molecules with strongly antioxidant activity and anti-inflammatory potential (Bubonja-Sonje et al., 2011; Cai, Luo, Sun, & Corke, 2004; Gu, House, Wu, Ou, & Prior, 2006; Pérez-Berezo, Franch, Castellote, Castell, & Pérez-Cano, 2012). The compounds from cocoa also have been shown to have numerous health-promoting properties such as reducing blood pressure, increasing the formation of endothelial nitric oxide, and promoting vasodilation (Ried, Fakler, & Stocks, 2012). Previous reports found that *Lactobacillus* strains were one of the predominating bacterial species of cocoa bean fermentations (Lefeber, Janssens, Camu, & De Vuyst, 2010; Papalexandratou, Camu, Falony, & De Vuyst, 2011), and there is also evidence that cocoa components like flavanols, multiple carbohydrates, and dietary fibers that reach the large intestine may have prebiotic-like benefits by promoting the growth of select beneficial gut microflora (Massot-Cladera, Pérez-Berezo, Franch, Castell, & Pérez-Cano, 2012; Tzounis et al., 2011).

Synbiotics and synergistic effects

The concept 'synbiotic' was first 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'. Briefly, several related studies have revealed the co-administration 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 & Gibson, 2002). Likewise, *bifidobacteria* in together with trans-galacto-oligosaccharides could protect mice from *S. Typhimurium* and following lethal infections (T. 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.* in vivo study (Bomba et al., 2002). 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 (Adebola, Corcoran, & Morgan, 2014; Saulnier, Gibson, & Kolida, 2008) as well as the quality of life in patients suffering from foodborne illness and UC (Fujimori et al., 2009).

Intestinal production of beneficial metabolites by probiotics

Undigested dietary components from consumed food that reach the large intestine are fermented and/or metabolized by the gut microbial community, resulting in the generation of a diverse bank of beneficial metabolites (Flint, Scott, Louis, & Duncan, 2012; Marcobal et al., 2013). During fermentation, beneficial bacteria (probiotics), particularly the lactic acid bacteria *Lactobacillus*, produce a wide range of secondary metabolites (byproducts) that are associated with health-promoting benefits to the host. Major byproducts include lipid molecules of various chemical structures such as SCFAs and PUFAs (Louis et al., 2014; Serini, Piccioni, Merendino, & Calviello, 2009). The combined concentration of lipid byproducts in the human colon is approximately 50-150 mM, and these beneficial molecules assist in modulation of host immune responses and regulation of cell apoptosis (Louis et al., 2014). Other bio-functional metabolites from probiotics include bio-active proteins or polypeptides with antimicrobial and immune-modulatory properties and vitamin B, essential for human growth, metabolism, and reproduction (Stanton, Ross, Fitzgerald, & Van Sinderen, 2005).

Antimicrobial polypeptides (bacteriocins)

Bacteriocins are proteins or polypeptides with antimicrobial activities produced by certain bacteria especially probiotics for the purpose of inhibiting the growth of their competitive bacterial strains in the environment. Such antimicrobial proteins are able to inhibit the growth of major foodborne bacterial pathogens like *Salmonella*, *Listeria*, and *Campylobacter* (Patton, Dickson, Lonergan, Cutler, & Stahl, 2007; Stahl, Callaway, Lincoln, Lonergan, & Genovese, 2004). For example nisin, produced by *L. lactis*, has already been found to be effective in spoilage bacteria reduction in meat and milk, and encapsulated nisin is able to inhibit the growth of *L. monocytogenes* (da Silva Malheiros, Daroit, & Brandelli, 2010). Reaching or secreting in the lower gut, nisin exerts its antimicrobial activities by disrupting the cell membranes of target foodborne pathogens. Likewise, the mixture of related cyclic polypeptides called ‘bacitracin’ produced mainly by *Bacillus subtilis* also has antimicrobial properties by interfering with bacterial cell wall and peptidoglycan bio-synthesis in both Gram-positive and Gram-negative bacteria (Weston, Wahab, Roberts, & Mason, 2001). Furthermore, reuterin, a broad-spectrum antimicrobial substance produced by *L. reuteri*, can inhibit the growth of *L. monocytogenes*, *C. jejuni*, *Yersinia enterocolitica*, *S. aureus*, and EHEC, and it has been used as bio-preservative in different food products like beef sausage, milk, and cottage cheese (Arqués, Rodríguez, Nuñez, & Medina, 2011).

Essential vitamin B

The B-group vitamins including thiamine (B1), riboflavin (B2), niacin (B3), pantothenic acid (B5), pyridoxine (B6), biotin (B7), folate (B11), and cobalamin (B12) act synergistically to maintain the body's homeostasis by regulating cell metabolic processes such as energy production, red blood cell formation, and nucleic acids synthesis (LeBlanc et al., 2011). Most of these vitamins cannot be synthesized by humans and animals, but they could be produced by LAB fermentation (A. Patel, Shah, & Prajapati, 2013). For instance, folic acid as an essential cofactor for bacterial metabolism could be used by *L. lactis* for biosynthesis of folate which is essential for reproduction and reducing coronary heart disease as well as cancer risks (Sybesma et al., 2003). Similarly, multivitamin production such as riboflavin and cobalamin has recently been achieved by single strain of *L. lactis* (Sybesma, Burgess, Starrenburg, Van Sinderen, & Hugenholtz, 2004). These two B-group vitamins are essential cofactors in fatty acids, amino acids, carbohydrates and nucleic acids metabolism (Burgess, O'Connell-Motherway, Sybesma, Hugenholtz, & Van Sinderen, 2004).

Intestinal production of PUFAs/ SCFAs and their major contributors

The common PUFAs are summarized in Table 1-4. The major bio-function of PUFA linoleic acid conjugation in bacteria is to detoxify the inhibitory effects of fatty acids on growth (Coakley et al., 2003; Jiang, Björck, & Fondén, 1998). The rumen anaerobic bacteria *Butyrivibrio fibrisolvens* was the first to be recognized as a CLA producer (Kepler, Hirons, McNeill, & Tove, 1966). In 1998, it was found that *Propionibacterium freudenreichii* could also convert linoleic acid to CLA (Jiang et al., 1998). It has since been shown that numerous genera of dairy and human/animal intestinal bacteria, including lactic acid bacteria *Lactobacillus* and *Bifidobacterium*, are able to produce CLA during normal metabolic activities (Alonso, Cuesta, & Gilliland, 2003a; Y. J. Kim & Liu, 2002; Tung Y. Lin, 2000; Ogawa, Matsumura, Kishino, Omura, & Shimizu, 2001; Soo et al., 2008). Among these bacteria, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactococcus lactis*, and *Streptococcus thermophilus*, most of which are known to be common gut microflora, have been revealed as CLA producers (Farooq, Mohsin, Liu, & Zhang, 2013; Van Nieuwenhove, Cano, Pérez-Chaia, & González, 2011), however the levels of CLA produced varies. Moreover, isomers of PUFAs formed are also strain-dependent (Van Nieuwenhove et al., 2011). Several strains, such as *Lactobacillus*

paracasei and *S. thermophilus*, each only produce a single isomer, whereas two or more CLA isomers are generated by other strains such as *L. acidophilus*, *L. casei*, and *L. plantarum*.

Linoleate isomerase (LI) is the enzyme responsible for linoleic acid isomerization and CLA production in several bacteria, especially *Lactobacillus* and *Bifidobacterium* (Martin Macouzet, Robert, & Lee, 2010). The mechanisms for *Lactobacillus* production of CLA were found to involve hydroxyl fatty acids as intermediates (Kishino, Ogawa, Yokozeki, & Shimizu, 2011); later analysis revealed that CLA formation is composed of three distinct steps: linoleic acid hydration into 10-hydroxy-octadecenoic acid, followed by isomerization and subsequent dehydration into CLA (B. Yang et al., 2014). The LI is a myosin cross-reactive antigen (MCRA) originally discovered in *Streptococcus pyogenes* as a 67 kD protein (Kil, Cunningham, & Barnett, 1994). Previously, Peng *et al.*, (2007) showed that MCRA-like proteins contain more than 50% sequence similarity with LI from *L. acidophilus* and *Lactobacillus reuteri*. Recently, it was reported that MCRAs comprise a family of proteins present in a wide range of bacteria, especially lactic acid bacteria. MCRAs from multiple species of LAB were confirmed as fatty acid hydratase (B. Yang et al., 2014). A BLAST search using the MCRA protein sequence revealed more than 148 conserved sequences across different Gram-positive and Gram-negative bacteria

species (Volkov et al., 2010). In 2011, Kishino *et al.* reported that the linoleic acid isomerase in *L. plantarum*, as a multi-component enzyme system, was associated with both membrane and soluble fractions, and a hydration step from linoleic acid to 10-hydroxy-12-octadecenoic acid was also involved in the isomerization reaction. Thereafter, Rosberg-Cody *et al.*, (2007) reported that the recombinant *Lactococcus* and *Corynebacterium* with heterologous gene of MCRA (*mcra*) from *B. breve* are more resistant to heat and stress compared to wild-type strains. Moreover, O'connell *et al.*, (2013) demonstrated that MCRA enzyme in *B. breve* was an oleate hydratase and it might provide detoxification activities on bacteria by catalyzing hydration of multiple unsaturated fatty acids. Additionally, MCRA found across a wide range of taxa including LAB have been shown to promote blood survival and cell adherence (O'Flaherty & Klaenhammer, 2010; Volkov et al., 2010).

SCFAs are released in the anaerobic gut lumen as fermentation byproducts of human intestinal microflora when indigestible carbohydrates are incompletely oxidized. The type and structures of available complex carbohydrates play a critical role in determining the complement of fermented products produced by the microbiota. Therefore, the level and composition of intestinal SCFAs are heavily influenced by diet and the endogenous microbial community structure (Harrison, Balan, & Babu, 2013). Generally, organic acids are transformed from complex carbohydrates by the primary

fermenting microbes such as the Bacteroidetes, to be subsequently utilized for generation of SCFAs by secondary fermenting microbes such as *Clostridium* and other butyrate-producing gut microorganisms (Nava, Friedrichsen, & Stappenbeck, 2011). The resultant hydrogenous byproducts are taken up by acetogens for the production of acetate, the dominant type of SCFA in the human gut (Rey et al., 2010). Another major intestinal SCFA, butyrate, is thought to be produced via one of two transformation pathways, acetyl-CoA condensation and butyryl-CoA formation; it has been proposed based on previous research which led to the development of metabolic enzyme molecular markers for use in revealing the functional dynamics of microbial intestinal ecology (Hosseini, Grootaert, Verstraete, & Van de Wiele, 2011). In the proposed pathway, catalyzed by butyrate kinase and phosphotransbutyrylase from *Clostridium acetobutylicum*, butyryl-phosphate was surmised to be an intermediate in conversion of butyryl-CoA to the final product butyrate (Hartmanis & Gatenbeck, 1984). In the recent molecular and enzymatic *in vitro* studies on major intestinal butyrate sources, butyryl-CoA-acetate-CoA transferase is recognized as an alternative butyrate-producing pathway, in which the coenzyme itself catalyzes the transformation from acetate to butyrate (Duncan, Barcenilla, Stewart, Pryde, & Flint, 2002; Louis et al., 2004).

Quantity and composition of intestinal SCFAs can also be determined by spatial organization of the human endogenous microbial community (Table 1-5) (Pédron et al., 2012; Roy, Kien, Bouthillier, & Levy, 2006; Topping & Clifton, 2001). For example, large spatial variation has been found in the relative ratio of different SCFAs, resulting in different pH environments between the small and large intestine, as the small intestine contains a relatively lower microbial distribution (Walter & Ley, 2011). However, diet also plays a critical role in SCFA production, since the type of SCFAs produced by fermenting gut microbiota is dictated by the structural complexity of the carbohydrate substrates available. Some examples are summarized in Table 1-6.

Beneficial effects of CLA on human health

CLA is the generic term to describe a mixture of positional and geometric isomers of linoleic acid (C18:2, c9, c12). These isomers contain double bond system in either cis or trans configuration at several 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 associated with multiple health and nutritional benefits on human beings (Alonso et al., 2003a). Structures of linoleic acid and its common isomers are listed in Table 1-7. Dietary sources of linoleic acid and CLA include dairy products, vegetable oils, animal meats, mixed nuts (including peanut), as well as cocoa

butter (Kris-Etherton et al., 2000; Sonwai, Kaphueakngam, & Flood, 2012), whereas the major source for human is through milk consuming. Therefore, CLA especially c9, t11-CLA (represents 80-90%) as the intermediate during dietary linoleic acid biohydrogenation is accumulated predominantly in milk fat (Jensen, 2002). Due to their biological benefits, CLA has received great attention in recent years, and most research was conducted for the purpose to evaluate the health effects of mixed isomers of CLA (Kelley, Hubbard, & Erickson, 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, Cotter, Stanton, Ross, & Hill, 2012). CLA produced during fermentation by probiotic bacteria possess potential beneficial roles including anti-cancer, anti-inflammatory, and anti-pathogenic activities (Ewaschuk, Walker, Diaz, & Madsen, 2006; Bo Yang et al., 2015).

CLA is well documented in numerous studies as an efficient inhibitor for all stages of carcinogenesis including initiation, promotion, and metastasis (Belury, 2002a; B. Q. Chen et al., 2003; Kelley et al., 2007; Lau & Archer, 2010; Soel, Choi, Bang, Park, & Kim, 2007). In vitro studies suggested that both c9, t11-CLA and t10, c12-CLA as well as their mixture have anti-proliferative effects against multiple cancer cell lines, such as colon cancer cells, breast cancer cells, and prostate cancer cells (Cho et al.,

2006; Flowers & Thompson, 2009; Lau & Archer, 2010; Ochoa et al., 2004; Rakib et al., 2013). In vivo animal researches revealed that 0.05-1% (w/w) mixture of CLA isomers c9, t11-CLA and t10, c12-CLA could significantly inhibit chemically-induced colon, forestomach, and mammary gland tumors (Bassaganya-Riera, Viladomiu, Pedragosa, De Simone, & Hontecillas, 2012; McGowan et al., 2013; Rosberg-Cody et al., 2007). By preferentially affecting arachidonic acid metabolism and down-regulating cyclooxygenase and 5-lipoxygenase expression, c9, t11-CLA reduces the level of prostaglandin E2 and thromboxane B2 (Ochoa et al., 2004) and disrupts Hsp90/IKK complex, thus prevents the phosphorylation of I κ B by IKK, and further blocks NF- κ B activation (D. I. Kim et al., 2011; Perdomo, Santos, & Badinga, 2011; Rakib et al., 2013). The down-regulation of NF- κ B signaling reduces the proliferation of cancer cells and weakens them to be vulnerable to TNF-induced apoptosis (D. I. Kim et al., 2011; Park et al., 2007; Perdomo et al., 2011; Rakib et al., 2013). On the other hand, t10, c12-CLA isomer works preferentially through apoptosis modulation by activation of caspase-3 and caspase-9, induction of p21, p53, and p27, inhibition of anti-apoptotic bcl-2, and activation of pro-apoptotic protein Bax (Cho et al., 2006; K. P. Kim et al., 2006; S. H. Lee et al., 2006; Ochoa et al., 2004).

The anti-inflammatory properties of CLA has been reported both *ex vivo* in cell culture assays and *in vivo* in human clinical trials. The overall anti-inflammatory

mechanism of CLA is through cyclooxygenase-2 inhibition and thus reducing prostaglandin E2 release (Flowers & Thompson, 2009; Li, Barnes, Butz, Bjorling, & Cook, 2005; Y. K. Nakamura & Omaye, 2009; Stachowska et al., 2007). Then by activating peroxisome proliferator-activated receptors, CLA could manage to inhibit the activation and translocation of NF- κ B into nucleus (Borniquel, Jadert, & Lundberg, 2012; D. I. Kim et al., 2011), and followed by reducing the level of pro-inflammatory cytokines formation such as TNF- α (Akahoshi et al., 2004), IL-1 β (Albers et al., 2003; Tricon et al., 2004), and IL-8 (Jaudszus, Foerster, Kroegel, Wolf, & Jahreis, 2005) which are normally activated by NF- κ B (Clarke et al., 2010; Song, Sneddon, Heys, & Wahle, 2006). Moreover, CLA has also been reported to stimulate the expression levels of anti-inflammatory cytokine TFG- β 1 (Bassaganya-Riera et al., 2004, 2012) and protective immunoglobulins IgA, IgG, and IgM, whereas suppressing IgE production (Martinez et al., 2010). On the other side, bacterial pathogenic challenged macrophage dendritic cells could rapidly produce and secret IL-12 which acts as the signal for T_h cells - Th1 (INF- γ , IL-12, and TNF- α) and Th2 (IL-4 and IL-10) (Bassaganya-Riera et al., 2003). *In vitro* and *in vivo* studies revealed that 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. However, the

anti-inflammatory effects of CLA will not impair cellular immunity to intracellular pathogens or alter resistance to bacterial infection, though the mechanism under it is still unknown (Turnock, Cook, Steinberg, & Czuprynski, 2001).

The antimicrobial activity of most long-chain saturated and unsaturated fatty acids was revealed during years to be dependent on both the bacterial strains and types of the fatty acids (Mbandi, Brywig, & Shelef, 2004; S. Y. Shin, Bajpai, Kim, & Kang, 2007; Zheng et al., 2005). Among those fatty acids, long-chain unsaturated fatty acids especially linoleic acid has been well documented to be bactericidal to *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Micrococcus Kristinae* (Zheng et al., 2005), however no/minimum antimicrobial effect was found to be against Gram-negative bacteria such as *E. coli* and *Salmonella*, except for *Pseudomonas aeruginosa* (Dilika, Bremner, & Meyer, 2000; C. Q. Sun, O'Connor, & Robertson, 2003). Whereas, CLA has also shown considerable promise as an antimicrobial bio-active agent on foodborne and pathogenic bacteria for both Gram-positive and Gram-negative, which added value for any linoleic acid-containing functional food products as well as CLA-producing probiotics. To be specific, 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 (such as *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 both bacterial membrane and cultural medium (Byeon et al., 2009), since CLA in this study had been found to be present in the membranes of all tested microorganisms in the cultural medium and their disrupted cell membrane surfaces were observed.

However, animal and human studies have demonstrated that an estimated daily intake of 2.2-4.2 g of CLA may be required to realize these multiple health benefits mentioned above (Moloney, Yeow, Mullen, Nolan, & Roche, 2004; Mullen et al., 2007; Smedman & Vessby, 2001), but the real consumption of CLA in different countries (less than 500 mg) is much lower than this recommended dose (Dhiman, Nam, & Ure, 2005). Although such health benefits are probiotic strains dependent, a significantly higher CLA producing bacterial CFU in fecal is required for the optimal amount of CLA production (Gionchetti et al., 2007; Helwig et al., 2006; Larsen et al., 2006; Mimura et al., 2004). Therefore, increasing of CLA production by per bacterial cells might be a feasible plan to maintain the efficacious dosage of CLA.

Production of free CLA by bacteria

Multiple factors including culture media, temperature, oxygen availability, period of fermentation, as well as substrate concentration influence the CLA production (Pandit, Anand, Kalscheur, & Hassan, 2012). For certain examples, *L. acidophilus* is only able to form CLA in microaerophilia instead of aerophilia conditions (Kishino et al., 2011); Yadav *et al.* (2007) informed that dahi-containing fermentation could stimulate the CLA production by *L. acidophilus*; Lin *et al.* (2005) demonstrated that immobilized *L. bulgaricus* and *L. acidophilus* in chitosan and poly-acrylamide matrix exhibited higher CLA productivity 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 productivity than obtained in growth cultures with extended incubation. Moreover, the isomers formed are strain-dependent (Van Nieuwenhove et al., 2011), indicating that several strains like *L. paracasei* and *S. thermophilus* only form single isomer, whereas two or more CLA isomers could be produced by other strains such as *L. acidophilus*, *L. casei*, and *L. plantarum* (Table 1-8).

LI has been found to present in *Lactobacillus* by two major forms: either as a membrane-bound protein or as a soluble enzyme which allows possible extraction primarily from bacterial cell-free cultural supernatant (Gorissen et al., 2010; M.

Macouzet, Lee, & Robert, 2010; Martin Macouzet et al., 2010). Few studies were conducted focusing on the optimal enzymatic conditions for LI. It was suggested that the optimal conditions for linoleic acid isomerization by LI in washed cells of multiple *Lactobacillus* are pH 6.5 and 34-37 °C (Kishino et al., 2011; S. O. Lee et al., 2003). However for growing culture of *Lactobacillus*, the optimal temperature condition turned out to be between 10 and 22 °C (Hernandez-Mendoza, Lopez-Hernandez, Hill, & Garcia, 2009). During past years, putative LI proteins have been characterized and sequenced for research (Farmani et al., 2010), which include LI enzymes from *Bifidobacterium breve*, *B. dentium*, *Clostridium sporogenes*, *L. acidophilus*, *L. plantarum*, *L. lactis*, *L. reuteri*, *Propionibacterium acnes*, and *Rhodococcus erythropolis* strains (S. S. Peng et al., 2007). The mechanism for the *Lactobacillus* production of CLA has been demonstrated to involve hydroxyl fatty acids as intermediates (Kishino et al., 2011). Furthermore, the production/conversion of CLA in *Lactobacillus* was hypothesized as 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, followed by isomerization and dehydration of 10-hydroxy-octadecenoic acid into CLA (B. Yang et al., 2014).

Prospective applications in human health and food safety

In patients which lack the appropriate microbiota, prebiotics cannot selectively increase intestinal levels of fatty acids and confer the resulting health benefits. This has led to the introduction of synbiotics, ‘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’ (G R Gibson & Roberfroid, 1995; Y. Sun & O’Riordan, 2013). Briefly, several related studies have suggested that the co-administration of probiotics and prebiotics could yield synergistic health benefits, and are more effective than either probiotics or prebiotics alone in promoting SCFA and PUFA production and establishing and maintaining the correct composition and balance of gut microbiota (Adebola et al., 2014; Saulnier et al., 2008), thereby significantly improving the quality of life of patients suffering from foodborne illnesses and UC (Fujimori et al., 2009).

On the other hand, livestock including chickens, turkeys, swine, cattle, and sheep as well as wild birds in and around farm environments are principal reservoirs for foodborne bacterial pathogens like *Salmonella*, EHEC, and *C. jejuni* (Oliver et al., 2009). These foodborne pathogens further contaminate produce and meat products during food processing and production (McEwen & Fedorka - Cray, 2002).

Considering the limitations PUFAs and SCFAs place on enteric bacterial survival, especially certain pathogenic foodborne bacteria, they have the potential to be added into food products as preservatives to greatly reduce pathogenic bacterial contamination (Carpenter & Broadbent, 2009; Harrison et al., 2013). Moreover, SCFAs have been shown to restrict the shedding of pathogenic bacteria, specifically *Salmonella*, *in vivo* (Filip Van Immerseel et al., 2004; F. Van Immerseel, De Buck, et al., 2004; F. Van Immerseel, Fievez, et al., 2004), and are now extensively used as animal feed additives in order to control *Salmonella* colonization in broilers and reduce the risk of cross-contamination during animal slaughtering and processing (Cox & Pavic, 2010; Defoirdt, Boon, Sorgeloos, Verstraete, & Bossier, 2009; F. T. Jones, 2011; Ricke, 2003; Wales, Allen, & Davies, 2010). However, as Van Immerseel *et al.* (2004) pointed out, elimination of foodborne pathogenic bacteria colonization in poultry cannot be achieved solely through SCFA and PUFA feed additives. Their use in combination with best hygienic practices and cross-contamination-preventing activities is necessary to thoroughly minimize the risk of foodborne infections (Y. Sun & O’Riordan, 2013).

However, excessive levels of SCFAs and PUFAs may also induce adverse effects on human health. Dietary carbohydrate mal-absorption and occasional overgrowth of commensal bacteria lead to over-production and accumulation of SCFAs

in human colon which could notably lower the bowel pH and further induce concentration-based injuries (J. Lin, 2004). Evidences include *in vivo* studies on newborn rats and piglets, in which intraluminal administration of 300-600 mM acetic acid caused intestinal injuries (Di Lorenzo, Bass, & Krantis, 1995; Jing Lin et al., 2002; Nafday et al., 2002). Moreover, it was hypothesized but without direct evidence that the over-secretion and accumulation of 200-300 mM SCFAs in bowel of premature infants, normally vulnerable and immature, might also induce intestinal mucosal injury which finally leading to neonatal necrotizing enterocolitis (Cheu, Brown, & Rowe, 1989; Jing Lin et al., 2002). However, further study is needed due to the limitation on premature infants' *in vivo* measurement of local intestinal SCFAs concentration. Similarly, daily PUFAs intake of >10% total energy might also induce few side effects in metabolic disorders, but systematic studies are necessary for evidence in cholesterol and glucose metabolism or homeostatic functions (Eritsland, 2000).

Overall Hypothesis and Specific Aims

By over-producing metabolites genetically or with supplement of prebiotic-like components, *Lactobacillus (L. casei)* can significantly reduce the colonization and infection of enteric bacterial pathogens (*S. Typhimurium* and EHEC) while improve the gut health.

To investigate the hypothesis, the following aims need to be fulfilled,

Aim 1: Characterization and *in vitro* evaluation of the functional properties of cocoa and peanut on normal microbial flora and enteric bacterial pathogens

Aim 2: Assessment of the synergistic effects of cocoa/peanut and *Lactobacillus* on enteric pathogenic bacterial growth *in vitro* and host-pathogen interactions *ex vivo*

Aim 3: Overexpression of linoleate isomerase gene in *Lactobacillus* as well as both *in vitro* and *in vivo* investigation on its functions in combating enteric pathogenic bacterial infections and improving overall gut health

Table list

Table 1-1. Foodborne diseases and the causative bacterial pathogens

Clinical symptoms / Diseases	Causative bacterial pathogens
Diarrhea, dysentery, and vomiting	<i>Salmonella, Campylobacter, EHEC, Shigella, Bacillus, Vibrio, Staphylococcus aureus</i>
Arthritis (reactive arthritis and rheumatoid arthritis)	<i>Salmonella, Campylobacter, Shigella, Yersinia</i>
Hemorrhagic uremic syndrome	<i>EHEC, Shigella</i>
Guillain Barre syndrome	<i>Campylobacter</i>
Meningitis and encephalitis	<i>Listeria</i>
Miscarriage, stillbirth, and neonatal infections	<i>Listeria</i>
Paralysis	<i>Clostridium botulinum, Campylobacter, Staphylococcus aureus</i>
Allergic responses	<i>Staphylococcus</i>

Table 1-2. Health benefits of probiotics and their proposed mechanisms

Beneficial attributes	Potential mechanism
Resistance to enteric bacterial pathogens	Colonization resistance Competitive exclusion Growth inhibition by producing organic acids, bacteriocins, and antimicrobial peptides
Modulation of gut flora	Growth stimulation by producing vitamins, SCFAs, and antioxidants Decrease toxic metabolites production
Modulation of immune system	Enhancement of mucosal barrier functions Activation of non-specific and antigen-specific defense Regulation and activation of Th1/Th2 cells
Anti-inflammation	Production of anti-inflammatory cytokines Reduction of pro-inflammatory cytokines

Table 1-3. The top-10 prebiotic-containing foods with their prebiotic fiber content and recommended serving amount per day

Food	Prebiotic fiber content (% by weight)	Recommended serving amount (g for 6 g prebiotic intake)
Chicory Root	64.6	9.3
Jerusalem Artichoke	31.5	19
Dandelion Greens	24.3	24.7
Garlic	17.5	34.3
Leek	11.7	51.3
Onion	8.6	69.8
Asparagus	5	120
Wheat bran	5	120
Whole Wheat flour	4.8	125
Banana	1	600

Table 1-4. Common PUFAs, their sources, and functions

Common name	Lipid name	Major sources		Bio-functions
		Food sources	Major microbial producer	
Alpha-linolenic acid	18:3 (n-3)	Flaxseeds, canola, soybean, pumpkin, tofu, walnuts	Essential fatty acid - must be acquired through diet	Lower heart disease; lower cholesterol; reduce high blood pressure; improve asthma
Docosahexaenoic acid	22:6 (n-3)	Salmon, tuna, sardine, shellfish, human breast milk	Fish gut microbes (<i>Shewanella</i>); microalgae (<i>Chlorella</i> , <i>Nannochloropsis</i> , etc.)	Slow down Alzheimer's disease; increase efficacy of chemotherapy; assist infant development; benefit nervous system; benefit cardiovascular health
Docosapentaenoic acid	22:5 (n-3)	Salmon, menhaden, beef, human breast milk	Fish gut microbes (<i>Shewanella</i>); microalgae (<i>Chlorella</i> , <i>Nannochloropsis</i> , etc.)	Slow down Alzheimer's disease; increase efficacy of chemotherapy; assist infant development; inhibit thrombosis; benefit wound-healing responses
Eicosapentaenoic acid	20:5 (n-3)	Cod liver, salmon, tuna, sardine, seaweed, human breast milk	Fish gut microbes (<i>Shewanella</i> , <i>Pneumatophoru</i> , etc.); microalgae (<i>Thraustochytrium</i> , <i>Pavlova</i> , etc.)	Slow down Alzheimer's disease; increase efficacy of chemotherapy; assist infant development; reduce depression; alleviate lupus
Arachidonic acid	20:4 (n-6)	Peanut	Mammalian cells enzymes	Assist muscle growth; slow down Alzheimer's disease
Gamma-linolenic acid	18:3 (n-6)	Spirulina, primrose, black currant, borage, fungal oils	Mammalian cells enzymes	Alleviate allergy; reduce high blood pressure; improve breast cancer; improve osteoporosis
Linoleic acid	18:2 (n-6)	Olive, cocoa, peanut, canola, almonds, sesame, corn	<i>Bifidobacterium</i> , <i>Lactobacillus</i> , <i>Lactococcus</i> , and <i>Streptococcus</i> isomerize linoleic acid	Reduce body fat; improve blood lipids; antioxidant defense; normalize glucose tolerance; improve hyperinsulinemia
Oleic acid	18:1 (n-9)	Olive	<i>Bifidobacterium</i> , <i>Lactobacillus</i>	Reduce cholesterol; reduce high blood pressure; decrease risk of breast cancer

Table 1-5. Representative gut microflora with their distribution and production of PUFAs/SCFAs

Distribution in gut	pH	Major microbes	Functions or activities	PUFA/SCFA produced	
Small intestine	Duodenum	5-7	<i>Helicobacter pylori</i> , <i>Lactobacillus</i> spp. ^a , <i>Streptococcus</i> spp.	Structure the intestinal barrier;	
	Jejunum	7-9	<i>Lactobacillus</i> spp., <i>Streptococcus</i> spp., <i>Staphylococcus</i> spp.	synthesize vitamins;	Uptake unsaturated fatty acids;
			<i>E. coli</i> , <i>Bacteroides</i> spp., <i>Clostridium</i> spp., <i>Coprococcus</i> spp., <i>Enterococcus</i> spp., <i>Klebsiella</i> spp., <i>Lactobacillus</i> spp. <i>Ruminococcus</i> spp.	metabolize carcinogens; stimulate intestinal immunity;	accumulate isomerized PUFAs;
	Ileum	7-8	<i>Coprococcus</i> spp., <i>Enterococcus</i> spp., <i>Klebsiella</i> spp., <i>Lactobacillus</i> spp. <i>Ruminococcus</i> spp.	produce anti-bacterial compounds; compete nutrients with pathogens	produce few SCFAs
	Cecum	5-7	<i>Firmicutes</i> Gr. ^b , <i>Bacteroidetes</i> Gr., <i>Actionobacteria</i> Gr., <i>Verrucomicrobia</i> Gr., <i>Proteobacteria</i> Gr. *	Break down indigestible fibers; produce numerous fatty acids; synthesize vitamins; neutralize toxic food ingredients	Digest reaching dietary fibers; higher amount of SCFAs produced; continue isomerizing alpha-linolenic acid, linoleic acid, oleic acid, etc.
Large intestine	Colon	5-7			
	Rectum	6-7			

^a Species; ^b Group; * Non-specified distribution in large intestine.

Table 1-6. Dietary intervention on SCFAs production

Substrates^a	Amount of substrate^b	SCFAs^c production (mg/mL)	Ratio of butyrate, acetate, and propionate
Cellulose		38.3	36:9:55
Methyl cellulose		43.1	51:25:24
Fructooligosaccharide		38.6	59:16:25
Glucose		44.0	70:10:20
Hydrolyzed huar gum	0.5g/100mL	54.6	35:29:36
Inulin		44.7	44:25:31
Hydrolyzed inulin		41.4	52:20:28
Psyllium husk		43.6	29:38:33
Soy oligosaccharide		41.8	43:29:28
Diet for mice	Daily intake (g/day)	Cecal SCFAs^d (mmol/L)	Ratio of butyrate, acetate, and propionate
Barley hulls	2	62	78:17:5
Cellulose	1	36	72:14:14
Fructooligosaccharide	1	61	74:17:9
Inulin	2	57	63:19:18
Oligofructose	1	50	71:19:10
Pectin	3	71	84:5:11
Resistant starch	5	92	76:17:7
Xylooligosaccharides	1	46	76:13:11
Diet for pig	Daily intake (g/day)	Cecal SCFAs^d (mmol/L)	Ratio of butyrate, acetate, and propionate
White rice	33	69	68:26:6
Brown rice	33	63	62:32:6
Oat bran	44	92	59:32:9
Rice bran	36	68	67:25:8
Wheat bran	42	131	60:31:9
Baked beans	45	124	65:30:5

^a Substrates used *in vitro* batch-culture fermentation with human faecal microflora

^b 0.5 g of each study substrates were added into 100 mL human faecal homogenate solution

^c Total concentration of SCFAs was measured with acetate, butyrate, propionate, and isobutyrate

^d Total cecal SCFAs included acetate, butyrate, propionate, and lactate

Table 1-7. Structures and formal names of linoleic acid and its common isomers


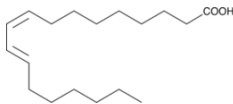
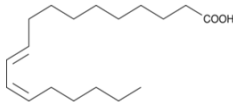
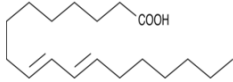
Synonyms	Formal name	Structure	Molecular formula	Formula weight
Linoleic acid; Telfairic acid	9Z,12Z- octadecadienoic acid			
c9, t11-CLA; Rumenic acid; Bovinic acid	9Z,11E- octadecadienoic acid		C ₁₈ H ₃₂ O ₂	280.5
t10, c12-CLA	10E,12Z- octadecadienoic acid			
t9, t11-CLA; Isolinoleic acid; Mangold's acid	9E,11E- octadecadienoic acid			

Table 1-8. LAB strains with their linoleic acid conversion ratio and converted isomers

LAB strain	Linoleic acid conversion (%)	Converted isomers		
		c9, t11-CLA	t10, c12-CLA	t9, t11-CLA
<i>L. acidophilus</i>	20.0	+	+	+
<i>L. brevis</i>	1.1	+	+	+
<i>L. bulgaricus</i>	7.6	+	+	+
<i>L. casei</i>	4.8	+	+	+
<i>L. curvatus</i>	1.6	+	+	-
<i>L. gasseri</i>	3.7	+	+	+
<i>L. helveticus</i>	4.7	+	+	+
<i>L. paracasei</i>	NA	+	-	-
<i>L. plantarum</i>	4.6	+	+	+
<i>L. reuteri</i>	26.0	+	+	+
<i>L. rhamnosus</i>	34.0	+	+	+
<i>L. lactis</i>	NA	+	+	-
<i>S. thermophilus</i>	33.0	+	-	-

NA: data not available; +: positive CLA production detected; -: no CLA production detected

Chapter 2: Functional properties of cocoa and peanut on common flora/probiotics and enteric bacterial pathogens

Introduction

Probiotics are live non-pathogenic microorganisms that could improve gut intestinal microbial balance as well as protect the host from infective agents once administered in host (Callaway et al., 2003). Major probiotics are essential part for human gut flora and often found as resident bacteria in milk and other dairy products. They can be divided into 2 main categories - colonizing species, such as *Lactobacillus*, *Lactococcus*, and *Enterococcus*, and free floating non-colonizing species including *Bacillus* and *Saccharomyces* (M. Sharma & Devi, 2014). These beneficial microbes colonizing in the gut are able to ameliorate the overall health of humans by restructuring the gut microbial balance (Nueno-Palop & Narbad, 2011). The possible characteristics of probiotic microorganisms include production of bacteriocins, propionic acid, and vitamin B12, and other various effects include increasing the villous length and nutrient absorption, immuno-stimulatory activities, and competitive exclusion of pathogenic microorganisms (West, Hammarström, & Hernell, 2009).

Gibson and Roberfroid (1995) first defined the term ‘prebiotics’ as referring to ‘non-digestible food ingredients which improve host health by selectively stimulating the growth and activity of limited numbers of bacteria in the colon’. Nine years later, in 2004, they reclassified the term as indicating ‘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’ (G.R. Gibson, 2006). Common

prebiotics include inulin, lactulose, and oligosaccharides, and their major properties are that they are amenable to colonic microflora fermentation while resistant to digestive enzyme metabolization in the human gut (Bielecka, Biedrzycka, & Majkowska, 2002b; Kolida, Tuohy, & Gibson, 2002b). Moreover, preliminary studies have revealed that several bifidogenic prebiotics could provide native microflora with rate-limiting nutrients and energy for fermentation, either by increasing levels of SCFAs and PUFAs for modulation of intestinal microflora composition or increasing production of antimicrobial byproducts as a competitive advantage promoting the exclusion of pathogenic bacteria (J. H. Cummings & Macfarlane, 2002; M. Fukuda et al., 2002; Videla et al., 2001).

Cocoa is a rich source of various polyphenols, including flavanols and procyanidins, which are free radical cleaning molecules with strongly antioxidant activity and anti-inflammatory potential (Bubonja-Sonje et al., 2011; Cai et al., 2004; Gu et al., 2006; Pérez-Berezo et al., 2012). The compounds from cocoa also have been shown to have numerous health-promoting properties such as reducing blood pressure, increasing the formation of endothelial nitric oxide, and promoting vasodilation (Ried et al., 2012). Previous reports found that *Lactobacillus* strains were one of the predominating bacterial species of cocoa bean fermentations (Lefeber et al., 2010; Papalexandratou et al., 2011), and there is also evidence that cocoa components like flavanols, multiple carbohydrates, and dietary fibers that reach the large intestine may have prebiotic-like benefits by promoting the growth of select beneficial gut microflora (Massot-Cladera et al., 2012; Tzounis et al., 2011). In addition, novel films of ethylenevinyl alcohol copolymer containing flavonoid-rich cocoa have been

developed and demonstrated to have an antimicrobial effect against several pathogens (Calatayud et al., 2013).

In 2003, the US FDA approved a qualified health claim that suggests consuming 1.5 ounces (42 g) of most nuts, including peanuts (approximately 0.5 to 1.0 g of peanut skin and 15 to 20 g of peanut flour), as part of a diet low in saturated fat and cholesterol may reduce the risk of heart disease. The benefits on reducing cardiovascular diseases (Bao et al., 2013; Isanga & Zhang, 2007; J. B. Jones et al., 2014; Ozcan, 2010) with daily intake of peanut or other nut products has been suggested to associate with their phenolic compound Resveratrol (3,4,5-trihydroxystilbene) (Bubonja-Sonje et al., 2011; Wang et al., 2011). Additionally, Resveratrol once reached large intestine may exert prebiotic-like benefits in large intestine by promoting the growth of selective beneficial gut microflora, especially *Lactobacillus* (Calatayud et al., 2013; Tzounis et al., 2011).

It has been tested that the composition of gut microbiota and its functions are affected by daily intake of various foods and beverages (Martin et al., 2010), and are under attack by foodborne enteric pathogens. Among these pathogens, EHEC, *Salmonella*, and *Listeria* have drawn the most attention (D. Adams et al., 2017; K. J. Cummings et al., 2012; Dussurget, 2008; Teunis et al., 2008). The human enteric bacterial pathogens interaction and their infection process is usually initiated by intestinal epithelial cell adhesion and following by cell invasion through site-specific ligands (Ahn & Biswas, 2014). Infections with these foodborne enteric pathogens and their severity are also highly influenced by normal gut microbiota, and the immunity of the host. In general, normal microflora colonization in human's gut is effective in competitively preventing foreign bacterial pathogens from attachment (Steinhoff,

2005). As a consequence, there is an increasing interest in the use of diet especially probiotics to modulate the composition of the colonic beneficial microflora for health-promoting effects such as immuno-stimulation and inhibition of pathogen while diminishing the negative effects which include carcinogen production and infection (Glenn R. Gibson et al., 2004a).

The aim of this work is to *in vitro* assessment of the effects of cocoa/peanut on probiotics from two folds: first, investigating the promotive effect on growth of beneficial bacteria including four strains of *Lactobacillus* and three resident bacteria in milk, and meanwhile detecting bio-active components in cocoa/peanut for explaining the noted growth promotion; second, assessing the effect of cocoa/peanut on stimulation of bio-active metabolites production by *Lactobacillus*. The findings of this research will provide insight into the beneficial properties of cocoa on human health based on gut microbes.

Material and Methods

Bacterial strain and growth condition. Four *Lactobacillus* strains, *Lactobacillus casei* (ATCC334), *L. rhamnosus* (ATCC11443), *L. plantarum* (ATCC39542) (gift from Dr. John A. Lindquist, University of Wisconsin Madison), and *L. acidophilus* (ATCC4356) were used as probiotics in this study. *Lactobacillus* strains were grown on de Man-Rogosa-Sharpe (MRS) agar at 37 °C overnight in the presence of 5% CO₂ (Forma™ Scientific CO₂ water jacketed incubator, Thermo Scientific, Massachusetts, USA). Three resident bacterial strains including *B. subtilis* (PIC 620), *E. faecalis* (PIC 522A), and *Streptococcus Salivarius* subsp. *thermophilus* (ATCC19258) were also tested in

this study. *B. subtilis*, *E. faecalis*, and *S. thermophilus* were grown on Braine-Heart-Infusion (BHI) agar at 37 °C overnight under aerobic conditions (Thermo Scientific MAXQ 4450, Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.). Foodborne bacterial pathogens Enterohemorrhagic *Escherichia coli* EDL933 (ATCC700927), *Salmonella enterica* serovar Typhimurium LT2 (ATCC19585), and *Listeria monocytogenes* LM2 (ATCC19115) were used in this study. EHEC, *S. Typhimurium*, and *L. monocytogenes* were grown on MacConkey agar, Luria-Bertani (LB) agar, and BHI agar (EMD Chemicals Inc., Gibbstown, N.J., U.S.A.), respectively, for 18 h at 37 °C under aerobic conditions (Thermo Scientific MAXQ 4450) (Thermo Fisher Scientific Inc., Waltham, Mass., U.S.A.).

Cocoa powder and peanut flour/skin preparation. Commercial, non-alkali treated cocoa was purchased at the local supermarket. Cocoa powder was defatted with hexane for 18 h and the residual hexane was evaporated from the cocoa powder before use. The defatted cocoa powder was stored at 4 °C and sterilized 2 h under ultraviolet light before experimental use. In shell Jumbo Virginia raw peanut was purchased from a local market and shelled by hand to isolate the kernel fractions. Peanut skin was removed by hand and the white kernel was ground to form peanut flour. Both peanut skin and peanut flour were defatted by 2 extractions with n-hexane (n-hexane: peanut portion 10 mL/g) for 12 h at room temperature (25 °C). Peanut flour and peanut skin fraction suspensions were prepared in sterilized distilled water (pH adjusted to 8.0 with 1 N NaOH), mixed well and sterilized with UV irradiation for 2 h. Cocoa powder or

peanut portion was mixed in MRS broth. The cocoa/peanut-MRS solution was used for HPLC-MS analysis.

Viability assay of *Lactobacillus* strains, milk resident bacteria, and foodborne bacterial pathogens in presence of cocoa and peanut. *L. casei*, *L. rhamnosus*, *L. plantarum*, and *L. acidophilus* on MRS agar plates were collected in phosphate buffer saline (PBS) and the optical density (OD) of the bacterial suspension was adjusted by PBS to an absorbance value of 0.2 at 600 nm which contains 10^7 CFU/mL bacterial cells using a LAMBDA BIO/BIO λ spectrophotometer (PerkinElmer, Beaconsfield, UK). A 400 mL aliquot of the bacterial suspension was added into either 3.6 mL MRS broth, whole milk, or skim milk in the presence or absence of 3% (w/v) cocoa powder in sterilized culture tube (Thermo Fisher Scientific Inc.) and incubated for different time points (0, 24, 48 and 72 h) at 37 °C under aerobic conditions with 5% CO₂ (Forma™ Scientific CO₂ water jacketed incubator). Serial dilutions were performed in PBS after each incubation period, followed by plating on MRS agar in triplicate. Bacterial CFUs on plates were counted after 24 h incubation and transformed into log values, and results were expressed as average number of CFU in log-scale. *B. subtilis*, *E. faecalis*, and *S. thermophilus* were grown and plated on BHI agar, and their viability in whole milk was investigated following the method described above. *L. acidophilus* was also recognized as well as resident bacteria in milk. Likewise, bacterial suspensions of EHEC, *S. Typhimurium*, and *L. monocytogenes* in PBS were adjusted to absorbance value of 0.100 at 600 nm to get 10^7 CFU/mL. LB broths with and without 3% (w/v) cocoa powder was used as media for growth comparison on different time periods (0, 3, 6, 9,

12, 24, 48, and 72 h). After incubation, foodborne pathogens were plated and counted on strain-specific agar plates (MacConkey agar for EHEC, Xylose Lysine Deoxycholate agar for *S. Typhimurium*, and Oxford *Listeria* agar base for *L. monocytogenes*) overnight at 37 °C under aerobic conditions (Thermo Scientific MAXQ 4450).

Determination of total phenolic contents in cocoa. The total phenolic content in each extract was estimated by Folin-Ciocalteu reagent described previously (Serajus Salaheen, Nguyen, Hewes, & Biswas, 2014). Briefly, to construct standard curve, 0, 0.25, 0.5, 1.0, 1.5, 2.0 mg/mL Gallic acid solutions were prepared for individual standard points. Supernatant containing soluble contents in cocoa was collected from 3% cocoa-water suspension by passing sterile 0.2 mm syringe filter (VWR). 20 mL of each Gallic acid solution or 3% cocoa sample supernatant was dissolved in 1.58 mL water and mixed with 100 mL Folin-Ciocalteu reagents (MP; CAT NO.195186). 300 mL of 7.5% Na₂CO₃ was added to the mixture and incubated at room temperature for 2 h. The absorbance was measured with spectrophotometer at wavelength of 765 nm. The total phenolic content was expressed as gallic acid equivalent (GAE).

Reversed-phase HPLC-tandem mass spectrometry (LC-MS/MS) analysis of metabolites. A Thermo Fisher LTQ Orbitrap XL (ThermoFisher Scientific, San Jose, CA) instrument coupled with an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA) via an electrospray ionization source was used for the analysis of metabolites extracted from the bacterial cultures. The HPLC was equipped with a micro

well plate autosampler and binary pumping device. Metabolites were separated using a Waters Atlantis T3 reversed-phase column (Waters Corp., Milford, MA) with 2.1×150 mm, 3.0 mm dimensions. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile, with a flow rate of 300 mL/min. A volume of 10 mL was loaded onto the column and separated using a 60 min gradient. Mobile phase B started at 0% and reached 95% at 41 min and remained at that level until 50 min, before reverting to 0% B at 60 min. The Orbitrap was operated in positive ion mode. The source voltage was 3.9 kV, capillary voltage was 29 V, and the tube lens voltage was 150 V. The capillary temperature was 275 °C, sheath gas flow was set to 30 (arb), auxiliary gas was set to 18 (arb), and sweep gas was set to 0. Data were acquired using data dependent scanning mode and dynamic exclusion was enabled. Fourier Transform Mass Spectrometry (FTMS) resolution of 60,000 with a mass range of 70e1100 m/z was used for full scan analysis and the FTMS was used for MS/MS data acquisition with a resolution of 7500. The top two most intense ions were acquired from scan 1 with a minimum signal of 1000, isolation width of 2, normalized collision energy of 35, default charge state of 1, activation Q of 0.250, and an activation time of 30 min. The samples were evaluated with Thermo Xcalibur software (version 2.1.0) and Agilent Mass Profiler Professional (version B.12). Signal peak intensities of metabolites from *L. casei* in cocoa-MRS solution were compared with the peak intensities values in MRS broth. Glutamic acids were used and analyzed as control. Relative intensity of each component was converted by normalized peak values. Peak annotations were made by querying the Human Metabolite Database (HMDB, <http://www.hmdb.ca/>), using a mass tolerance of <4 ppm.

Statistical Analysis. Data were analyzed by the Statistical Analysis System software. The one-way analysis of variance following by Tukey's test were used to evaluate the treatments and determine the significant differences on single time point among the control and the treatments based on significant level of 0.05.

Results

Comparative growth of *L. casei*, *L. plantarum*, and *L. rhamnosus* in MRS broth with and without cocoa powder. The stimulatory effect of cocoa powder on beneficial *Lactobacillus* strains was evaluated in liquid cultures and measured by the plate counting method. The effect of cocoa powder on the growth of *L. casei*, *L. rhamnosus*, and *L. plantarum* is shown in Figure 2-1. In the presence of 3% cocoa powder, the growth of all three *Lactobacillus* strains significantly increased (0.27 CFU/mL for *L. casei*, 0.61 CFU/ mL for *L. rhamnosus*, and 0.36 CFU/mL for *L. plantarum*) in MRS broth at 24 h. However, as the living numbers of *Lactobacillus* in control condition decreased after 24 h, cocoa showed different effects depending on the strains. The growth of *L. casei* (Figure 2-1A) and *L. rhamnosus* (Figure 2-1B) was continuously increased after 24 h, whereas growth stimulation of cocoa powder on *L. plantarum* (Figure 2-1C) became insignificant. Among the *Lactobacillus* strains, *L. casei* was most affected by cocoa powder in MRS broth as it was stimulated by more than 1 log CFU/mL at 48 and 72 h in time-dependent manner.

Growth stimulation of *Lactobacillus* strains and various milk resident bacteria in whole milk and skim milk supplemented with cocoa powder. The effect of 3% cocoa powder in whole milk and skim milk on the growth of three *Lactobacillus* strains (*L. casei*, *L. rhamnosus*, and *L. plantarum*) as well as different resident bacterial strains including *L. acidophilus*, *B. subtilis*, *E. faecalis*, and *S. thermophilus* were investigated. The stimulatory effect of cocoa powder on *Lactobacillus* strains is shown in Figure 2-2. Cocoa powder (3%) in both whole milk and skim milk exhibited a significant and continuous growth stimulatory effect on all three *Lactobacillus* strains at 24, 48 and 72 h time points. We observed a greater than 1 log CFU/mL increase at the 24 h time point for all three strains in whole milk. Furthermore, cocoa powder in milk had a longer lasting growth stimulating effect (more than 1 log CFU/mL) on *L. plantarum* (Figure 2-2C) compared with it in MRS broth (Figure 2-1C).

The overall effect of 3% cocoa powder on all four resident bacteria is shown in Figure 2-3. We found significant and continuous growth stimulatory effect of cocoa in whole milk on all these four resident bacterial strains at different time points (24, 48 and 72 h). Among these bacterial strains, the maximum growth promotions were observed for *L. acidophilus* (Figure 2-3A) which was promoted by 1 log CFU/mL within 72 h and *E. faecalis* (Figure 2-3C) whose growth was also stimulated by 1 log CFU/mL within 48 h in the presence of cocoa in milk.

Growth inhibition of EHEC, *S. Typhimurium* and *L. monocytogenes* by cocoa powder. Antimicrobial activity of cocoa powder against selected foodborne bacterial pathogens was assessed in liquid cultures by plate counting. The inhibitory effect of 3% cocoa

powder in LB broth on three of the most common foodborne pathogens, EHEC, *S. Typhimurium*, and *L. monocytogenes* is shown in Figure 2-4. The growth of each pathogen was significantly ($p < 0.05$) reduced during the first 9 h of incubation with cocoa powder. With 3 more hours extended incubation, cocoa powder continues to exhibit significant inhibitory effect on EHEC (Figure 2-4A). Cocoa reduced the growth of EHEC (Figure 2-4A) and *S. Typhimurium* (Figure 2-4B) by more than 1 log CFU/mL during the first 6 h, however after 12 h of incubation, the effect was attenuated. Growth of *L. monocytogenes* was reduced by cocoa powder by 0.3 logs CFU/mL ($p < 0.05$) during the first 9 h, however after 9 h this reduction was minimized to be neglected (Figure 2-4C). No statistically significant growth promotion was found in three pathogens within 72 h.

Comparative growth of *L. casei*, *L. rhamnosus*, and *L. plantarum* in the presence and absence of peanut flour and skin extract in MRS broth. Growth conditions of 3 beneficial *Lactobacillus* strains were evaluated in MRS broth in the presence and absence of 0.5% peanut flour or peanut skin extract. Comparative growth of *L. casei*, *L. rhamnosus*, and *L. plantarum* between control and treatments is shown in Figure 2-5. In the presence of 0.5% peanut flour, out of the 3 strains of *Lactobacillus*, *L. casei* (Figure 2-5A) and *L. rhamnosus* (Figure 2-5B) were stimulated at 24, 48, and 72 h time points of incubation in MRS broth. Out of these 2 strains, the most intensive stimulatory effect was found on *L. casei*, whose growth was promoted by more than 1 log CFU/mL within 72 h. However, peanut flour had no significant effect on the growth of *L. plantarum* and it grew similarly as it did in MRS broth in absence of peanut flour

(Figure 2-5C). In the same study, it was found that *L. casei*, *L. rhamnosus*, and *L. plantarum* were significantly inhibited by >2 log CFU/mL at 24, 48, and 72 h time points in MRS broth supplemented with 0.5% peanut skin extract (Figure 2-5).

Growth of *Lactobacillus* strains in milk supplemented with peanut skin extract. In order to further investigate the growth inhibitive effect of 0.5% peanut skin extract, the growth of 3 beneficial *Lactobacillus* strains were also examined in whole milk which serves as a natural reservoir for *Lactobacillus*. Time-dependent comparative growth performance of *L. casei*, *L. rhamnosus*, and *L. plantarum* in the presence of peanut skin extract between controls and treatments are shown in Figure 2-6. *Lactobacillus* strains showed overall better and longer growth in whole milk compared to MRS broth after 48 h. However, the growth of all 3 strains of *Lactobacillus* was significantly inhibited by 0.5% peanut skin extract in whole milk. The growth of *L. casei* (Figure 2-6A), *L. rhamnosus* (Figure 2-6B), and *L. plantarum* (Figure 2-6C) was inhibited by >2 log CFU/mL at 24, 48, and 72 h time points in the presence of 0.5% peanut skin extract in whole milk, compared to growth in whole milk alone.

Effect of peanut flour or peanut skin extracts on growth of EHEC, *S. Typhimurium*, and *L. monocytogenes*. The antimicrobial properties of 0.5% peanut flour and 0.5% peanut skin extract against selected foodborne bacterial pathogens (EHEC, *S. Typhimurium*, and *L. monocytogenes*) were evaluated in liquid cultures. Among these selected 3 enteric bacterial pathogens, 0.5% peanut flour only showed inhibitory effect on EHEC (Figure 2-7A). At 24, 48, and 72 h time point of incubation, the growth of

EHEC in the presence of 0.5% peanut flour was significantly inhibited by a range of 0.5 to 1 log CFU/mL. However, 0.5% peanut flour exhibited no significant negative effect on the growth of *S. Typhimurium* (Figure 2-7B) or *L. monocytogenes* (Figure 2-7C) within 72 h. On the contrary, EHEC (Figure 2-7A) and *S. Typhimurium* (Figure 2-7B) were stimulated slightly by 0.5% peanut skin extract within 72 h. Peanut skin extract only showed inhibitory effect on *L. monocytogenes* (Figure 2-7C).

Total phenolic compounds concentration in 3% cocoa. Total phenolic content was estimated by using Folin-Ciocalteu reagent, determined from the supernatant part obtained from 3% cocoa-water solution, and expressed as mg/mL GAE. Based on triplicate measurement, concentration of phenolic compounds in 3% cocoa was 0.848 ± 0.068 mg/mL GAE.

HPLC-MS based analysis of metabolites from *L. casei* in cocoa solution. After the structural match based on HMDB database, total of 350 compounds in 3% cocoa-MRS solution, 284 compounds from supernatant of *L. casei* overnight culture in MRS broth, and 284 compounds from supernatant of *L. casei* overnight culture in MRS broth supplemented with 3% cocoa were identified by LC-MS/MS analysis (lists showed in supplemental data). Table 2-1 summarizes some of the bio-active metabolites of *L. casei* identified in 3% cocoa solution with their classes, known biological functions, and relative intensities. They belong to steroids (Androsterone and Corchoroside B), disaccharides (1-(3-Methylbutanoyl)-6-apiosylglucose, and Methyl cellulose), indoles (1H-Indole-3-carboxaldehyde, and 3-Methylene-indolenine), indazole (13-OxoODE as

lineolic acid, and Nigellidine), and Glycerophospholipid (LysoPC (18:0)) classes and were potential nutrients. Six possible antimicrobial compounds (Epicatechin, Quercetin 3-galactoside, Rubraflavone D, 2⁰,4-Dihydroxy-4⁰,6⁰-imethoxy-3⁰-prenylchalcone, Glicoisoflavanone, and 3-Hydroxyglabrol) which belong to the class of flavonoids were also identified. Two compounds (Benzoic acid and Benzonatate) are benzoic acid and its derivative, among which Benzoic acid and 3-Hydroxyglabrol have already been recognized as antimicrobials. Citrusin E originated from lemon peel were also detected.

Discussion

Previous reports have demonstrated that phenolic compounds from plant products could inhibit the growth of several harmful bacteria such as *S. Typhimurium* (Puupponen-Pimiä et al., 2005) and EHEC (Bubonja-Sonje et al., 2011). Resveratrol, one of the known compound of polyphenol, has been shown to extend the doubling time of multiple bacterial pathogens including *S. Typhimurium*, *S. Enteritidis*, *E. coli*, and so on (C. M. Jung, Heinze, Strakosha, Elkins, & Sutherland, 2009). In our study, the growth of the Gram-negative foodborne pathogen EHEC was inhibited by nearly 1 log CFU/mL within 48 h of incubation in the presence of peanut flour, and the Gram-positive foodborne pathogen *L. monocytogenes* was inhibited by more than 1 log CFU/mL at 24 h by use of peanut skin extract. Moreover, significant growth inhibition against both EHEC and *L. monocytogenes* was found within 72 h. This study indicates that components of peanut flour and peanut skin exhibit both different scopes and efficacies of antimicrobial effects depending on bacterial species. However, the growth stimulatory effect of peanut skin extract on *S. Typhimurium* might provide *Salmonella*

opportunities to survive or multiply in peanut products especially unblanched peanut butter.

We also observed that the growth of *L. casei* and *L. rhamnosus* were continuously and significantly stimulated up to 72 h by 0.5% peanut flour. This result agreed with the previous research of Salaheen *et al.*, (2014), which suggested that the production of fatty acids, one growth factor for *Lactobacillus*, was promoted by water-soluble peanut flour fractions. However, in our study, no significant stimulatory effect on *L. plantarum* was observed within 72 h incubation in MRS broth supplemented with peanut flour. The possible explanation might be based on *padA* gene, a substrate-inducible gene responsible for phenolic acid decarboxylase enzyme expression, which counteract the promotive effect on this special *Lactobacillus* (Gury, Barthelmebs, Tran, Diviès, & Cavin, 2004). In the same study, we also evaluated the growth of 3 *Lactobacillus* strains in both MRS broth and whole milk supplemented with 0.5% peanut skin extract. Our data indicated that the growth of all 3 *Lactobacillus* strains was drastically reduced by peanut skin extract within 72 h, no matter whether in broth or milk compared with in controls. Based on study in 2005, peanut skins are considered as having no adverse effects and would qualify as a GRAS (generally recognized as safe) product (Yu, Ahmedna, & Goktepe, 2010). Beside safety, peanut skins extract possess even much greater *in vitro* antioxidant properties than vitamin C and vitamin E (Yu *et al.*, 2010). However, no previous study has examined the role of peanut skins play on growth of beneficial bacteria. In general, our finding indicates that components in peanut skin might impair the *in vitro* growth of *Lactobacillus*. Further investigation

is needed to know how peanut skin extract and its compounds inhibit the growth of *Lactobacillus*.

This study also describes the potential role of cocoa in promoting growth of probiotics. Previous study on polyphenolic compounds-containing beverages like berry juices demonstrated the promotive effect on the growth of probiotic bacteria including *Lactobacillus* (Puupponen-Pimiä *et al.*, 2005; Lacombe *et al.*, 2012; H. Yang *et al.*, 2014). Similarly, in this study, we observed that the growth of 4 *Lactobacillus* strains and 3 other milk resident bacteria were all generally promoted by cocoa powder. Study on the relationship between polyphenol-rich diet and gut microbiota composition (Etxeberria *et al.*, 2013; Hervert-Hernández, Pintado, Rotger, & Goñi, 2009; Parkar, Stevenson, & Skinner, 2008), and research about cocoa on modulating fecal microbiota (Massot-Cladera *et al.*, 2012) give some clues that the presence of polyphenol compounds as well as nutritious components like disaccharides and indoles in cocoa detected by LC-MS/MS might be the reason for probiotic bacterial growth stimulation in our observation. No significant stimulatory effect on *L. plantarum* was observed after 24 h incubation in MRS broth supplemented with cocoa powder. A substrate-inducible *padA* gene in *L. plantarum* encoding for a phenolic acid decarboxylase enzyme capable of converting phenolic compounds into several derivatives (Gury *et al.*, 2004) might be one possible explanation for no significant growth stimulation after 24 h in MRS broth supplemented with cocoa. Although milk is known to provide more abundant nutrients, the reason for different stimulatory abilities of cocoa in MRS broth and milk medium warrants further investigation. In the same study, four resident bacteria (*L. acidophilus*, *B. subtilis*, *E. faecalis*, and *S. thermophilus*) in milk showed significant and continuous

growth promotion in whole milk supplemented with 3% cocoa powder. These results supported the idea of using cocoa as a probiotic growth stimulus in milk and dairy products, which could benefit human health after consuming.

Flavonoid compounds of plant origin have gained considerable attention due to their functional and nutritional benefits including anti-inflammatory, antimicrobial activity and some other beneficial effects (Cooper et al., 2007; Cushnie & Lamb, 2005; M. Kim et al., 2010). Cocoa rich in flavonoids were believed to act as antimicrobials by depriving substrates required for microbial growth, inhibiting extracellular microbial enzymes, or inhibiting oxidative phosphorylation (Kanazawa et al., 2005). This study demonstrates the short-time inhibitory effects of cocoa against two Gram-negative foodborne pathogens (EHEC and *S. Typhimurium*) and a Gram-positive pathogen (*L. monocytogenes*), which is parallel with previous reports that have already indicated the role of flavonoids and phenolic compounds in cocoa as well as other plant products in inhibition of the growth of harmful bacteria (Bubonja-Sonje et al., 2011; Puupponen-Pimiä et al., 2005; Vargas et al., 2013). However, the evidence in our study only suggests that cocoa powder may be active against both Gram-negative and Gram-positive foodborne pathogens for the first 9 h. In accordant with previous study, the minimum inhibitory concentrations (MICs) of blueberry and blackberry pomace extract on *Campylobacter jejuni* are 0.4 and 0.6 mg/mL GAE (Serajus Salaheen, Nguyen, et al., 2014). Based on total phenolic content estimated by Folin-Ciocalteu in this study, 3% cocoa contains 0.848 ± 0.068 mg/ mL GAE, which is high enough for antimicrobial activities. However, attenuated inhibitory ability against bacterial pathogens after 9 h could be due to nutritious components in cocoa powder were

provided as extra sources for pathogens with limited carbon sources, which acts as growth promoting agents, neutralizing the effect of antimicrobials (including phenolic compounds and flavonoids) in cocoa. In addition, in accordance with the potential antimicrobial agents in cocoa analyzed by LC-MS/MS in this study, synergistic application of flavonoids, benzoic acids and derivatives, and citrus-derivative compounds are promising to be combined in order to reduce the level of foodborne illness as well as minimizing the wide spread of multidrug resistant bacteria.

Conclusions

1. Bio-active steroids, indoles, indazoles, and glycerophospholipid ingredients found in cocoa/peanut provided extra nutrients on probiotics; antimicrobial components like flavonoids, benzoic acids, and citrussin were detected in cocoa/peanut.
2. Cocoa and peanut flour stimulated the growth of multiple *Lactobacillus* strains. However, peanut skin induced negative effects on growth of *Lactobacillus*.
3. Cocoa and peanut induced no change or short time period-reduction on enteric bacterial pathogens.

Figure and Table list

Figure 2-1. Effect of cocoa on bacterial growth in MRS broth. *L. casei* (A), *L. rhamnosus* (B), and *L. plantarum* (C) strains were grown with 3% cocoa powder in MRS broth, and sampled at 0, 24, 48, and 72 h for the growth measurements. Asterisks (*) indicate significant difference between control and treatment at $p < 0.05$.

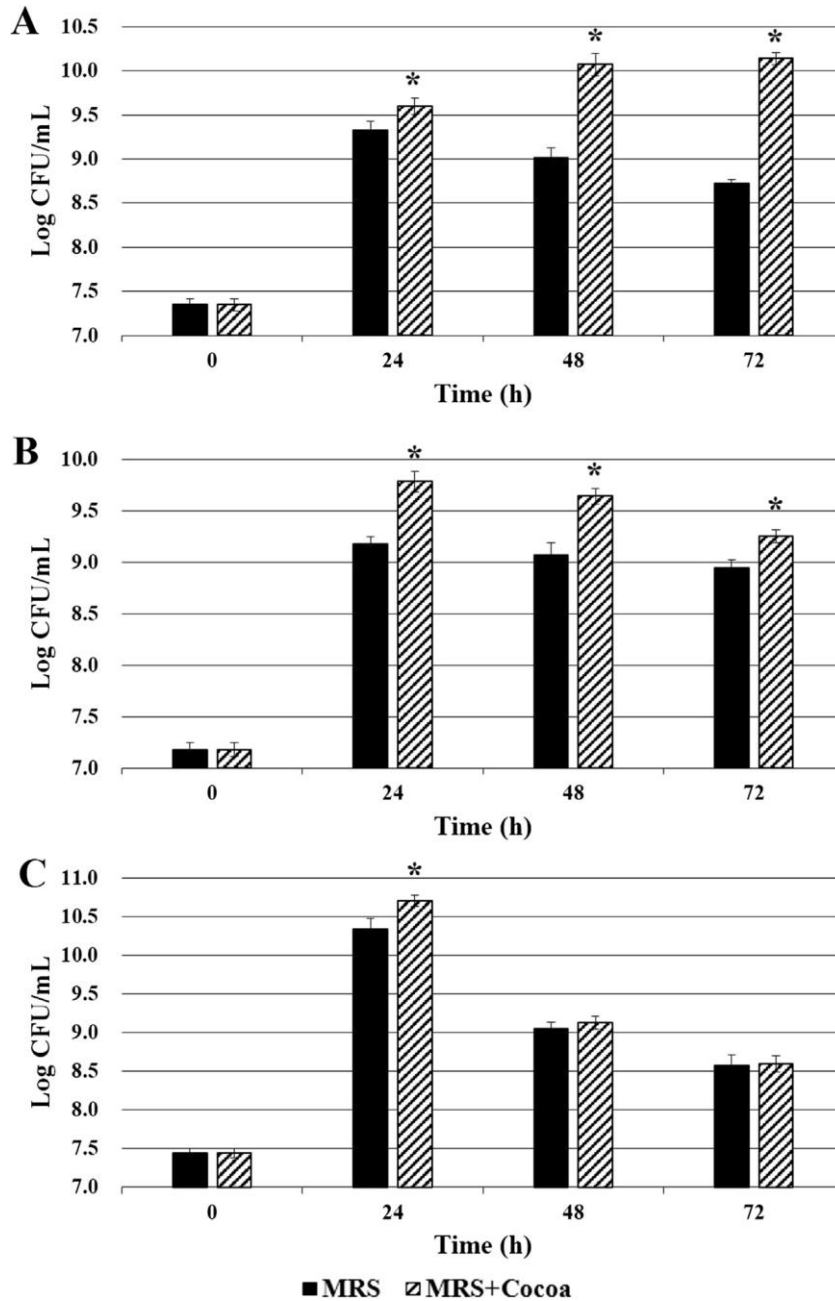


Figure 2-2. Effect of cocoa on bacterial growth in milk. *L. casei* (A), *L. rhamnosus* (B), and *L. plantarum* (C) were grown in either whole milk or skim milk with or without 3% cocoa powder, and sampled at 0, 24, 48, and 72 h. Asterisks (*) indicate significant difference at $p < 0.05$.

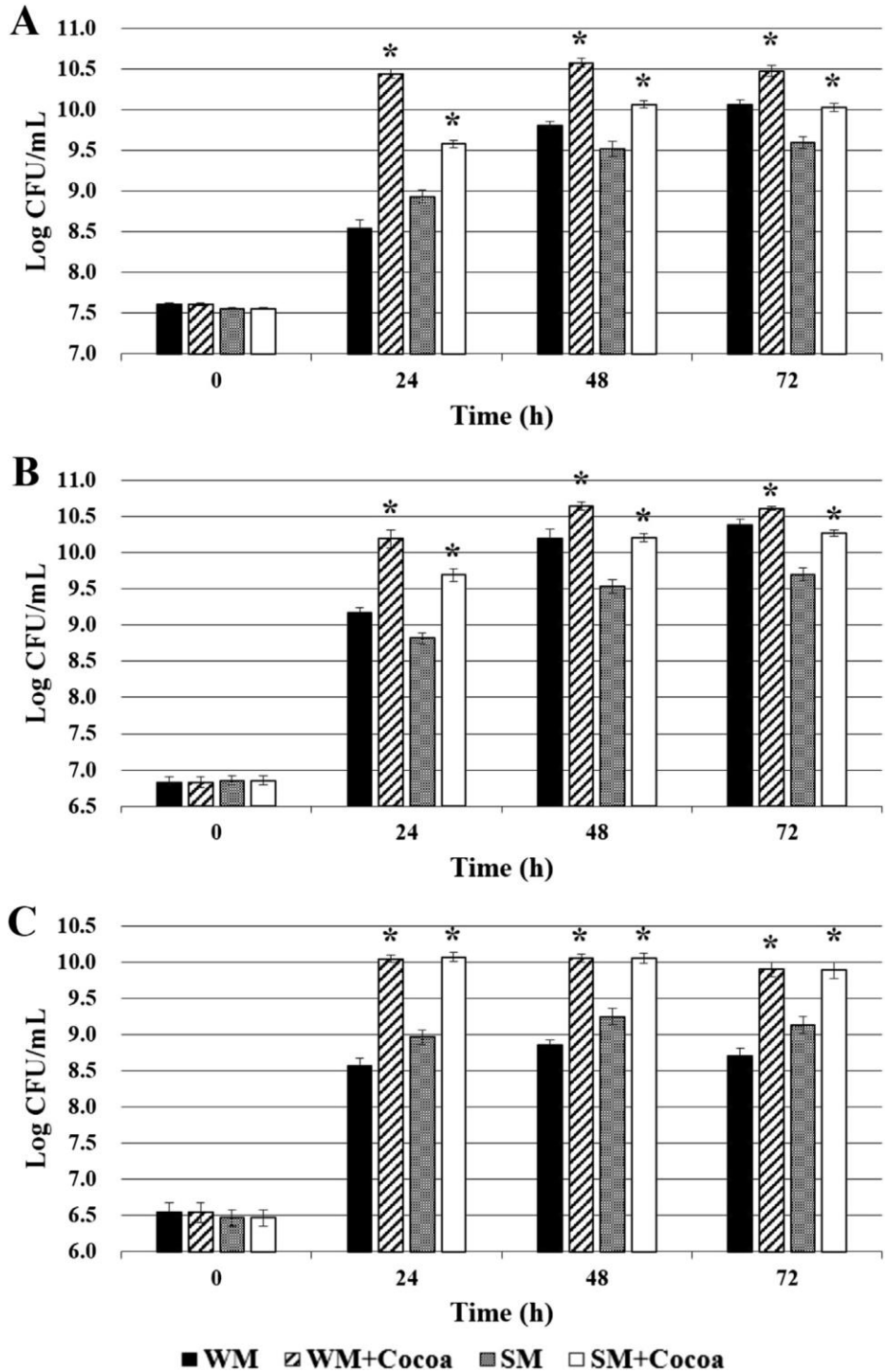


Figure 2-3. Growth stimulation of *Lactobacillus acidophilus* (A), *Bacillus subtilis* (B), *Enterococcus faecalis* (C), and *Streptococcus thermophilus* (D) in whole milk with or without 3% cocoa powder. Cultures were sampled at 0, 24, 48, and 72 h for growth measurements. Error bars indicate standard deviation. Asterisks (*) indicate significant difference at $p < 0.05$.

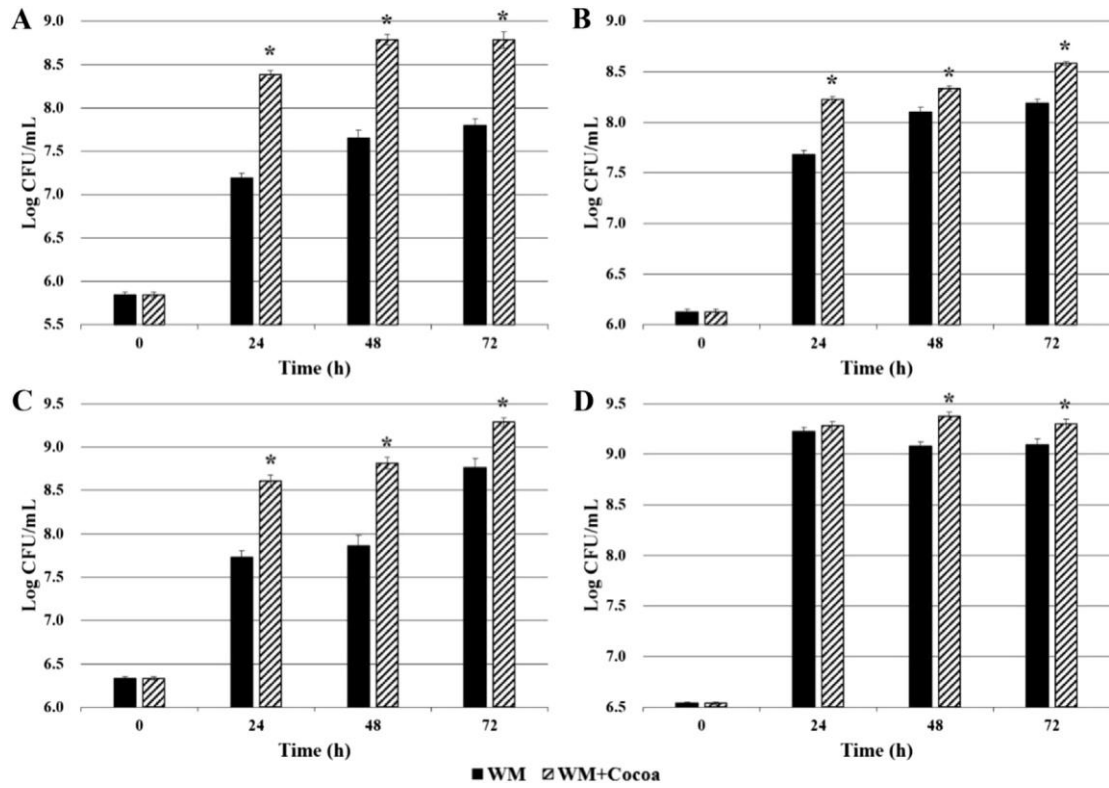


Figure 2-4. Cocoa inhibits growth of enteric pathogenic bacteria. EHEC (A), *S.* Typhimurium (B), and *L. monocytogenes* (C) were grown in strain-specific broth with or without 3% cocoa powder. Bacterial CFU were counted at 0, 3, 6, 9, 12, 24, 48, and 72 h time points. Asterisks (*) indicate significant difference at $p < 0.05$.

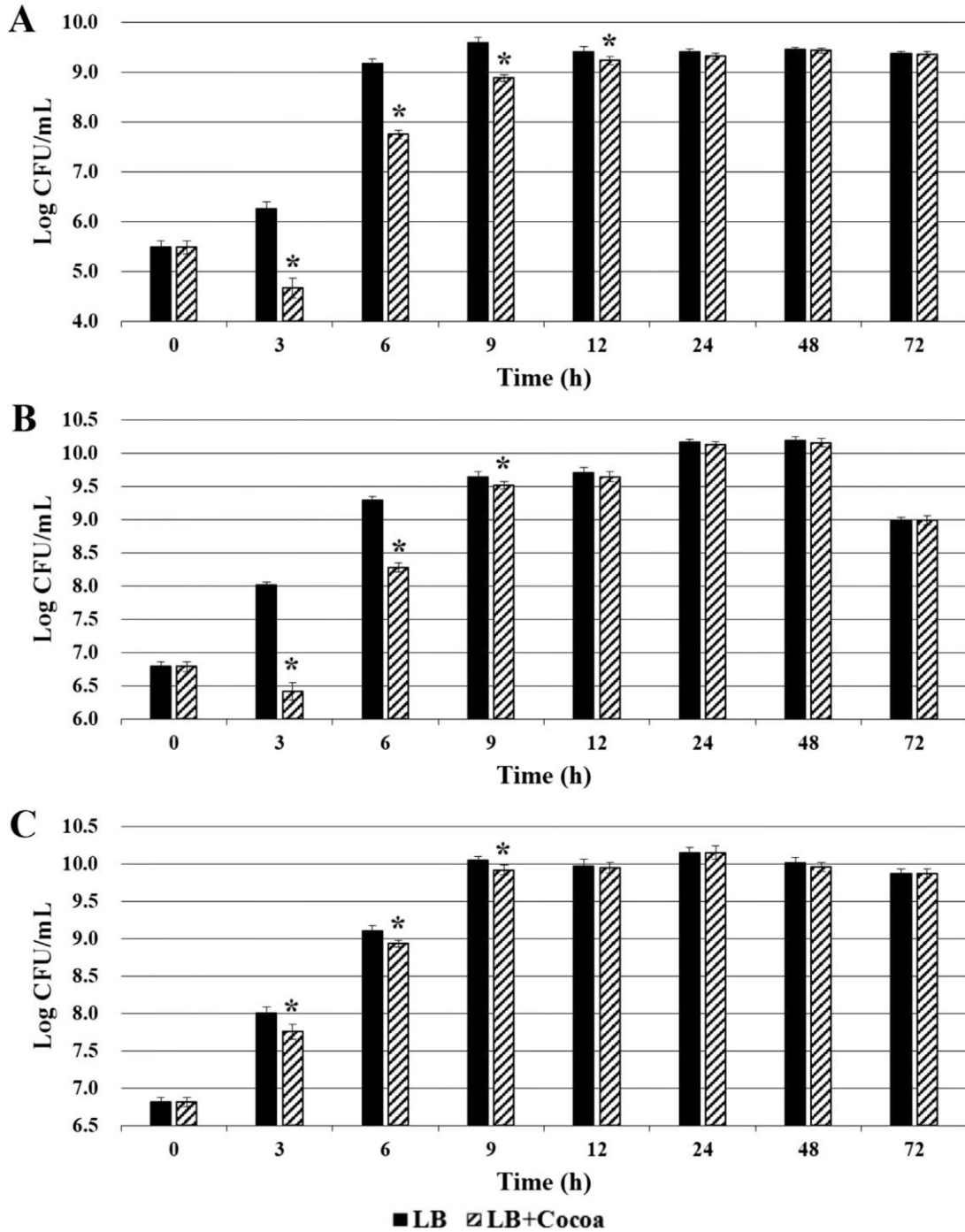


Figure 2-5. Growth stimulation by 0.5% peanut flour and inhibition by 0.5% peanut skin extract on *L. casei* (A), *L. rhamnosus* (B), and *L. plantarum* (C) in MRS broth at 0, 24, 48, 72 h. Error bars indicate standard deviation. Different letters within a time point indicate significant difference between control and treatment at $p < 0.05$.

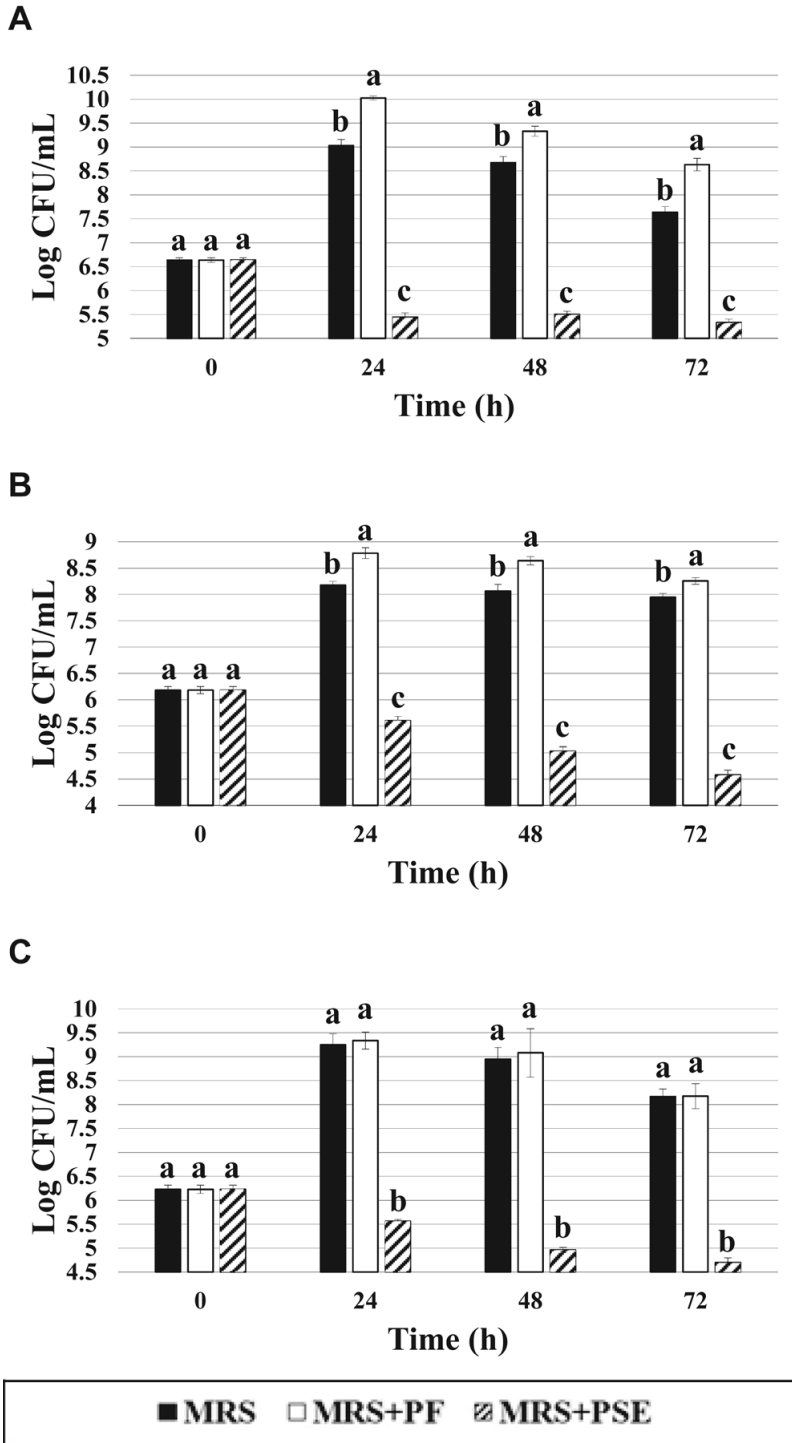


Figure 2-6. Growth inhibition of *L. casei* (A), *L. rhamnosus* (B), and *L. plantarum* (C) in MRS broth, whole milk, and whole milk with 0.5% peanut skin extract at 0, 24, 48, 72 h. Error bars indicate standard deviation. Different letters within a time point indicate significant difference between control and treatment by Tukey's test at $p < 0.05$.

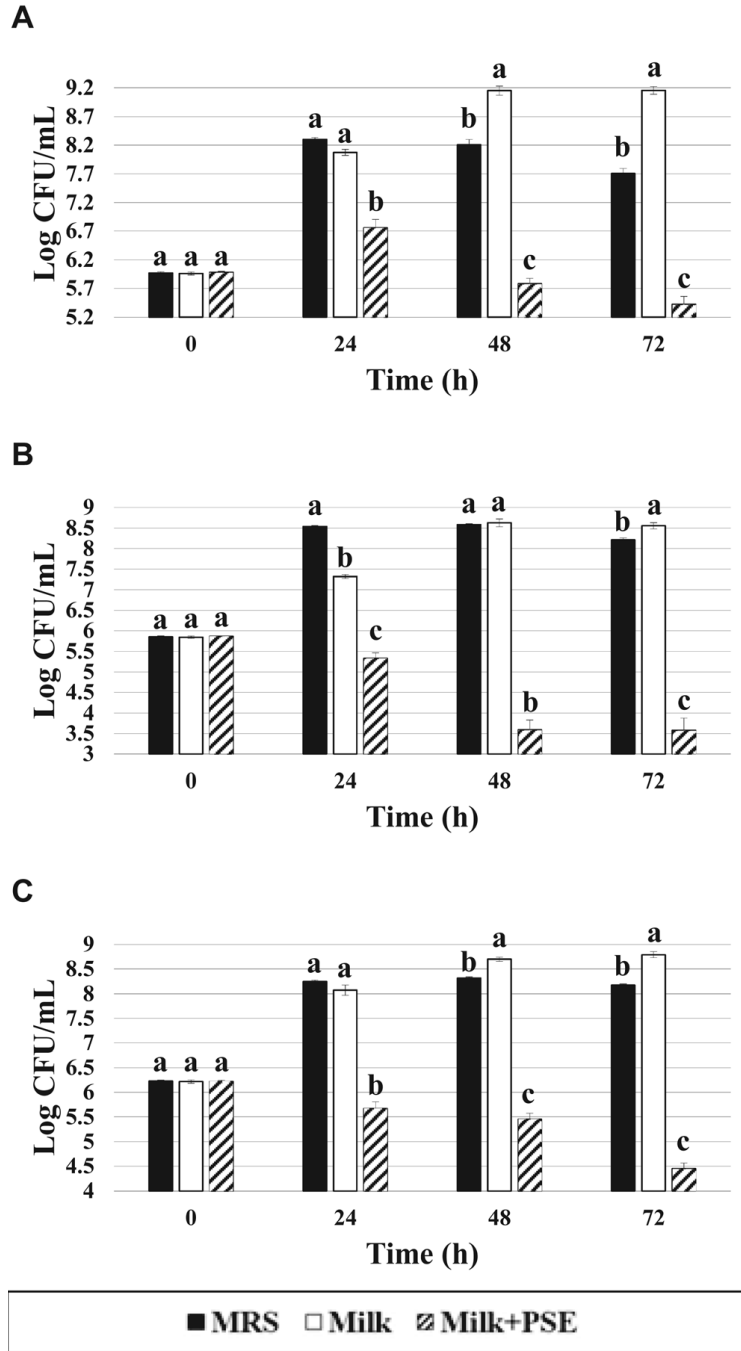


Figure 2-7. Growth conditions of EHEC (A), *S. Typhimurium* (B), and *L. monocytogenes* (C) in LB broth, with 0.5% peanut flour, and with 0.5% peanut skin extract at 0, 24, 48, 72 h. Error bars indicate standard deviation. Different letters within a time point indicate significant difference between control and treatment at $p < 0.05$.

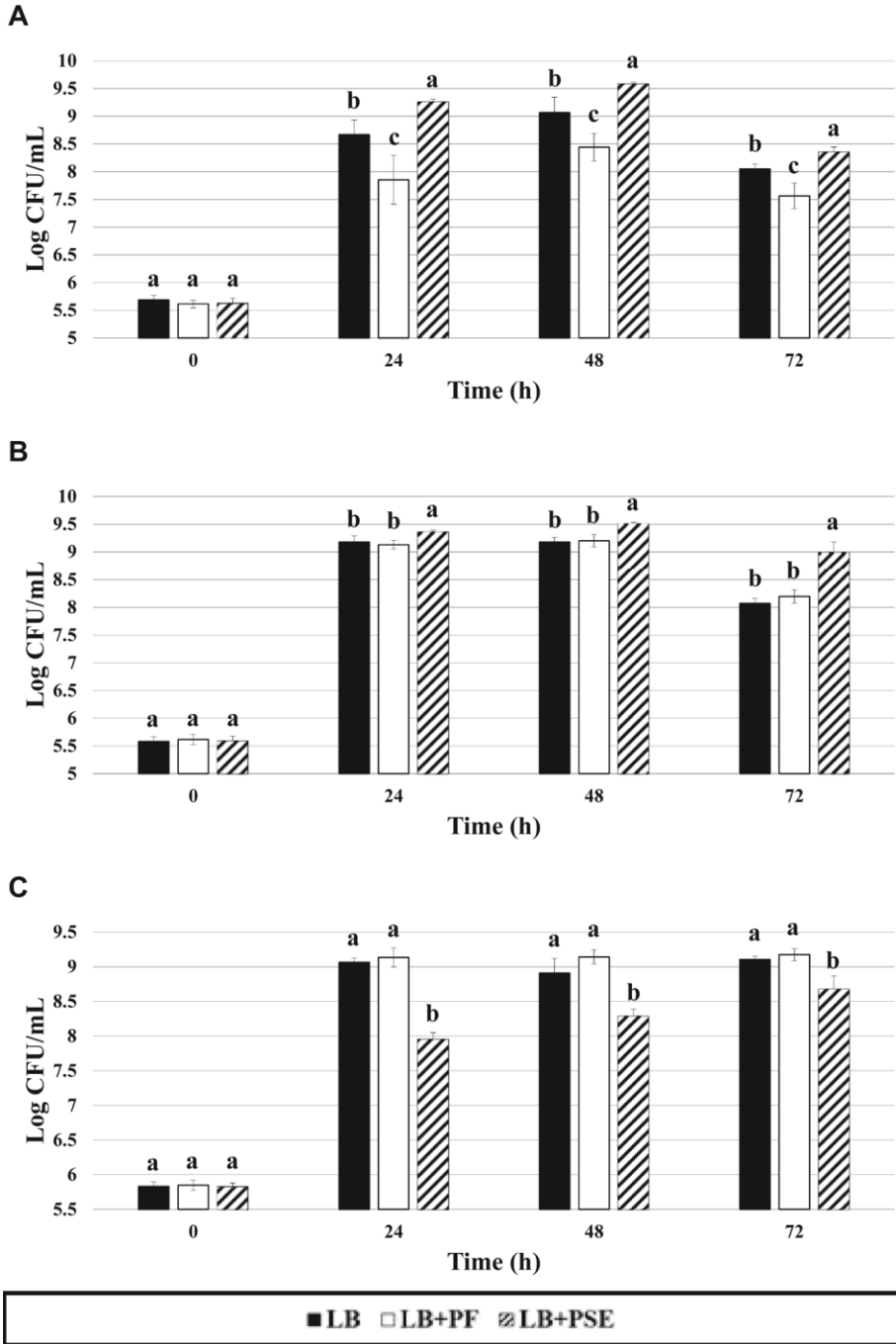


Table 2-1. Some putative bio-active components identified in cocoa/peanut by HPLC-MS

Compound name	Class	Biological Functions	Relative Intensities*
Epicatechin	Flavonoids	Not available	0.016
Quercetin 3-galactoside	Flavonoids	Not available	0.066
Rubraflavone D	Flavonoids	Not available	0.069
2',4-Dihydroxy-4',6'-imethoxy-3'-prenylchalcone'	Flavonoids	Nutrient supply	0.075
Benzoic acid	Benzoic Acid and Derivatives	Fungistatic	0.111
Benzonate	Benzoic Acid and Derivatives	Antitussives	0.251
Citrusin E	Glycosyl Compounds	Nutrient supply	0.438
Androsterone	Steroids and Steroid Derivatives	Energy source Membrane integrity maintenance	0.610
1-(3-Methylbutanoyl)-6- <i>apiosyl</i> glucose	Disaccharides	Nutrient supply	0.881
13-OxoODE	Lineolic Acids and Derivatives	Energy source Membrane integrity maintenance	0.702
Methyl cellulose	Disaccharides	Nutrient supply	1.035
Corchoroside B	Steroids and Steroid Derivatives	Energy source Membrane integrity maintenance	1.526
1H-Indole-3-carboxaldehyde	Indoles	Nutrient supply	1.630
3-Methylene-indolenine	Indoles	Nutrient supply	1.658
Nigellidine	Indazoles	Nutrient supply	1.935
Glicoisoflavanone	Flavonoids	Nutrient supply	7.845
3-Hydroxyglabrol	Flavonoids	Antimicrobial	16.204
LysoPC(18:0)	Glycerophospholipids	Energy source Membrane integrity maintenance	47.844

* Relative intensity of each compound is normalized by peak intensity value of glutamic acids

Chapter 3: Synergistic effects of prebiotic-like cocoa/peanut and *Lactobacillus* in control of enteric bacterial pathogens

Introduction

The Center for Disease Control and Prevention estimated that 48 million illnesses, 128000 hospitalizations, and 3000 deaths are caused by foodborne pathogens (D. Adams et al., 2017). Among bacterial pathogens, EHEC, *Salmonella*, and *L. monocytogenes* are the leading causes of foodborne illness and deaths in the US (K. J. Cummings et al., 2012; Dussurget, 2008; Teunis et al., 2008). Consequently, their prevention and growth inhibition are of great concern to public health professionals, farmers, and food producers. Antibiotics are commonly used either orally through drinking water or as feed additives (M Peng et al., 2014) for control of zoonotic pathogen colonization in gut of farm animal including cattle, swine, and chicken (Scallan et al., 2011). However, it was demonstrated to worsen the niche for beneficial gut microflora (Andersson & Hughes, 2010) and accelerate the drug-resistance in human pathogens (DeWaal et al., 2013). Therefore, there is increasing interest in use of dietary supplement, especially prebiotics, to modulate the composition of the colonic microflora. The promotion of the growth of probiotics by prebiotics including prebiotic-like components like cocoa and peanut, in turn, is hypothesized to inhibit or even exclude the harmful bacteria like foodborne bacterial pathogens (Glenn R. Gibson, Probert, Loo, Rastall, & Roberfroid, 2004b).

To colonize in the human GI tract, all foodborne pathogens must compete with gut microflora specifically in the large intestine, where a huge amount of resident

microbiota are colonized (Sullivan & Nord, 2002). Probiotic such as lactic acid bacteria (LAB) are known to play crucial roles in maintaining the microbial ecosystem of human GI tract by preventing colonization and infection of incoming bacterial pathogens (Campana, Federici, Ciandrini, & Baffone, 2012; Maldonado Galdeano & Perdigón, 2006; Servin & Coconnier, 2003). Though the molecular basis has not been fully understood, possible mechanisms of the protection against pathogens by probiotics include stimulating innate and acquired immune response of human intestinal cell (Neeser et al., 2000; Reid & Burton, 2002), direct antimicrobial effects (Van de Guchte, Ehrlich, & Maguin, 2001), and competition in receptor mediated colonization to cell hosts (Sherman, Bennett, Hwang, Sherman, & Bevins, 2005). Moreover, (Medellin-Peña *et al.* (2007) hypothesized that intestinal bacteria in different genera may all use quorum sensing as regulatory system for the control of virulent genes of foodborne pathogens. They also found that secreted compounds of *L. acidophilus* inhibited the production of AI-2 molecules of EHEC O157 as well as altered flagella synthesis and motility of the pathogen. Therefore, it is possible that probiotics in the human intestine, particularly in the large intestine, influence the virulence gene expression of other non-resident bacteria through their quorum sensing system, and thus prevent the pathogenic bacterial infection by affecting or limiting bacterial motility, flagella assembly, specific protein synthesis and secretion, and other pathogenic mechanisms (Sperandio, Li, & Kaper, 2002). Meanwhile, increased interests have been put on investigating the role of probiotic bacteria for maintaining human health and personal hygiene, including oral health, GI tract health and vaginal hygiene, as well as preventing wound infection during operation (T. H. Lin & Pan,

2014; Parvez, Malik, Ah Kang, & Kim, 2006; Wannun, Piwat, & Teanpaisan, 2014). These health promoting activities by LAB have been proposed to be associated with their growth inhibiting effect against human pathogens.

Recent research on LAB revealed their abilities of multiple antimicrobial production (D. Sharma & Singh Saharan, 2014), which suggests the selection of certain *Lactobacillus* strains as promising biological preservatives against foodborne bacterial pathogens. Antimicrobial compounds produced by LAB include organic acids, hydrogen peroxide, diacetyl, SCFAs, small peptide inhibitors, bacteriocins, and bio-surfactants, among which bacteriocins have been recognized as the most potent agent (Miao et al., 2014; D. Sharma & Singh Saharan, 2014). Nisin for example, produced by *Lactococcus lactis*, has been approved by the US FDA since last decade for food preservation and shelf life extension (Collins, Guinane, Cotter, Hill, & Ross, 2012). The most common application of *L. casei*, the resident bacteria in human intestine and mouth, is for dairy production such as yogurt, cheese, and ice cream. Recently, an extensive study has been focused on the use of *L. casei* in preventing AAD and *Clostridium difficile* infections (McFarland, 2009). In addition, *L. casei* has been suggested to be effective in alleviating GI pathogenic bacterial infections both *in vitro* and *in vivo* (Chung & Yousef, 2010; Forestier, De Champs, Vatoux, & Joly, 2001; Wong et al., 2014), and there is no evidence of any pathogenic behavior on human and animals.

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and *Clostridium difficile* infections (McFarland, 2009). In addition, *L. casei* has been suggested to be effective in alleviating GI pathogenic bacterial infections both *in vitro* and *in vivo* (Chung & Yousef, 2010; Forestier et al., 2001; Wong et al., 2014), and there is no evidence of any pathogenic behavior on human and animals. Furthermore, our previous study also suggested that potential components (disaccharides and indoles) in cocoa powder could stimulate the growth of *L. casei* in both MRS broth and milk media (Mengfei Peng, Aryal, Cooper, & Biswas, 2015a).

Based on the latent beneficial properties reported previously, we aimed to examine the combined growth inhibitory effect of *L. casei* and cocoa on common foodborne pathogens and to investigate the capacities of this probiotic strain combined with prebiotic-like cocoa/peanut in reducing host cell (INT-407) and pathogen interactions at cellular level. In addition, for better understanding of the reduced host cell-pathogen interactions, investigation of their role on expression of foodborne bacterial virulent genes related to flagellation, motility, and cell-specific binding is included as well.

Material and Methods

Bacterial strains and growth conditions. *L. casei* (ATCC 334) was grown on MRS agar overnight at 37 °C under aerobic condition with 5% CO₂ (Thermo Fisher Scientific Inc., Waltham, MA, USA). Three foodborne bacterial pathogens Enterohemorrhagic *E. coli* EDL933 (ATCC700927), *Salmonella enterica* serovar Typhimurium LT2 (ATCC19585), and *Listeria monocytogenes* LM2 (ATCC19115) were grown on

MacConkey agar, LB agar, and BHI agar (EMD Chemicals Inc., Gibbstown, NJ, USA), respectively, overnight at 37 °C under aerobic conditions (Thermo Fisher Scientific).

Cocoa powder and peanut flour/skin preparation. Commercial, non-alkali treated cocoa was purchased at the local supermarket. Cocoa powder was defatted with hexane for 18 h and the residual hexane was evaporated from the cocoa powder before use. The defatted cocoa powder was stored at 4 °C and sterilized 2 h under ultraviolet light before experimental use. In shell Jumbo Virginia raw peanut was purchased from a local market and shelled by hand to isolate the kernel fractions. Peanut skin was removed by hand and the white kernel was ground to form peanut flour. Both peanut skin and peanut flour were defatted by 2 extractions with n-hexane (n-hexane: peanut portion 10 mL/g) for 12 h at room temperature (25 °C). Peanut flour and peanut skin fraction suspensions were prepared in sterilized distilled water (pH adjusted to 8.0 with 1 N NaOH), mixed well and sterilized with UV irradiation for 2 h. Cocoa powder or peanut portion was mixed in MRS broth. The cocoa/peanut-MRS solution was used for HPLC-MS analysis.

Cell lines and culture conditions. Human intestinal epithelium cell line (INT-407 CCL-6) was purchased from American Type Culture Collection and cultured following the method described by Peng, Bitsko and Biswas (2015) with slight modification. Briefly, cells were grown at standard condition (37 °C, 5% CO₂, 95% humidity) in Dulbecco's modified Eagle medium (DMEM; HyClone Laboratories Inc., Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) and 100 g/mL gentamicin. The

cultured cells were seeded at approximately 2×10^5 cells/mL into 24-well tissue culture plates (BD Falcon, Franklin Lakes, NJ, USA) to reach 80-90% confluence monolayer at standard condition. The post-confluent INT-407 epithelial cell monolayers were rinsed with PBS and stabilized in antibiotic-free DMEM for 1 h prior to the invasion assay.

Mixed culture of *L. casei* with foodborne pathogens. *L. casei*, EHEC EDL933, *S. Typhimurium* LT2, and *L. monocytogenes* LM2 bacterial cells were collected from overnight agar plate culture. A volume of 400 μ L *L. casei* bacterial suspension containing 10^7 CFU/mL was mix-cultured with same amount of EHEC EDL933, *S. Typhimurium* LT2, or *L. monocytogenes* LM2, respectively, in 3.6 mL LB/MRS (1:1, v/v) broth in the presence or absence of 3% cocoa powder at 37 °C under aerobic condition. Serial dilutions were performed in PBS, followed by plating on MRS agar (*L. casei*), MacConkey agar (EHEC EDL933), Xylose Lysine Deoxycholate (XLD) agar (*S. Typhimurium* LT2), and Oxford *Listeria* agar base (*L. monocytogenes* LM2) at 0, 12, 24, 36, and 48 h time points.

Cell free culture supernatant (CFCS) of *L. casei* on growth of foodborne pathogens. Fresh overnight (18 h) liquid cultures of *L. casei* in MRS with or without 3% cocoa were centrifuged at $4000 \times g$ for 20 min. CFCSs were collected and filtered by sterile syringe 0.2 μ m filter (VWR). Filtered CFCS from *L. casei* (CFCS1) and filtered CFCS from cocoa supplemented *L. casei* (CFCS2) were collected and stored at 4 °C. A cell suspension (400 μ L) containing 10^7 CFU/mL of EHEC EDL933, *S. Typhimurium* LT2,

or *L. monocytogenes* LM2 was inoculated in separate culture tubes with 3.6 mL LB/MRS broth (1:1, v/v), LB/MRS broth (1:1, v/v) with 3% (w/v) cocoa powder, LB/CFCS1 (1:1, v/v), or LB/CFCS2 (1:1, v/v), respectively and cultured at 37 °C under aerobic condition. Serial dilutions were performed in PBS, followed by plating on specific agars mentioned above for different pathogens at 0, 4, 8, 24, and 48 h time points.

CFCS sensitivity to pH, heat, and enzymes. The pH of CFCS was adjusted from 4.87 either to 1.0, 2.0, 3.0, and 4.0 with 10 M HCl or to 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 with 10 M NaOH measured by FiveEasy™ pH meter (Mettler Toledo, Columbia, MD, USA). After overnight incubation at 4 °C, all pH was adjusted back to 4.87 for further assay. Thermal treatment of CFCS was conducted by incubating CFCS at 40, 60, 80, or 100 °C in a water bath (Isotemp, Thermo Fisher Scientific) for 30 min, and then cooled down at room temperature (25 °C) for further assay. Enzymatic treatments on CFCS were conducted at original pH value (4.87) by using of catalase (25 °C), proteinase K (50 °C), and/or trypsin (25 °C) (Sigma-Aldrich) at a final enzyme concentration of 1 mg/mL. EHEC EDL933 was used as indicator strain for multiple antimicrobial activity tests. The MIC was evaluated using broth micro-dilution method described previously (Nkanwen, Gatsing, Ngamga, Fodouop, & Tane, 2009). Briefly, CFCS ranging from 1:1 to 1:128 dilutions in LB broth were used as medium for growth of bacteria. An aliquot of 2 µL of bacterial suspension containing approximately 10⁷ CFU/mL was added into 198 µL medium in 96-well plate (Greiner Bio-One Inc., Monroe, NC, USA). The plate was incubated for 24 h at 37 °C under aerobic conditions.

MIC was determined as the lowest dilution of CFCS that prevented visible growth of EHEC EDL933 compared with control, and it was recorded in the form of arbitrary unit per mL (AU/mL).

Cell adhesion and invasion assay. We performed the adherence and invasion assays following the methods described previously by Peng, Bitsko and Biswas (2015) with some modification. Briefly, the INT407 cells grown in 24-well plate with 800 μ L DMEM containing 10% FBS were pre-treated with 100 μ L DMEM (control), 3% cocoa, 2×10^6 CFU/mL *L. casei*, CFCS1, or CFCS2 separately for 1 h, with each treatment in triplicate. A 100 μ L aliquot of EHEC EDL933, *S. Typhimurium* LT2, or *L. monocytogenes* LM2 with multiplicity of infection (MOI) of about 10 (2×10^6 CFU/mL) were inoculated into triplicate wells. Infected cells were incubated at standard condition for 2 h and washed three times with DMEM containing 10% FBS. The monolayers were lysed by 0.1% Triton X-100 for 15 min, serial diluted, and plated on specific agars for adhesive bacterial CFU counting. Cell invasive activity was measured by further 1 h incubation of the washed monolayers in DMEM containing 10% FBS and 100 g/mL gentamicin followed by three-times washing, Triton X-100 lysis, serial dilution, and plating on specific agars.

Evaluation of hydrophobicity of foodborne pathogens treated with CFCSs. Cell surface hydrophobicity was determined in accordance with the methods described previously (Ahn, Almario, Salaheen, & Biswas, 2014; Serajus Salaheen, Nguyen, et al., 2014) with some modification. Briefly, EHEC EDL933, *S. Typhimurium* LT2, or *L.*

monocytogenes LM2 was cultured overnight (18 h) in 5 mL LB broth and treated with equal volume (5 mL) of LB broth, MRS broth, CFCS1, and CFCS2 separately for 4 h at 37 °C under aerobic condition. Cells were collected and resuspended in pH 7.4 PBS to adjust the OD to 0.5 (H_{t_0}) under 570 nm wavelength. One volume cell suspension was mixed with 2 volumes of n-hexadecane (Sigma-Aldrich), vigorously vortexed, and incubated at room temperature for 5 min. The aqueous phase was collected and the OD (H_{t_5}) was measured at 570 nm by microplate reader (Multiskan FC, Thermo Scientific Inc., Odessa, TX, USA). The hydrophobicity value was calculated by the equation: $\text{Hydrophobicity (\%)} = (1 - H_{t_5}/H_{t_0}) \times 100$.

RNA extraction and cDNA synthesis. The bacterial cell suspensions were rinsed three times with 5 mL ice-cold PBS. The cell pellets were lysed with 1 mL TRIzol reagent (Life Technologies Co., Carlsbad, CA, USA) for 5 min at room temperature. The cell lysates were mixed with 200 μL chloroform, vortexed vigorously, and then kept at room temperature for 3 min. After centrifugation at $13,000 \times g$ for 15 min at 4 °C, the aqueous phase was collected and gently mixed with 500 μL of isopropanol, allowed to stand for 10 min at room temperature, and then centrifuged at $13,000 \times g$ for 15 min at 4 °C. The gel-like RNA pellet was washed with 1 mL 75% ethanol, vortexed 10 s, centrifuged at $7000 \times g$ for 5 min at 4 °C, and then air-dried in bio-safety cabinet for 10 min to remove the remaining ethanol. The RNA pellet was dissolved in 50 μL RNase-free water and quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific). Amount of 1 μg of extracted RNA was mixed with 1 μL of RTS DNase and 5 μL of DNase buffer (MO BOI Laboratories, Inc., Carlsbad, CA, USA) and incubated at 37 °C for 20 min

to remove genomic DNA. Then 5 μ L RTS DNase Removal Resin was added and resuspended every 1 min up to 10 min to remove DNase. Supernatant containing RNA was transferred after $13,000 \times g$ centrifugation for 1 min. The synthesis of cDNA was performed according to the qScript cDNA SuperMix protocol (Quanta Biosciences, Gaithersburg, MD, USA). The extracted RNA (1 μ g) was mixed with qScript cDNA SuperMix (containing optimized concentrations of $MgCl_2$, deoxyribonucleotide triphosphates, qScript reverse transcriptase, and RNase inhibitor protein). The reaction mixture was incubated subsequently at 25 $^{\circ}C$ for 5 min, 42 $^{\circ}C$ for 30 min, and 85 $^{\circ}C$ for 5 min.

Quantitative RT-PCR assay. The PCR reaction mixture containing 10 μ L of PerfeCTa SYBR Green FastMix, 2 μ L of each primer (100 nM), 2 μ L of cDNA (10 ng), and 4 μ L of RNase-free water were amplified using an Eco Real-Time PCR system (Illumine, San Diego, CA, USA) with 30 s denaturation at 95 $^{\circ}C$, followed by 40 cycles of 95 $^{\circ}C$ for 5 s, 55 $^{\circ}C$ for 15 s, and 72 $^{\circ}C$ for 10 s. The custom-synthesized oligonucleotide primers (Erofin's MWG Operon, Huntsville, AL, USA) for EHEC EDL933, *S. Typhimurium* LT2, and *L. monocytogenes* LM2 are summarized in Table 3-1. The relative transcription levels of target genes were estimated by the comparative fold change. The C_T 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. The one-way analysis of variance 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

Competitively exclusion of foodborne pathogens in mixed culture. To determine the effect of probiotic and its byproducts on the growth of enteric bacterial pathogens, we co-cultured EHEC EDL933, *S. Typhimurium* LT2, or *L. monocytogenes* LM2 with *L. casei* in medium to support the growth of both bacteria. In this co-cultured or mixed culture condition, the growth of *L. casei* was only promoted slightly at a negligible level ($p < 0.05$) (Figure 3-1). In the same study, we found the growth of each of these bacterial pathogens drastically inhibited in the presence of *L. casei* (Figure 3-1). Two pathogens EHEC EDL933 and *S. Typhimurium* LT2 were completely excluded from the cultural medium by *L. casei* at 48 and 36 h, respectively (Figure 3-1A and B). Growth of *L. monocytogenes* LM2 was also reduced by 5.26 log CFU/mL at 48 h (Figure 3-1C).

Distinct effects of peanut flour and peanut skin extract on growth of EHEC, *S. Typhimurium*, and *L. monocytogenes* in the mixed culture with *L. casei*. Growth conditions of these foodborne pathogens were determined in mixed culture with *L. casei* in the presence and absence of 0.5% peanut flour or 0.5% peanut skin extract. We found that EHEC (Figure 3-2A) and *S. Typhimurium* (Figure 3-2B) were completely excluded (below the limit of detection) from the medium by *L. casei* after 48 h. In the same study, *L. monocytogenes* (Figure 3-2C) was drastically inhibited by *L. casei* by >4

logs after 48 h of incubation in mixed culture with *L. casei*. In mixed culture with *L. casei* in the presence of 0.5% peanut flour, the same inhibitory patterns on the growth of these enteric bacterial pathogens were found, but peanut flour, by stimulating growth of *L. casei*, assisted in quickly reducing more EHEC (>0.5 log CFU/mL at 24 h) and *S. Typhimurium* (>1.0 log CFU/mL at 24 h). In contrast, in the presence of 0.5% peanut skin extract, *L. casei* with same amount of inocula only showed reduced inhibitory effects on growth of EHEC (Figure 3-2A) and *S. Typhimurium* (Figure 3-2B), whereas *L. monocytogenes* (Figure 3-2C) was completely excluded (below the limit of detection) after 48 h of incubation.

Effect of CFCS on growth inhibition of enteric bacterial pathogens. Both CFCS1 and CFCS2 showed growth inhibitory effects on EHEC EDL933, *S. Typhimurium* LT2, and *L. monocytogenes* LM2 in a dose-dependent manner based on the amount of *L. casei* cells inoculated for collecting CFCSs. CFCS1 (pH = 4.87) and CFCS2 (pH = 5.24), obtained from overnight culture started with the initial inocula of 10^6 CFU/mL (OD 0.1) and ended with 2×10^9 CFU/mL (CFCS1) and 10^{10} CFU/mL (CFCS2) final concentration of *L. casei* overnight inoculation, reduced 3.27 and 4.73 log CFU/mL EHEC EDL933 (Figure 3-3A), 4.31 and 5.12 log CFU/mL *S. Typhimurium* LT2 (Figure 3-3B), 3.16 and 4.33 log CFU/mL *L. monocytogenes* LM2 (Figure 3-3C), respectively, at 48 h, compared with control in LB/MRS (1:1, v/v). In the same study, CFCS1 (pH = 4.61) and CFCS2 (pH = 5.12), collected from the overnight culture started with initial inocula of 10^7 CFU/mL (OD 1.0) and ended with 5×10^9 CFU/mL (CFCS1) and 3×10^{10} CFU/mL (CFCS2) final concentration of *L. casei*, showed higher

antimicrobial effects on the growth of all three enteric foodborne bacterial pathogens and both CFCS1 and CFCS2 were able to exclude all three foodborne pathogens within 48 h (Figure 3-3). However, 3% cocoa only exhibited significant inhibitory effect on growth of EHEC EDL933 and *S. Typhimurium* LT2 within short time period (0.82 log CFU/mL EHEC reduction at 4 h, 0.70 log CFU/mL EHEC EDL933 reduction at 8 h, and 0.70 log CFU/mL *S. Typhimurium* at 4 h).

Characterization of CFCS antimicrobial activity. The effects of pH, heat, and enzyme treatments on antimicrobial activity of CFCS are summarized in Table 3-2. Original collected CFCS with pH value of 4.87 at room temperature (25 °C) (control) exhibited strong (>50 AU/mL) antimicrobial activity on EHEC. HCl treatment on CFCS maintained its antimicrobial activity, whereas NaOH treatment reduced the antimicrobial activity of CFCS. CFCS showed moderate (>5 AU/mL) antimicrobial activity when first adjusted to pH from 6.0 to 8.0 for incubation and then converted back to pH 4.87, whereas no antimicrobial property was detected when pH value first adjusted higher than 9.0. CFCS remained its strong (>50 AU/mL) antimicrobial activity with 30 min incubation at both 40 and 60 °C. However, when the thermal treatment increased to 80 and 100 °C, antimicrobial activity of CFCS was reduced to moderate (>5 AU/mL). Moreover, three kinds of enzymatic treatments (catalase, proteinase K, and trypsin) on CFCS all decreased its antimicrobial activity but remained within detectable level (>5 AU/mL). Combined enzymatic treatments of catalase and protease(s) further reduced the antimicrobial activity of CFCS to non-detectable level.

Alteration of bacterial cell surface hydrophobicity. In the presence of 3% cocoa, CFCS1, and CFCS2, the cell surface hydrophobicity values of EHEC EDL933, *S. Typhimurium* LT2, and *L. monocytogenes* LM2 were decreased significantly (Table 3-3). In comparison with control, *L. monocytogenes* LM2 cells treated with 3% cocoa significantly reduced the cell surface hydrophobicity value by 28.50%, whereas no significant change in EHEC EDL933 and *S. Typhimurium* LT2 was observed. In the same study, EHEC EDL933, *S. Typhimurium* LT2, and *L. monocytogenes* LM2 cells treated with CFCS1 showed significant reduction of cell surface hydrophobicity by 55.63, 52.06, and 55.72% folds, respectively; similarly, EHEC EDL933, *S. Typhimurium* LT2, and *L. monocytogenes* LM2 cells with CFCS2 treatment decreased the hydrophobicity values much effectively by 79.62, 74.30, and 78.37% folds, respectively.

Reduction in pathogen-host cell interactions. Pre-treatment with 3% cocoa, *L. casei* cells, CFCS1, and CFCS2 reduced the pathogenic cell adhesive and invasive activities of three enteric bacterial pathogens (Figure 3-4). In this study, we observed that 3% cocoa reduced the adherence and invasive abilities of EHEC EDL933 by 77.58 and 97.23%, *S. Typhimurium* LT2 by 93.41 and 100%, and *L. monocytogenes* LM2 by 37.41 and 100%, respectively. In the same study, we found that pre-treatment with *L. casei* cells could also competitively inhibit the pathogen-host cell interactions. For EHEC EDL933, *S. Typhimurium* LT2, and *L. monocytogenes* LM2, 62.12, 68.51, and 58.53% of cell-adhesive activities as well as 77.50, 93.97, and 79.85% of cell-invasive activities were reduced. Likewise, CFCS1 significantly inhibited adhesion abilities of

three foodborne pathogens (56.40% for EHEC EDL933, 85.28% for *S. Typhimurium* LT2, and 24.11% for *L. monocytogenes* LM2) and invasion abilities of *S. Typhimurium* LT2 (92.25%) and *L. monocytogenes* LM2 (95.86%). Furthermore, CFCS2 showed more intensive effect compared with CFCS1, in which adhesion abilities of EHEC EDL933, *S. Typhimurium* LT2, and *L. monocytogenes* LM2 were reduced by 80.81, 97.35, and 30.47%, respectively. We also found that in the presence CFCS2, invasion abilities of EHEC EDL933, *S. Typhimurium* LT2 and *L. monocytogenes* LM2 were reduced by 98.98, 100, and 100%, respectively.

Pretreatment with peanut fractions or in the presence of *L. casei* reduced significantly the adhesion to and invasion into INT-407 cells by these enteric bacterial pathogens (Figure 3-5). We observed that pretreatment with 0.5% peanut flour significantly ($p < 0.05$) reduced the adherence ability of EHEC, *S. Typhimurium*, and *L. monocytogenes* to INT-407 cells by 89.9%, 86.5%, and 40.3%, respectively. Likewise, 0.5% peanut skin extract also significantly ($p < 0.05$) reduced the adhesion abilities of these 3 foodborne pathogens by 90.1%, 84.7%, and 54.9%, respectively. Pre-incubation of *L. casei* showed attenuated inhibitive effects, which reduced the adhesive level of EHEC, *S. Typhimurium*, and *L. monocytogenes* by 51.5%, 66.7%, and 61.0%, respectively. However, the combine effect, pretreated with *L. casei* and 0.5% peanut flour, further enhanced the inhibitive capability on pathogens' adhesion ability up to 94.2% for EHEC, 97.2% for *S. Typhimurium*, and 83.8% for *L. monocytogenes*. When compared with single effect of *L. casei*, the combined effect of *L. casei* and pretreatment of 0.5% peanut skin extract did not inhibit more cell adhesive activity at significant level of 0.05. In the same study, we also found that cells treated with 0.5%

peanut flour or 0.5% peanut skin extract showed significant reduction (96.8% to 98.6%) in invasion ability by these foodborne pathogens (Figure 5D, 5E, and 5F). We also found that combined effect of pretreatment with *L. casei* and 0.5% peanut flour or 0.5% peanut skin extract enhanced the inhibitory effect of these pathogens by 100% (below the limit of detection) for EHEC, 93.9% for *S. Typhimurium*, and 83.4% for *L. monocytogenes*.

Effects on virulent gene expression. To further investigate the effects of 3% cocoa, *L. casei* CFCS1, and *L. casei* CFCS2 on each of these enteric bacterial pathogens (EHEC, *S. Typhimurium*, or *L. monocytogenes*) and host intestinal epithelial INT-407 cell interactions, the relative expression levels of major virulent genes related to attachment and invasion were determined using qRT-PCR (Figure 3-6).

Fold-change of EHEC EDL933 virulence genes were shown in Figure 3-6A. In the presence of 3% cocoa, CFCS1, and CFCS2 significantly reduced the relative expression level of *eaeA* gene by 1.68, 1.79, and 4.57-fold, respectively. In the same study, relative expression level of *fliC* gene of EHEC EDL933 were up-regulated by 3% cocoa (6.89-fold), CFCS1 (7.95-fold), and CFCS2 (8.25-fold). Whereas compared with control, the relative expression level of *tir* gene in EHEC EDL933 showed no significant fold change when treated by cocoa or CFCSs.

For *S. Typhimurium* LT2 (Figure 3-6B), in the presence of 3% cocoa, CFCS1, and CFCS2, significant down-regulation of the relative expression level of *nmpC* gene by 3.31, 100.75, and 28.57-fold, respectively, was found. Meanwhile, *fliC* gene was up-regulated by 2.00, 2.54, and 7.61-fold by 3% cocoa, CFCS1, and CFCS2,

respectively. When compared with control, expression level of *fliD* gene was only increased by CFCS1 (1.97-fold) and CFCS2 (4.38-fold), whereas expression level of *motB* gene was increased by 3% cocoa (1.61-fold) and CFCS2 (4.68-fold), respectively.

Fold-change of *L. monocytogenes* LM2 virulence genes were shown in Figure 3-6C. The expression level of *fbp* gene was reduced significantly by 3% cocoa (1.49-fold), CFCS1 (3.73-fold), and CFCS2 (6.13-fold). Meanwhile treatment of 3% cocoa, CFCS1, and CFCS2 also significantly up-regulated the relative expression level of *flaA* gene of *L. monocytogenes* LM2 by 1.61, 2.97, and 5.24-fold, respectively. Additionally, the expression level of *iap* gene in *L. monocytogenes* LM2 was also decreased by CFCS1 (5.49-fold) and CFCS2 (11.36-fold), but no meaningful change was found by cocoa treatment.

Discussion

Probiotics can be found in various different foods as supplement, and they are believed to play very important roles in regulation of proper intestinal function, digestion by balancing intestinal microflora, and disease progressions including growth inhibition of bacterial pathogens to various degrees (Anas, Jamal Eddine, & Mebrouk, 2008; Coman et al., 2014; Rodríguez et al., 2012). In consistence with previous studies, we observed the antimicrobial property of *L. casei* against EHEC EDL933, *S. Typhimurium* LT2, and *L. monocytogenes* LM2 in mixed cultures. In order to test our hypothesis that the antimicrobial activity of *L. casei* comes from its produced metabolites or byproducts, we further investigated the effects of CFCS collected from overnight culture of *L. casei* on growth of three foodborne pathogens. In agreement

with former researchers (Coconnier, Liévin, Bernet-Camard, Hudault, & Servin, 1997), we found that CFCS of *L. casei* inhibited the growth of EHEC EDL933, *S. Typhimurium* LT2, and *L. monocytogenes* LM2 strains. Additionally, we observed that 3% supplemented medium could stimulate the growth and increase the amount of byproducts of *L. casei* (Mengfei Peng, Aryal, Cooper, & Biswas, 2015b) and 3% cocoa containing CFCS exhibited stronger inhibitory effects on multiple foodborne bacterial pathogens. This study also indicated that the intensive antimicrobial effects of CFCSs depended on the amount of metabolites/byproducts *L. casei* produced overnight in the medium.

To characterize the CFCS's activity, CFCS of *L. casei* with pH, temperature, and enzymatic treatments on growth of EHEC was also examined. We detected attenuated antimicrobial property of CFCS at high pH treatment condition, which revealed the crucial role of the ionic state of lactic acids produced and secreted by *L. casei* in exclusion of foodborne pathogens in acidic conditions (Gyawali & Ibrahim, 2012; Serajus Salaheen, White, et al., 2014). Discounted antimicrobial activity of CFCS processed by high temperature (80 and 100 °C) as well as enzyme (catalase, proteinase K, or trypsin) treatments indicated that in spite of lactic acids, hydrogen peroxide and antimicrobial polypeptides in CFCS also contribute to antimicrobial activity (Atassi & Servin, 2010; Wannun et al., 2014; Xu et al., 2008). Simultaneously, combined enzymatic treatments with catalase + proteinase K, catalase + trypsin, and catalase + proteinase K + trypsin showed the minimum or no antimicrobial activity. With HPLC-MS/MS analysis, we also detected few potential bio-active metabolites from *L. casei* promoted by cocoa (Table 3-4), for example, citric acid and linoleic acid,

which further proved the synergistic antimicrobial effects of hydrogen peroxide and antimicrobial polypeptides in CFCS.

Combined with *L. casei* and peanut flour showed a drastically inhibitory effect against 3 major foodborne bacterial pathogens. Two possible explanations for this might be that compounds in peanut flour either induced the number of *L. casei* or the antimicrobial metabolites production by *L. casei*, both of which contribute to the competitive exclusion of foodborne pathogens. As antimicrobial resistance among foodborne pathogens especially *Salmonella*, *L. monocytogenes*, and *E. coli* growing rapidly recently, FDA has paid close attention on this emergent issue. This study has investigated the potential of combining probiotics and prebiotics in inhibiting foodborne pathogens. Our findings may suggest replacing the chemical antimicrobial use with bio-competitive inhibitors and natural growth promotive components such as peanut flour, as an alternative to minimize the spread of multidrug resistance bacteria as well as it may ensure food safety for human. The combinational use of *L. casei* and peanut skin extract only showed strong inhibition on growth of *L. monocytogenes*. The antimicrobial effect of peanut skin extract itself on *L. monocytogenes* might be one explanation of this, as a result of which, though the amount as well as the inhibitive effect of *L. casei* in the mixed culture were attenuated by peanut skin extract, *L. monocytogenes* still was not able to survive in the supplemented mixed culture.

In the same study, we also found that *L. casei*, peanut flour, and peanut skin extract can reduce foodborne pathogens colonization on human intestinal cells. In general, the enteric bacterial pathogens' infection processes usually initiate from intestinal epithelial cell adhesion and then followed by cell invasion through site-

specific ligands. However, normal microflora colonization in gut competitively prevents foreign bacterial pathogens from attachment. Both *Lactobacillus* and enteropathogens are known to express cell surface proteins and displays carbohydrate-binding specificities on human intestinal cells (Neeser et al., 2000). In order to attach and colonize host's gut, foodborne pathogens have to compete with normal gut microflora, as a consequence of which, we hypothesize that pretreatment of *L. casei* reduce the adhesive and invasive activities of pathogens by occupying the intestinal cell surface receptors. In addition, bio-active components in peanuts such as phenolic acids and flavonoids might inhibit the colonization of pathogens by reducing their flagellin and adhesin level. Simultaneously, the increased activities of cell attachment by *L. casei* in supplement with peanut flour may explain why combinational pretreatment with *L. casei* and peanut flour could exhibit much stronger adhesion and invasion inhibitive effect on 3 pathogens.

To investigate the role of *L. casei* cells in co-culture condition and CFCs on pathogen-host INT-407 cell interactions, we examined their inhibitory effects on cell adhesive and invasive activities, cell surface hydrophobicity, and cell attachment-related gene expression of EHEC EDL933, *S. Typhimurium* LT2, and *L. monocytogenes* LM2 strains. Both pre-treatment of *L. casei* cells and CFCs significantly reduced the cell adhesion and invasion abilities of three pathogens, which is in agreement with previous studies on anti-adherence properties of *Lactobacillus* strains against multiple bacterial pathogens (Bendali F, Hebraud M, & Sadoun D, 2014; Campana et al., 2012; Spurbeck & Arvidson, 2010; Tuomola, Ouwehand, & Salminen, 1999).

By sharing similar carbohydrate-binding specificities displayed by cell surface proteins, *L. casei* is hypothesized to decrease the adhesive and invasive activities of pathogens by pre-occupying the surface receptors on INT407 cells (Neesser et al., 2000; Serajus Salaheen, White, et al., 2014). Furthermore, antioxidant containing products like cocoa is known to block the level of host cell-pathogen interactions especially bacterial invasion abilities by inhibiting inflammatory responses in intestinal epithelial cells (M. Kim et al., 2010; Mengfei Peng, Aryal, et al., 2015b). In this study, CFCS2 combined the capabilities of both CFCS1 and antioxidants-rich cocoa and exhibited the most intensive properties on limiting pathogen-cell interactions. Additionally, Saran *et al.* (2012) demonstrated the positive correlation between bacterial cell surface hydrophobicity and cell attachment activities. Therefore, the noticeable reduction in cell surface hydrophobicity of three foodborne pathogens with CFCS treatments could be another identical indicator of their attenuated adhesion activities on human GI cells in this study.

We assessed the relative expression levels of multiple virulence genes of EHEC EDL933, *S. Typhimurium* LT2, and *L. monocytogenes* LM2 strains, which are summarized in Table 3-1. Based on our findings, with the exception of *tir* (EHEC intimin translocation) gene, all genes for specific cell attachment and infection including *eaeA* (EHEC intimin adherence protein synthesis), *fbp* (*L. monocytogenes* fibronectin-binding-protein synthesis), and *iap* (*L. monocytogenes* invasion-associated protein synthesis) were negatively affected by CFCSs. The significant down-regulations of these genes, in supporting previous studies (Dowd, Killinger-Mann, Blanton, San Francisco, & Brashears, 2007; Medellin-Peña et al., 2007), somehow

provide us an explanation for the reduced cell adhesion and invasion abilities. A significant increase (4-8 folds) in relative expression of all flagellin synthesis and bacterial motility associated genes (*fliC*, *fliD*, *flaA*, and *motB*) were detected when the bacterial cells were pretreated with CFCS2. Generally, flagella ensure bacterial motility and are involved in their initial interaction with intestinal cells of the host (La Ragione, Cooley, Velge, Jepson, & Woodward, 2003). It is likely that the observed up-regulation of flagella/ motility associated genes would be correlated with emergent flagellin synthesis and flagella production in response to induced stress in the presence of multiple antimicrobial components (peroxide, polypeptides, phenolic compounds, flavonoids and many other) containing CFCS2. This result agrees with previous study that under stressed condition, survival strategy such as up-regulating flagellar biosynthesis and motility will be induced by bacterial pathogens (Bradley, Beach, de Koning, Pratt, & Osuna, 2007; Serajus Salaheen, Nguyen, et al., 2014). The mechanism behind up-regulation of flagella/ motility associated genes remains unknown, but these differentially transcribed genes contribute to the pathogenesis of foodborne pathogens, especially bacterial motility and initial host cell attachment, as response to the attenuated specific cell surface binding activities. Finally, it is of note that significant down-regulation of *nmpC* gene (encodes *Salmonella* outer membrane-associated protein) is associated with bacterial membrane disruption and increased permeability.

Conclusions

1. Combining of cocoa/peanut and *L. casei* competitively excluded EHEC and *S. Typhimurium* in mixed-culture condition.

2. CFCS from *L. casei* supplemented with cocoa/peanut altered the enteric pathogenic bacterial cell surface hydrophobicity, disrupted their host-pathogen interactions, and down-regulated their virulence gene expressions.
3. Antimicrobial properties of CFCS from *L. casei* was attributed from multiple metabolites including hydrogen peroxide, organic acids, and polypeptides.
4. Cocoa/Peanut supported/stimulated the production and secretion of linoleic acids by *Lactobacillus*.

Figure and Table list

Figure 3-1. Comparison in growth conditions of both *L. casei* and enteric pathogens including EHEC (A), *S. Typhimurium* (B), and *L. monocytogenes* (C) between single and mixed culture at 0, 12, 24, 36, and 48 h. Error bars indicate standard deviation from parallel trials. Asterisks (*) at each time point indicate the significant growth in mixed culture when compared with single culture as a control at $p < 0.05$.

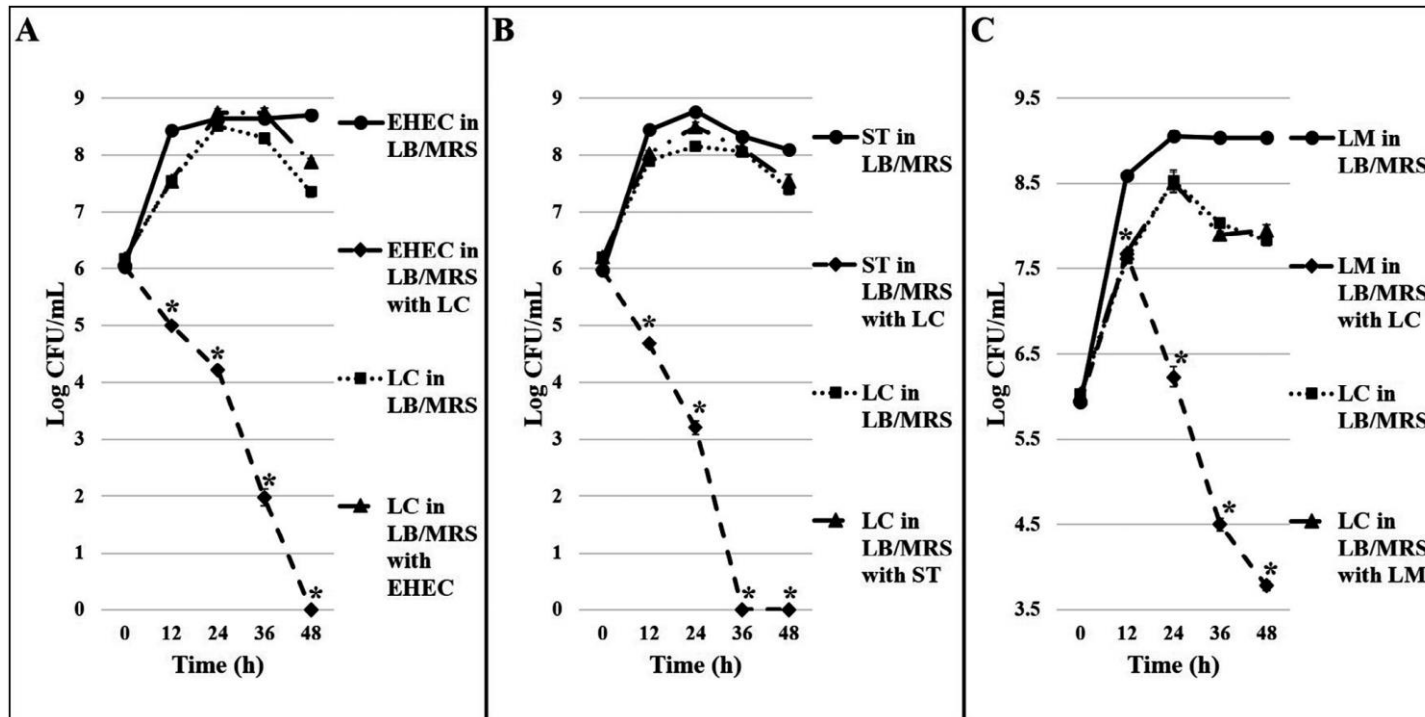


Figure 3-2. Comparative growth conditions of EHEC (A), *S. Typhimurium* (B), and *L. monocytogenes* (C) alone, in mix-culture with *L. casei*, and in mix-culture in the presence of 0.5% peanut flour or with 0.5% peanut skin extract at 0, 24, 48, 72 h. Error bars indicate standard deviation from parallel trials. Different letters within a time point indicate significant difference between control and treatment at $p < 0.05$.

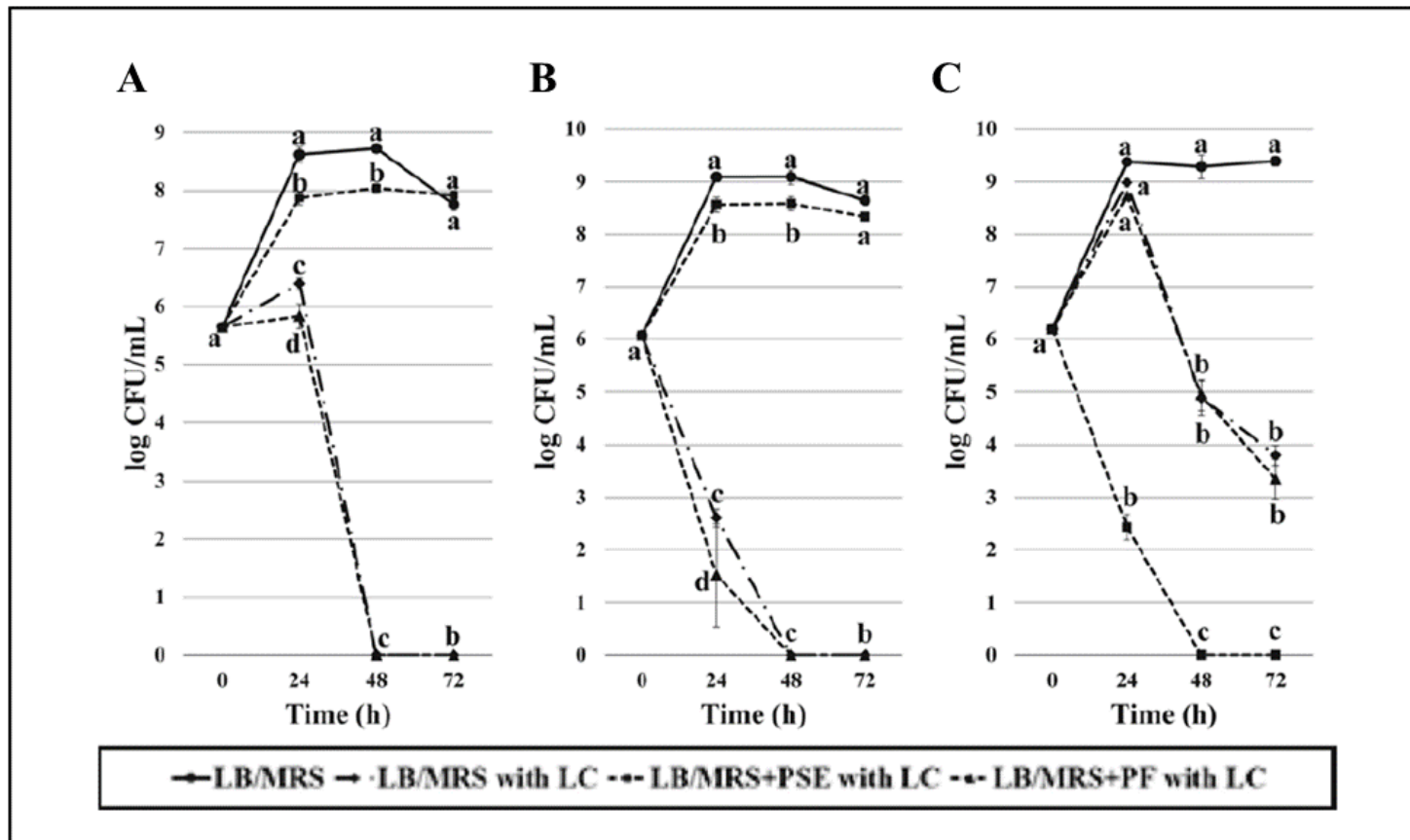


Figure 3-3. Inhibitory effects of CFCSs from *L. casei* on growth of EHEC (A), *S.* Typhimurium (B), and *L. monocytogenes* (C) at 0, 4, 8, 24, and 48 h. Error bars indicate standard deviation from parallel trials. Bars with different letters (a through d) at single time point within each strain are significantly different at $p < 0.05$.

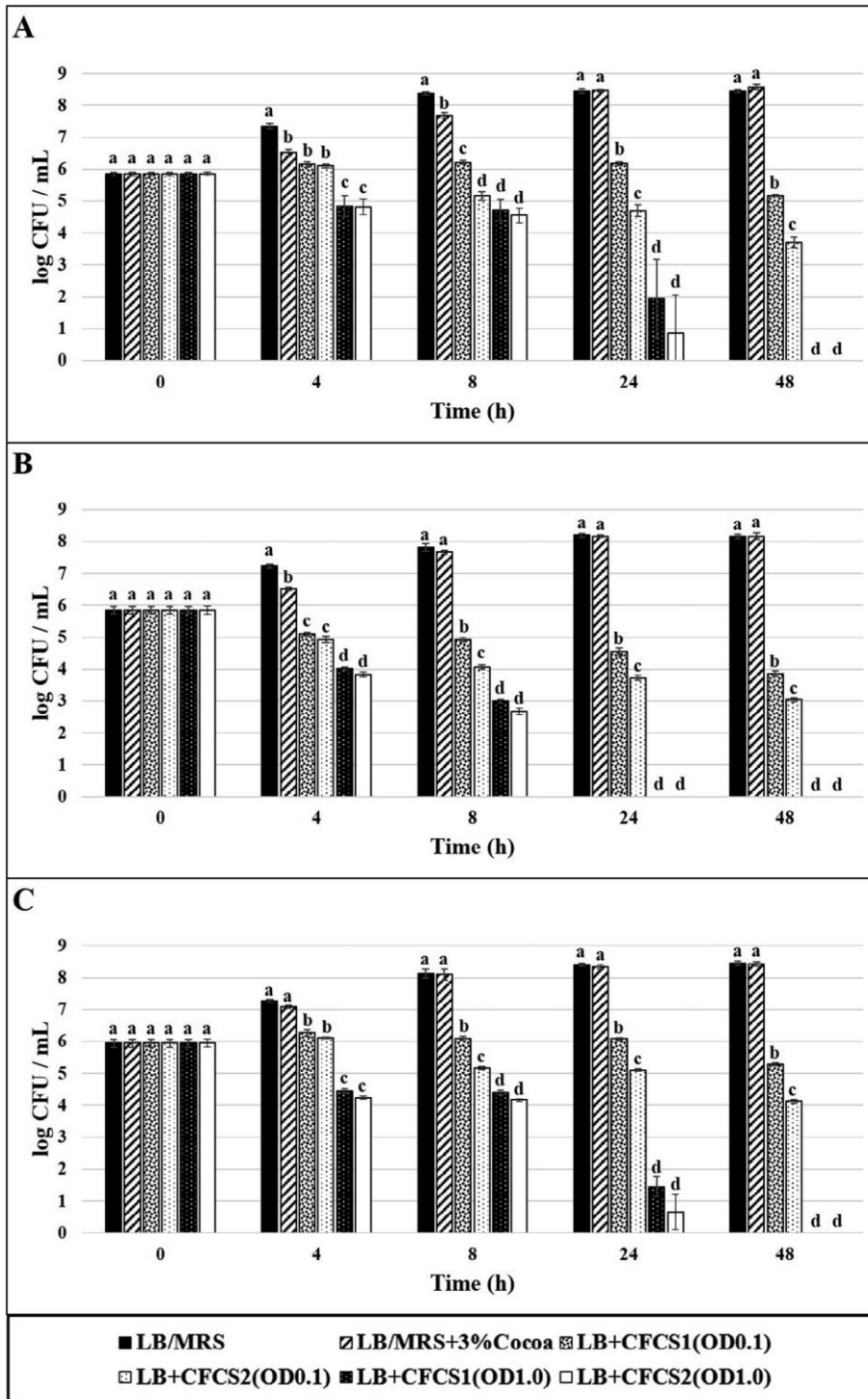


Figure 3-4. Cell adhesion (A, C, and E) and invasion (B, D, and F) levels of EHEC (A and B), *S. Typhimurium* (C and D), and *L. monocytogenes* (E and F) to INT407 cells with pre-treatment of 3% cocoa, *L. casei* bacterial cells, and CFCSs of *L. casei*. A constant MOI=10 is applied in each sub-figure. Error bars indicate standard deviation from parallel trials. Bars with different letters (a through d) within each pathogen are significantly different at $p < 0.05$.

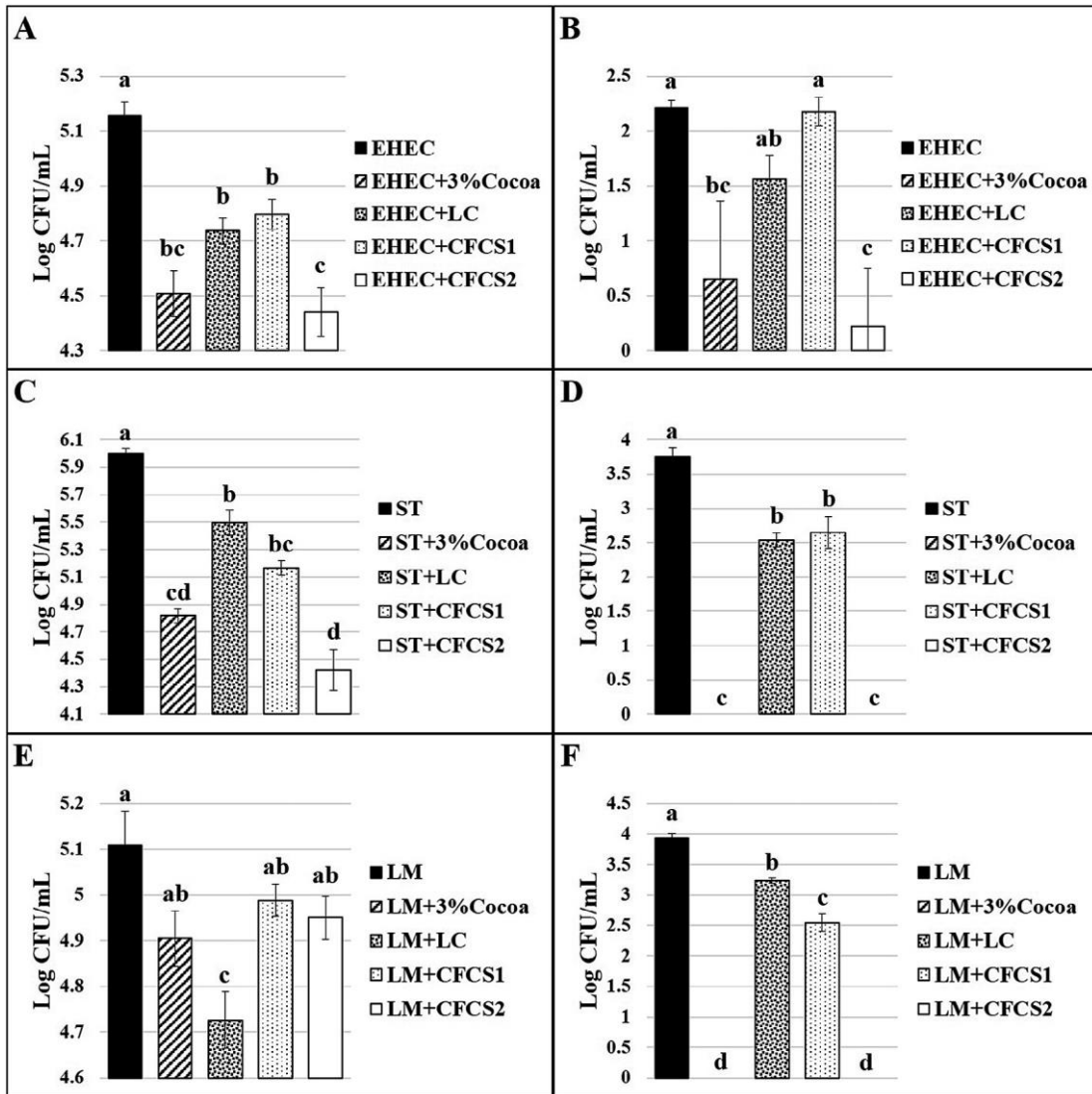


Figure 3-5. Reduction of adhesive activities of EHEC (A), *S. Typhimurium* (B), and *L. monocytogenes* (C) as well as invasive activities of EHEC (D), *S. Typhimurium* (E), and *L. monocytogenes* (F) on INT-407 cells by pretreatment with 0.5% peanut flour, 0.5% peanut skin extract, *L. casei*, 0.5% peanut flour plus *L. casei*, and 0.5% peanut skin extract plus *L. casei*. Error bars indicate standard deviation from parallel trials. Different letters within a time point indicate significant difference between control and treatment at $p < 0.05$.

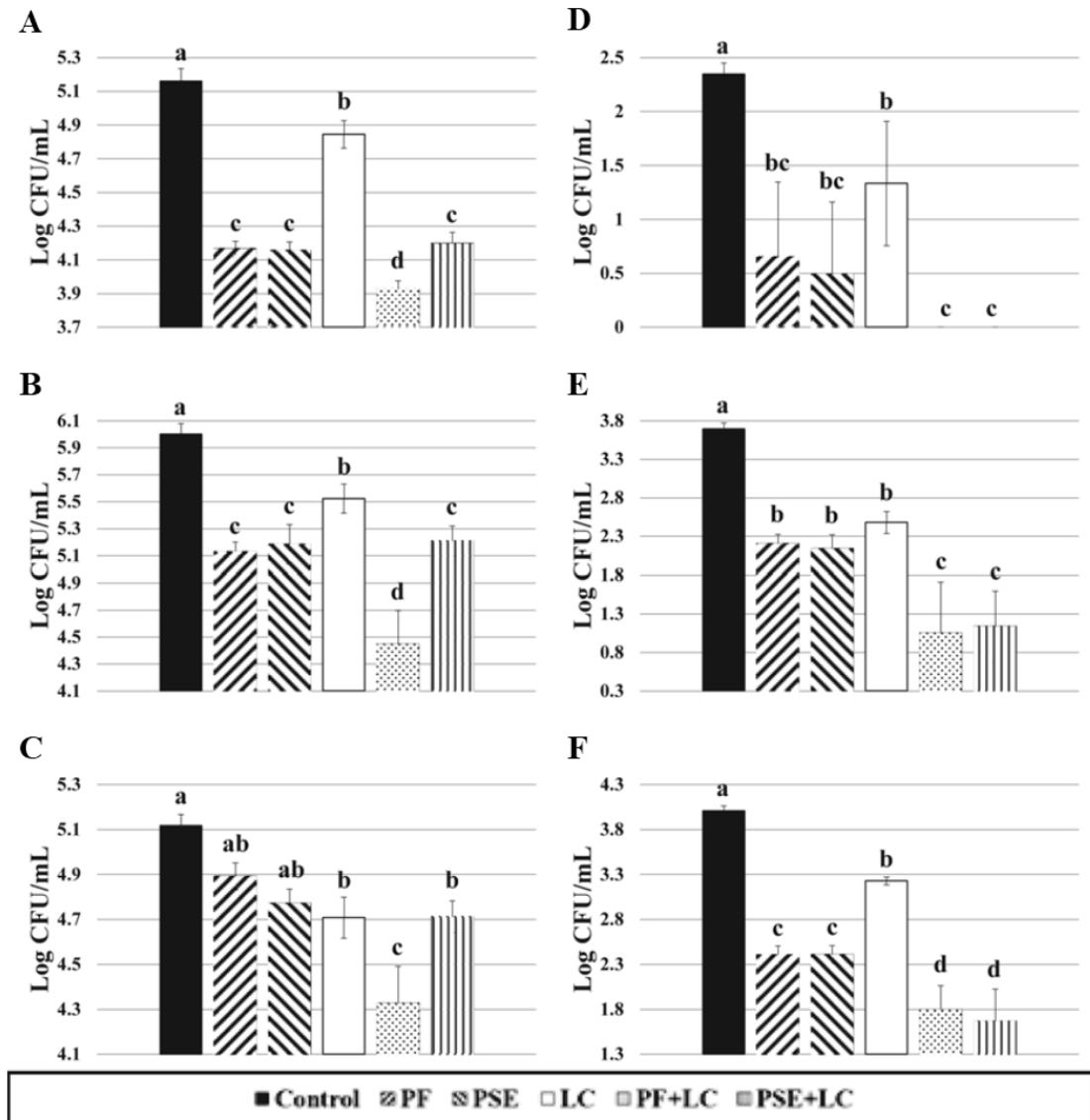


Figure 3-6. Relative expression of multiple virulence genes from EHEC (A), *S. Typhimurium* (B), and *L. monocytogenes* (C) when treated with 3% cocoa and CFCSs from *L. casei*. The relative transcription levels of target genes are shown in the form of comparative fold change with gene expression in control being 1. Error bars indicate standard deviation from parallel trials. Bars with different letters (a through d) are significantly different among treatments and control at $p < 0.05$.

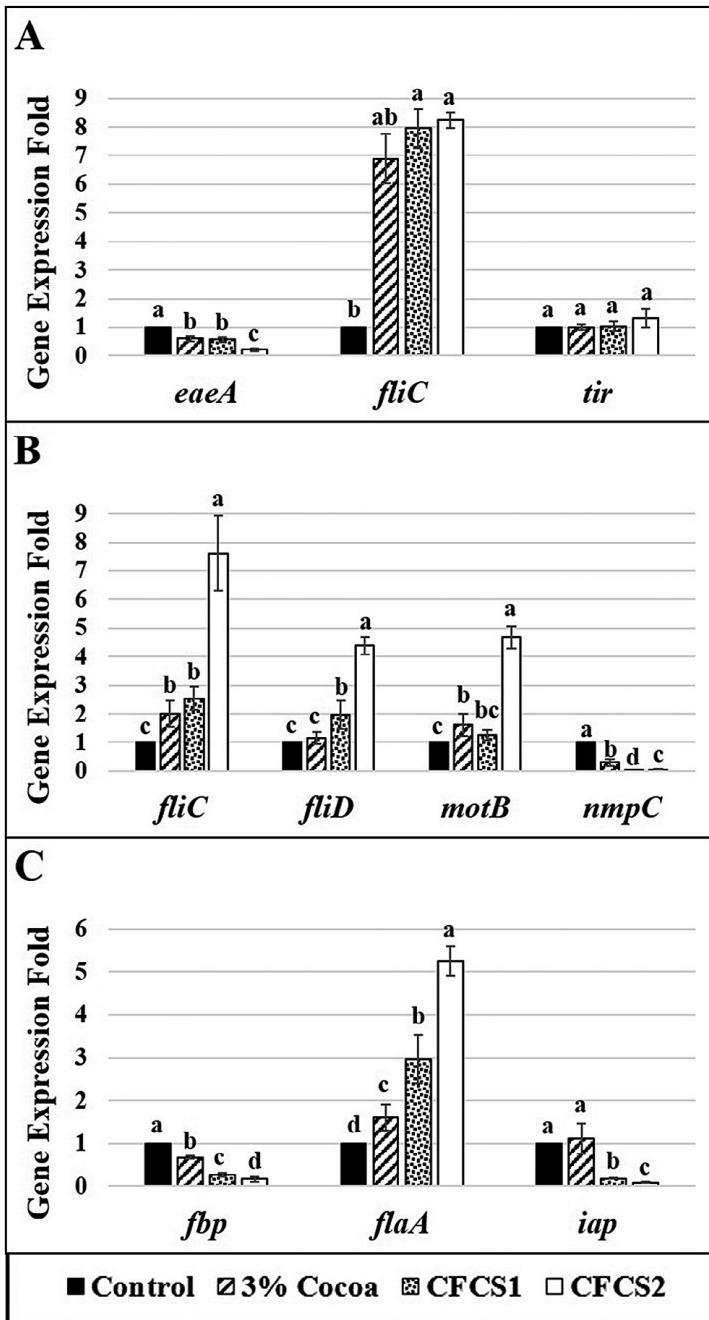


Table 3-1. Primers to be used for RT-qPCR analysis of virulent genes

Bacteria	Gene	Primer Sequence (5'-3')	Function
EHEC	<i>tufA</i>	F: ACTTCCCGGGCGACGACTC R: CGCCCGGCATTACCATCTCTAC	Housekeeping gene
	<i>eaeA</i>	F: CCCGAATTCGGCACAAGCATAAGC R: CCCGAATCCGTCTCGCCAGTATTTCG	Intimin gene
	<i>espA</i>	F: CGGTTATTTACCAAGGGATA R: TGGATACATCAAATGCAACA	LEE effector gene
	<i>espD</i>	F: TAAATTCGGCCACTAACAAT R: CTAAAGCGCTGGAGAATAAAA	LEE effector gene
	<i>fliC</i>	F: TACCATCGCAAAAGCAACTCC R: GTCGGCAACGTTAGTGATACC	Flagellin synthesis gene
	<i>ler</i>	F: ACTTCCAGCCTTCGTTTCAGA R: TTCTGGAACGCTTCTTTTCGT	LEE regulator gene
S. Typhimurium	16SrRNA	F: CAGAAGAAGCACCGGCTAAC R: AATGCAGTTCACAGGTTGAG	Housekeeping gene
	<i>fliC</i>	F: GCAGATGACGGTACATCCAA R: CCAGATCAGGCTGTGCTTTA	Filament structural protein synthesis gene
	<i>fliD</i>	F: ATGAAGATCACGGTGGAAGG R: TTGCTCTGACGCTCAATGTC	Flagellar synthesis gene
	<i>hilA</i>	F: CTGTACGGACAGGGCTATCG R: GCAGACTCTCGGATTGAACC	SPI-1 regulator gene
	<i>hilD</i>	F: TGGCGCTCTCTATGCACTTA R: AACGCCGTTTTTCAGATGTTTC	SPI-1 regulator gene
L. monocytogenes	16SrRNA	F: TTAGCTAGTTGGTAGGGT R: AATCCGGACAACGCTTGC	Housekeeping gene
	$\sigma\beta$	F: ATGCCAAAAGTATCTCAACCTGA R: TTACTIONCACTTCCTCATT	Stress-responsive factor gene
	<i>fbp</i>	F: ATGCAAACAAAATTGCACTG R: GAATTCGCCGACAACCTACT	Fibronectinbinding protein gene
	<i>flaA</i>	F: ATGAAAGTAAATACTAATATC R: TTAGCTGTTAATTAATTGAGT	Flagellin synthesis gene
	<i>iap</i>	F: GAATGTAAACTTCGGCGCAATCAG R: GCCGTGATGATTTGAACTTCATC	Invasion-associated protein gene

Table 3-2. Effects of pH, heat treatment, and enzyme on CFCS from *L. casei*

	Treatment	Antimicrobial activity	
Control	pH=4.87 25°C	++	
	1.0	++	
	2.0	++	
	3.0	++	
	4.0	++	
	5.0	++	
	pH	6.0	+
		7.0	+
		8.0	+
		9.0	-
10.0		-	
Heat (°C)	40	++	
	60	++	
	80	+	
	100	+	
Enzyme (1.0 mg/mL)	Catalase	+	
	Proteinase K	+	
	Trypsin	+	
	Catalase+Proteinase K	-	
	Catalase+Trypsin	-	
	Proteinase K+Trypsin	+	
Catalase+Proteinase K+Trypsin	-		

++: Antimicrobial activity >50 AU/mL.

+: Antimicrobial activity >5 AU/mL.

-: Non-detected antimicrobial activity.

Table 3-3. Cell surface hydrophobicity values of enteric bacterial pathogens *

Treatment	Bacteria		
	EHEC	<i>S.Typhimurium</i>	<i>L.monocytogenes</i>
Control	6.099±0.634 ^a	5.603±1.455 ^a	6.962±1.539 ^a
3% Cocoa	5.392±0.666 ^a	4.260±1.394 ^{ab}	4.978±0.474 ^b
CFCS1	2.706±0.592 ^b	2.686±0.338 ^b	3.083±0.678 ^c
CFCS2	1.243±0.578 ^c	1.440±0.534 ^c	1.506±0.618 ^d

Values with different letters (a–d) within an individual column are significantly different at $p < 0.05$.

* Data in the table represent ‘mean ± standard deviation’ of triplicate.

Table 3-4. Bio-active components production by *L. casei* with supplementary cocoa

Compound name	Class	Biological Functions	Fold change *
Citric acid	Tricarboxylic Acids and Derivatives	Component of glyoxylate; Dicarboxylate metabolism	514.29
Alpha-Linolenic acid	Lineolic Acids and Derivatives	Essential fatty acid; Membrane integrity/stability; Cell signaling	99.19
N-Phenylacetyl pyroglutamic acid	Pyrrolidones	Not available	28.36
2-Methylglutaric acid	Branched Fatty Acids	Membrane integrity/stability; Fuel or energy source; Cell signaling	24.06
Leucinic acid	Branched Fatty Acids	Membrane integrity/stability; Fuel or energy source; Cell signaling	5.28
L-Methionine	Alpha Amino Acids and Derivatives	Essential amino acids; Component of AAs metabolism	2.04
Butyl salicylate	Hydroxybenzoic Acid Derivatives	Nutrient	1.58
Cinnamyl alcohol	Phenylpropenes	Not available	1.25
Zalcitabine	Pyrimidine Nucleosides and Analogues	Reverse transcriptase inhibitors; Anti-HIV agents	1.10
Phenyllactic acid	Phenylpyruvic Acid Derivatives	Not available	1.01

* Fold change of each compound is calculated based on ratio of relative intensity values

Chapter 4: Combating enteric pathogenic bacterial infections with linoleic acids overexpressed *Lactobacillus casei*

Introduction

Human enteric microbial infections are principally characterized by diarrhea with or without other complications/consequences which causes 4 to 6 million deaths annually worldwide and possess huge economic burden (Christou, 2011; Viswanathan, Hodges, & Hecht, 2009). The dominant causative agents of enteric bacterial diseases include *Salmonella*, EHEC, *Campylobacter*, *Listeria monocytogenes* and *Shigella* (Forsythe, 2016; Huang et al., 2016; Mor-Mur & Yuste, 2010; Viswanathan et al., 2009). These enteric bacterial pathogens are typically acquired through contaminated foods and water, therefore daily risk associated with foodborne diseases for everyone living in this planet. According to the estimation of the Center for Disease Control and Prevention, in the US alone, 48 million illnesses (approximately 1 in 6 Americans), more than one hundred thousand hospitalizations, and thousands of death are caused by foodborne infections each year (D. Adams et al., 2015, 2017; D. A. Adams et al., 2016; Hoffmann et al., 2012). The most predominant causative foodborne infectious agents, *Salmonella* and EHEC are colonized in farm animal gut as a part of normal flora in swine, poultry, and cattle.

Poultry and cattle are the most common reservoirs for *Salmonella*, whereas EHEC mostly are shed in cattle and their products can be cross-contaminated directly or fruits and vegetables acquire these pathogens through water and/or soil contaminated with animal waste and infect human beings even exiting all possible prevention and

practices are enforced (García, Fox, & Besser, 2010; Mengfei Peng et al., 2016). The serious health concerns and enormous economic loss precipitated by enteric bacterial infections forced both scientists, and consumers to seek approaches defeating the pathogens by improving the host defense.

The majority of human gut epithelial surfaces are colonized by a tremendous number of microorganisms which are known as normal gut microflora and their role is crucial in forming a complex ecosystem with huge microbial diversity (Tlaskalová-Hogenová et al., 2011). Though viruses, fungi, and protozoans are also colonized along with predominant bacterial flora, and they build up a microbial genetic repertoire being approximately 100 times greater than the number cells that of human host (Fujimura et al., 2010). According to recent reports, human distal gastrointestinal (GI) tract houses more than 1000 distinct bacterial species and the total number was estimated to be larger than 10^{14} CFU/gm of fecal material. Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria are the prevalent phyla in human microbiota (Guarner & Malagelada, 2003; Shreiner, Kao, & Young, 2015; Vedantam & Hecht, 2003). In normal situation, most of the commensal bacteria colonize and survive symbiotically, whereas, under certain conditions such as immunodeficiency, malnutrition, and antibiotic-treatment causing dysbiosis, commensal bacteria can also induce pathology and diseases (Segal & Blaser, 2014; Wexler, 2007). For instance, taking broad-spectrum antibiotics or any other detrimental effect may cause dysbiosis of gut ecosystem that lead the irritating bowel, reducing the number of beneficial bacteria, and increase the number of opportunistic pathogens and further weaken the host defense and/or induce inflammation (Beaugerie & Petit, 2004). As a consequence of the imbalance gut

microflora and/or their produced metabolites, a very limited number of enteric bacterial pathogens and/or opportunistic pathogens can take over the gut ecosystem. In such situation, procommensal strategies by application of probiotics, and/or prebiotics, or synbiotics can be considered as priority in prevention and therapy of foodborne bacterial pathogen-induced enteric illness. With such promising scheme, it allows an establishment of the enteric microbial ecosystem by introducing native or genetically engineered beneficial probiotics without deleterious effects on human commensal gut bacteria (Mengfei Peng & Biswas, 2017).

The enormous microflora, specifically probiotics or beneficial bacteria, in the host GI tract ferment or metabolize undigested dietary components which reaches the small and large intestine, and generates/releases a tremendous treasury of secondary metabolites (byproducts), most of which have been associated with multiple health-beneficial effects (Flint et al., 2012; Marcobal et al., 2013). These metabolites from probiotics generally include bio-active polypeptides with antimicrobial and immunomodulatory properties as well as vitamin B which is essential for human growth, metabolism, and reproduction (Stanton et al., 2005). Whereas, the major byproducts are lipid molecules like fatty acids various in chemical structure from SCFAs to PUFAs (Louis et al., 2014; Serini et al., 2009). The mixed concentration of by-produced lipid molecules in human colon is approximately 50-150 mM, and these beneficial lipid molecules are active and involved in modulation of host's immune responses (Louis et al., 2014).

Linoleic acid is one of the most crucial beneficial metabolites produced from microbial sources including *Bifidobacterium*, *Lactobacillus*, and *Lactococcus*. (Rizos,

Ntzani, Bika, Kostapanos, & Elisaf, 2012). The mixture of positional and geometric isomers of linoleic acid (C18:2, c9, c12), as conjugated linoleic acids (CLA), distinguishes themselves from other fatty acids because of its wide range of benefits on human health including anti-obesity, anti-carcinogenesis, anti-inflammation, and anti-pathogenic activity (Benjamin & Spener, 2009; H. Y. Lee et al., 2006; O'Shea et al., 2012; Bo Yang et al., 2015). Dairy and human/animal intestinal originated bacteria including LAB and bifidobacteria were demonstrated to produce CLA during their metabolism (Alonso, Cuesta, & Gilliland, 2003b; Y. J. Kim & Liu, 2002; Tung Y. Lin, 2000; Ogawa et al., 2001; Soo et al., 2008). Among these bacteria, *L. acidophilus*, *L. casei*, *L. plantarum*, *L. rhamnosus*, *Lactococcus lactis*, and *Streptococcus thermophilus* have been revealed as CLA productive bacteria (Van Nieuwenhove et al., 2011), however their CLA productivity is various and usually limited by multiple factors including temperature, oxygen availability, substrate concentration etc. (Pandit et al., 2012). A growing number of researches are recently focusing on stimulating the productivities of linoleic acids and CLA from microbial sources especially probiotics both in human intestine and industry production level (Mengfei Peng & Biswas, 2017).

Through our previous research, we have observed relatively intense antimicrobial activities in *L. casei* against enteric bacterial pathogens such as *Salmonella enterica* serovar Typhimurium (ST) and EHEC (Mengfei Peng, Reichmann, et al., 2015). However, the linoleic acid productivity (conversion ratio) remains relative low as 4.8%. On the contrary, although *L. rhamnosus* possesses the highest CLA conversion ratio among all active *Lactobacillus* strains, its anti-pathogen capability is limited (Van Nieuwenhove et al., 2011). In this study, we cloned and over-expressed

the *mcra* (myosin-cross-reactive antigen gene), encoding LI, from *L. rhamnosus* GG into *L. casei*, and aimed to examine the role of this novel probiotic in prevention and control of enteric bacterial infections both *in vitro* and *in vivo* on BALB/cJ mice model.

Materials and Methods

Bacterial strain and growth conditions. Probiotic strains *Lactobacillus casei* ATCC 334 (LC-WT) and *L. rhamnosus* GG ATCC 53103 were purchased from American Type Culture Collection (ATCC, VA, USA). *Lactobacillus* strains were grown on MRS (EMD Chemicals Inc., Gibbstown, NJ, USA) agar at 37°C for 24 h in the presence of 5% CO₂ (Forma™ Scientific CO₂ water jacketed incubator, Thermo Fisher Scientific, Waltham, MA, USA). Enteric bacterial pathogens *Salmonella enterica* serovar Typhimurium (ATCC 14028) (ST) and enterohemorrhagic *Escherichia coli* EDL933 (ATCC 700927) (EHEC) were grown on LB agar (EMD Chemicals Inc., Gibbstown, NJ, USA) for 18 h at 37°C under aerobic conditions (Thermo Scientific, Thermo Fisher Scientific, Waltham, MA, USA).

Cell lines and culture conditions. Human intestinal epithelium cells (INT407, ATCC CCL-6) were purchased from ATCC and cultured at standard condition (37°C, 5% CO₂, 95% humidity) in DMEM) supplemented with 10% FBS and 100 g/mL gentamicin (HyClone Laboratories Inc., Logan, UT, USA). The cultured cells were seeded at approximately 2×10⁵ cells/mL/well into 24-well tissue culture plates (BD Falcon, Franklin Lakes, NJ, USA) to reach 80-90% confluence monolayer at standard condition for cell adhesion assay. The post-confluent INT-407 epithelial cell monolayers were

rinsed with PBS and stabilized in antibiotic-free DMEM for 1 h prior to the invasion assay.

Human macrophage cell line (U937, ATCC CRL3253) was purchased from ATCC and grown at standard condition in RPMI-1640 Medium supplemented with 10% FBS and 100 g/mL gentamicin. An aliquot of 6 mL cell suspension containing 1×10^6 cells will be transferred into 25 cm² flask (Greiner Bio-One, Monroe, NC, USA) and cultured at standard condition for 24-30 h. After time, the cell monolayer was washed for three times with RPMI for further bacterial infection.

Mice model and animal care. The 3-week-old BALB/cJ Mice (around 8-10 g) were purchased from the Jackson Laboratory (Bar Harbor, ME USA) and reared in static micro-isolating cages with cellulose Bio-Performance bedding and bio-huts as environmental enrichment. Teklad standard rodent diet and regular tap water were provided for mice feeding and drinking. A total of 90 mice (45 male and 45 female) were used for each trial experiment. Specifically, following completely randomized design, 90 mice were randomly assigned to 9 groups, thus 10 mice per group; two cages of the unit were assigned to each group, thus a total of 5 mice per cage.

Mice cages were changed weekly, and each individual mouse was weighed and monitored for health examination daily. At the end of the second, third, and fourth week, 3, 3, and 4 mice from each group were euthanized respectively with CO₂ inhalation in euthanasia chamber for organ samples collection.

Over-expression of myosin-cross-reactive antigen gene (*mcra*) in *L. casei* for developing LC-CLA. Plasmid pJET and *E. coli* DH5 α were purchased from Thermo Fisher Scientific (Waltham, MA, USA), pDS132 and *E. coli* β 2155 were donated by Dr. Fidelma Boyd (Delaware University, Newark, DE, USA), and pMSP3535 were purchased from Addgene (Cambridge, MA, USA). LC-WT and *L. rhamnosus* GG (ATCC 53103) were harvested from overnight culture in MRS broth, followed by three times sub-culture on MRS agar plate at 37°C for 24 h in the presence of 5% CO₂ incubator.

The entire cloning design was summarized in Figure 1. Briefly, the 1750 bp *mcra* from *L. rhamnosus* GG was PCR amplified and ligated into pJET vector through blunt-end cloning. Aliquot of 250 μ L *E. coli* DH5 α bacterial suspension in cold 50 mM CaCl₂ was mixed with 10 μ L ligated product (pJET-*mcra*) for 10 min incubation on ice, followed by 50 s incubation at 42°C in water bath. After further 2 min incubation on ice, 250 μ L LB broth was added into bacteria-plasmid mixture for 10 min incubation at room temperature followed by selection on LB agar with 100 μ g/mL ampicillin for transformation. The *E. coli* DH5 α -expressed *mcra* was double-excised from pJET-*mcra* with BamHI and XbaI and then ligated into pMSP3535 vector at 16°C overnight. Following the same condition, pMSP3535-*mcra* was further transformed into *E. coli* DH5 α and mixed with LC-WT at ratios of 1:1, 1:5, and 1:10 (donor cells: recipient cells) for bacterial mating. The *L. casei*-pMSP3535 was harvested through consecutive sub-culture and selection on MRS agars containing 300 μ g/mL erythromycin at 37°C under micro-aerophilic condition (Tabashsum, Peng, Salaheen, Comis, & Biswas, 2018).

Removal of antibiotic-resistance marker and *mcra* chromosomal recombination. The pMSP3535-*mcra* was isolated using Plasmid Mini Kit (Qiagen, Germantown, MD, USA). The gene sequence of *mcra* linked with transcription promoter P_{nis} was amplified by PCR using pMSP3535-*mcra* as the template. The upstream homologous arm *upp1* (208 bp) and downstream homologous arm *upp2* (211 bp) concatenated with Xba1 and Sac1 linkers were also PCR amplified using LC-WT genomic DNA as the template. Ligation of *upp1-mcra-upp2* was performed by PCR programmed for 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s. After pJET blunt-end cloning, pJET-*upp1-mcra-upp2* and pDS132 were double-digestion with Xba1 and Sac1, followed by sticky-end ligation for overnight at 16°C. The pDS132-*upp1-mcra-upp2* was then transformed into *E. coli* β 2155 following the same method described above but with 0.3 mM DAP selection. The transformed *E. coli* β 2155 was mixed with overnight cultured LC-WT at ratio of 1:1, 1:5, and 1:10 (donor cells: recipient cells) for bacterial mating. Aliquot of 1 mL of the mixed bacterial suspension was spread on MRS agar plate with 0.3 mM DAP, followed by 5 h incubation at 37°C under micro-aerophilic condition. The *L. casei*-pDS132 was harvested through sub-culture and selection on MRS agar with 30 μ g/mL chloramphenicol. Individual bacterial colony was consecutively sub-cultured in fresh MRS broth and selected on MRS agar containing 100 μ g/mL 5-fluorouracil (5-FU) for *upp1-mcra-upp2* chromosomal homologous recombination. Finally, the *mcra* chromosomal recombinant *L. casei* mutant was harvested and named it as LC-CLA.

In vitro mixed culture of *L. casei* with enteric bacterial pathogens. Bacterial cells from overnight agar plates were collected in 10 mL PBS using 10 μ L sterile disposable loops. Each concentrated bacterial suspension was adjusted using PBS and measured by LAMBDA BIO/BIO+ spectrophotometer (PerkinElmer, Beaconsfield, UK) for adjusting the bacterial concentration to approximately 7 log CFU/mL. Aliquots of 400 μ L adjusted *L. casei* and *S. Typhimurium* or EHEC suspension were added to sterilized test tubes containing 3.2 mL DMEM with 10% FBS and then incubated at 37 °C for different time points (0, 2, 4, 8, 24, 48, and 72 h). After incubation, serial dilutions were performed in PBS, and then plated on agar plates (MRS agar for *L. casei*, LB agar for *S. Typhimurium* and EHEC) in triplicate, followed by incubation for 18 h at 37 °C for growth. Bacterial CFUs were counted afterwards and results were expressed in unit of bacterial log CFU/mL as the average number from triplicate assays.

Isolation of CFCSs from *L. casei*. The fresh overnight (18 h) liquid cultures of *L. casei* ATCC 334 and *L. casei*-CLA in MRS were centrifuged at 4000 \times g for 15 min. CFCSs were collected and then passed through 0.2 μ m sterile syringe filter (VWR, PA, USA) for removal of bacterial cells. The filtered CFCSs were then collected and stored at 4 °C for further *in vitro* antimicrobial and anti-inflammatory investigations.

Evaluation of physicochemical properties of *S. Typhimurium* and EHEC. To determine the cell surface hydrophobicity of *S. Typhimurium* and EHEC, the enteric bacterial cells were grown in the presence or absence of CFCS from either *L. casei*-CLA or *L. casei* ATCC 334 at 37 °C for 18 h. Then bacterial cells were suspended in 2 mL of PBS for

OD adjustment into 0.5 (H_{t_0}) at wavelength at 570 nm. The adjusted bacterial suspension was mixed with 1 mL of n-hexadecane and incubated for 5 min at room temperature. The OD (H_{t_5}) of aqueous phase from the mixture was measured at 570 nm using microplate reader (Multiskan FC, Thermo Fisher Scientific Inc., Waltham, MA, USA). The hydrophobicity values were calculated based on the following equation: $\text{Hydrophobicity (\%)} = (1 - H_{t_5}/H_{t_0}) \times 100$.

The interactions between bacteria cell surfaces were determined by the auto-aggregation assay. The bacterial cells cultured in the presence or absence of *L. casei* CFCSs at 37 °C for 18 h were centrifuged at 3,000 g for 20 min, and then the bacteria were diluted with PBS (pH 7.2) for OD adjustment to 0.5 (A_{t_0}) at 570 nm. The bacterial suspensions (3 mL each) were incubated at 37 °C for 2 h. The OD of upper suspensions were further measured at 570 nm (A_{t_2}) using Multiskan microplate reader (Ahn et al., 2014). The ability of bacterial cells to aggregate was estimated based on following equation: $\text{Auto-aggregation (\%)} = (1 - A_{t_2}/A_{t_0}) \times 100$.

Enteric bacterial cell injury induced by *L. casei* CFCSs was evaluated according to the overlay method. Briefly, the control and CFCSs-treated *S. Typhimurium*/EHEC bacterial cells cultured at 37 °C for 18 h were serially (1:10) diluted with PBS (pH 7.2), followed by plating on Trypticase soy (TSA) agar and XLD- or MacConkey-overlaid TSA agar, respectively, and then they were incubated at 37 °C for 24h. The antimicrobial-induced bacterial cell injury rate was estimated by the difference between the bacterial cell CFU numbers obtained from TSA and XLD agar or MacConkey agar (Ahn et al., 2014).

Bacterial biofilm formation. Both *S. Typhimurium* and EHEC were inoculated at approximately 5×10^5 CFU/mL in 6-well plates (Corning, NY, USA) containing 22×22 mm² glass slides and LB broth in the absence or presence of *L. casei* CFCs in triplicate for 24, 48, and 72 h incubation at 37 °C without shaking. At each time point, the glass slides were rinsed with PBS for five times and bacterial cells were recovered from the glass slide surface using sterile cell scraper (VWR, PA, USA). The biofilmed bacterial cells were scrapped from glass slides and then serially diluted for plating on LB agar for enumeration.

Enteric bacterial adhesion and invasion activities on human intestinal cells. The INT407 cells grown in 24-well plate with 800 µL DMEM were pretreated with 100 µL DMEM (control), *L. casei* CFCs, or 2×10^8 CFUs *L. casei* bacterial cells, separately for 1 h, with each treatment in triplicate. A 100 µL aliquot of *S. Typhimurium* or EHEC with MOI=100 (2×10^8 CFUs) was inoculated into triplicate wells. Afterwards, the infected cells were incubated at standard condition for another 2 h, and then followed by three times washing with DMEM. The cell monolayers were lysed with 0.1% Triton X-100 for 15 min, serial diluted, and plated on specific agars (MRS agar for *L. casei*, Xylose Lysine Deoxycholate agar for *S. Typhimurium*, MacConkey agar for EHEC) to estimate the adhesive bacterial CFU. To measure bacterial cell invasive activity, DMEM washed cell monolayers after 2 h bacterial infection was incubated in DMEM containing 10% FBS supplemented with 250 µg/mL gentamicin for 1 h, then followed by three times DMEM washing, Triton X-100 lysis, serial dilution, and eventually plating on selective agars mentioned above.

Simulation of enteric bacterial inflammation in human macrophage cells. Enteric bacterial pathogen *S. Typhimurium* that provoke inflammation in human gut intestine was cultured on LB agar plate for 18 h. A 100 μ L aliquot of bacterial suspension, containing approximately 1×10^8 CFU (MOI=100) was inoculated into triplicate 25 cm^2 flasks containing U937 cell monolayer. In the test flasks, 500 μ L overnight (18 h) CFCSs from *L. casei* in DMEM with 10% FBS were added during *S. Typhimurium* infection period. The infected monolayers will be incubated for 24 h at standard condition, followed three times washing with ice-cold PBS for RNA extraction.

RNA extraction and cDNA synthesis. Extraction of bacterial cells, human macrophage cell line, and mice spleen RNA was carried out using TRIzol® Reagent (Life Technologies Co., Carlsbad, CA, USA). Briefly, host cell pellets or mice spleen smashed pieces were lysed with 1 mL TRIzol for 5 min at room temperature. The cell or tissue lysates were mixed with 200 μ L chloroform with 30 sec vigorous vortexes. After 3 min condensation at room temperature, the mixtures were centrifuged at $14,000 \times g$ at 4°C for 15 min. Then the aqueous phase was collected and gently mixed with 500 μ L of isopropanol for 10 min standing at room temperature and centrifugation at $14,000 \times g$ at 4°C for 15 min. The gel-like RNA pellet was washed with 1 mL 75% ethanol, vortexed 10 s, centrifuged at $7000 \times g$ at 4°C for 5 min, air-dried in bio-safety cabinet for 10 min, and dissolved in 50 μ L RNase-free water. The RNA concentration was quantified with a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Furthermore, 1 μ g of extracted RNA was mixed with 1 μ L of RTS

DNase and 5 μ L of DNase buffer (MO BIO Laboratories, Inc., Carlsbad, CA, USA) and incubated at 37 °C for 20 min; 5 μ L RTS DNase Removal Resin was added and re-suspended every 1 min up to 10 min. After 1 min 14,000 \times g centrifugation, the supernatant containing RNA was transferred and kept at -20 °C for cDNA reverse transcription.

The cDNA synthesis will be performed according to the protocol of qScript cDNA SuperMix. The eluted RNA (1 μ g) will be mixed with 4 μ L of 5X qScript cDNA SuperMix containing optimized concentration of MgCl₂, 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.

Quantitative RT-PCR for evaluation of gene expressions. The PCR reaction mixture containing 10 μ L PerfeCTa SYBR Green Fast Mix (Quanta Biosciences, Beverly, MA, USA), 2 μ L of each 100 nM primer (listed in Table 4-1, 4-2, and 4-4), 2 μ L of cDNA (10 ng), and 4 μ L of RNase-free water was amplified using an Eco Real-Time PCR system with 30 sec denaturation at 95 °C, followed by 40 cycles of 95 °C for 5 sec, 55 °C for 15 sec, and 72 °C for 10 sec. All the relative transcription levels of target genes were estimated by comparative fold change. The CT values of genes were normalized to the housekeeping gene, and the relative expression levels of target genes were calculated by the comparative method (Livak & Schmittgen, 2001). Quantitative RT-PCR was carried out in triplicate.

In vivo probiotic colonization and enteric bacterial challenge. Overnight culture of *L. casei* bacterial cells from MRS broth were 1:50 diluted in fresh 5 mL MRS broth for 3-4 h further growth. The exponential phase bacterial cells were harvested following centrifugation at $3,000 \times g$ for 15 min, PBS washing, and resuspension in 1.0 mL PBS. A final concentration of 10^{11} CFU/mL was adjusted with PBS. The design of *in vivo* trial was summarized in Table 3-3. Approximately 10^9 CFU *L. casei* bacterial cells were maintained daily in mice water bottle containing regular tap water for group B and C. Aliquot of 100 μ L *L. casei* ATCC 334 and *L. casei*-CLA suspensions were fed to mice in group B and C respectively, by oral gavage using fluid dispensing syringe (BD, Franklin Lakes, NJ, USA) at morning of Day 6 and 7. Mice in group A was orogastrically fed with 100 μ L PBS and served as control.

Overnight culture of *S. Typhimurium* and EHEC bacterial cells from LB broth were 1:50 diluted in fresh 5 mL MRS broth for 3-4 h further growth. The exponential phase bacterial cells were harvested following centrifugation at $3,000 \times g$ for 15 min, PBS washing, and resuspension in 1.0 mL PBS. A final concentration of 10^8 CFU/mL was adjusted with PBS. At Day 7 afternoon, Aliquot of 100 μ L *S. Typhimurium* or EHEC suspension was fed to mice in groups 2 or 3 respectively, with oral gavage, and the mice were reared thereafter for another 3 weeks. Mice in group 1 was orogastrically fed with 100 μ L PBS and served as control.

Mice sample collection and processing. In order to estimate the bacterial fecal shedding, fecal samples were collected from each mouse in sterile Whirl-Pak bags using sterile spoon at Day 1, 2, 3, 6, 7, 8, 9, 14, 21, and 28 for PBS serial dilution and plating on

specific agar plates (MRS agar for *L. casei*, XLT-4 agar for *S. Typhimurium*, MacConkey agar for EHEC).

In order to investigate the bacterial colonization in mice gut intestine, ileum, jejunum, and cecum from each euthanized mouse were separated and harvested. Then the ileum, jejunum, and cecal fluids were serial diluted with PBS, followed by plating on specific agar plates (MRS agar for *L. casei*, XLT-4 agar for *S. Typhimurium*, MacConkey agar for EHEC).

Mice spleen was harvested and kept in RNA Later for further RNA extraction, cDNA reverse transcription, and inflammation-related gene expression level analysis; Mice kidney, ileum, jejunum, and cecum were collected, washed with PBS, and preserved in 4% formaldehyde for further histopathology study.

Statistical analysis. All the data were analyzed by the Statistical Analysis System software. The one-way analysis of variance followed by Tukey's test was applied to determine the significant differences of bacterial counts, physicochemical values, and virulent gene expression levels among the control and treatments based on a significant level of 0.05.

Results

Phenotypical characterization of LC-CLA. In comparison with LC-WT, LC-CLA maintained an analogous growth/survival rate, improved the adhesion ability on human intestinal cells *in vitro* and *in vivo* colonization capability in mice gut (Figure 2). Due to over-expression of the *mcra* gene, LC-CLA secreted larger volume of linoleic acids

(Table 5). To be specific, primarily, LC-CLA showed slower growth (around 0.3-0.4 log CFU/mL less) compare to the LC-WT during exponential phase, first 24 h of inoculation, but after 24 h, LC-CLA showed higher survival ability (around 0.1-0.3 log CFU/mL more) during stationary and death phase, up to 96 h.

In addition, 0.8 and 1.5 log CFU/mL more LC-CLA attached to INT-407 cells both at 4 and 24 h of incubation, showing significant differences comparing to the adhesion ability of LC-WT. Furthermore, based on the duplicated mice trials, LC-CLA remarkably improved their colonization ability in mice cecum, jejunum, and ileum with 7-day-administration. The colonization amount of LC-CLA was found to be 1.0, 0.7, and 0.5 log CFU/g higher in cecal fluids, 0.6, 0.6, and 0.5 log CFU/g higher in jejunum fluids, and 0.1, 0.1, and 0.3 log CFU/g higher in ileum fluids than the LC-WT, at 2nd, 3rd, and 4th weeks respectively. Finally comparing with LC-WT, the genitival engineered strain, of LC-CLA induced 7.2-fold up-regulation on *mcra* (LI gene) mRNA level expression identified by qPCR; with HPLC-MS/MS analysis, we also detected 4.5-fold increment in relative total linoleic acids per 1 mL overnight cultural supernatant as well as 21.1-fold boost in relative total linoleic acids per bacterial cell.

Competitive exclusion of enteric bacterial pathogens. Probiotic *L. casei* strains and enteric bacterial pathogens ST and EHEC were grown in mixed-cultured condition *in vitro* to investigate their survival ability through competition between them in both short (4 and 8 h) and long (up to 72 h) period. The competitive inhibitory abilities of both LC-WT and LC-CLA against ST and EHEC were shown in Figure 3. With both of these pathogens outset with 10⁶ CFU/mL, LC-CLA rapidly started to phase out both

enteric bacterial pathogens, showing 0.8 and 2.0 log CFU/mL ST and EHEC, respectively, more reduction at 4 h, 1.3 and 2.3 log CFU/mL ST and EHEC, respectively, more reduction at 8 h comparing with LC-WT. Overall, LC-CLA competitively exclude ST at 72 h, in comparison with 8.3 and 2.5 log CFU/mL survival ST for control and wild type LC; similarly, EHEC were also eliminated by LC-CLA at 48 h, at which time point, 8.9 and 4.4 log CFU/mL EHEC were still detected in the cultural media.

Metabolites from LC-CLA in combating against enteric bacterial pathogens. Overnight CFCSs from both LC-WT (CFCS1) and LC-CLA (CFCS2), in terms of initial inocula of 10^6 CFU/mL overnight probiotic culture, were collected for examination the antimicrobial activities of their secreted byproducts. Comparing with control, both CFCSs from *L. casei* inhibited the growth of ST and EHEC, however, CFCS2 showed more intensive effects (Figure 4). To be specific, CFCS2 reduced 1.3 and 2.0 log CFU/mL more ST as well as 1.9 and 2.3 log CFU/mL more EHEC in the early stage at 4 and 8 h, when compared with CFCS1. The inhibitory activity of CFCS1 attenuated after 24 h, 3.2, 4.5, and 5.2 log CFU/mL ST and 4.2, 5.0, and 5.1 log CFU/mL EHEC were observed at 24, 48, and 72 h, respectively. Even, metabolites from LC-CLA exhibited a stable antimicrobial activity after 24 h, whereas CFCS2 were capable of ruling out all survival ST (9.2 log CFU/mL) at 72 h and EHEC (8.9 log CFU/mL) at 48 h.

Alterations in physicochemical properties of enteric bacterial pathogens. The entire metabolites from both LC-WT and LC-CLA alters multiple physicochemical properties of both ST and EHEC (Table 6). For example, CFCS1 decreased 7.2% and 3.2% bacterial surface hydrophobicity of ST and EHEC, respectively, whereas CFCS2 exhibited more profound effectiveness in significantly lowering hydrophobicity of both pathogens (11.7% for ST and 10.2% for EHEC). Following the same trend, metabolites from LC-CLA significantly reduced 9.5% and 3.8% bacterial auto-aggregation activities of ST and EHEC, respectively, which displayed more effective alterations compared with metabolites from wild type LC-WT (6.1% for ST and 1.6% for EHEC). Similarly, 30.9% and 38.3% injured ST and EHEC bacterial cells were observed with treatment of CFCS1, whereas CFCS2 significantly increased the injured cells of ST (11.9%) and EHEC (12.5%).

Effect on biofilm formation by ST and EHEC. The biofilm formation ability of ST and EHEC in absence or presence of CFCSs from LC-WT and LC-CLA has been presented in Figure 5. At 24, 48, and 72 h incubation under the inhibitory pressure of LC-CLA secreted metabolites in CFCS2, the biofilm formation of ST was significantly repressed by 1.4, 1.0, and 1.0 log CFU/mL respectively. Whereas CFCS1 from LC-WT exhibited less inhibitory effects and failed to decrease the ST biofilm formation significantly after 72 h of incubation. The biofilm formation ability of EHEC was significantly restrained by 1.9 log CFU/mL at 24 h treatment with CFCS2 from LC-CLA. At 48 and 72 h, both CFCS1 and CFCS2 exhibited significant reduction on EHEC biofilm formation by 1.0 and 0.8 log CFU/mL.

Disruption on host-ST/EHEC interactions. The host cell-ST or EHEC interactions were evaluated based on their adhesion to and invasion into human intestinal (INT-407) cells, and the results were shown in Figure 6. Wild type LC-WT pre-colonization before adding enteric bacterial pathogens significantly reduced the cell adhesive and invasive abilities of ST by 1.8 and 1.3 log CFU/mL, respectively. In the same experiment, cells were pretreated with LC-WT reduced the adherence and invasion abilities of EHEC by 0.7 and 1.1 log CFU/mL, respectively. With much effective performance was observed when INT-407 cells were allowed to pre-colonize with LC-CLA. The adhesive and invasive activities of ST were suppressed by 2.4 and 2.2 log CFU/mL, counting around 99.58% and 99.34% reductions separately, by LC-CLA. Similarly, LC-CLA also reduced EHEC host cell adhesion and invasion capabilities by 2.0 and 2.9 log CFU/mL, which equaled to 99.10 and 99.88% reductions separately.

Correspondingly, the pre-treatments of ST and EHEC with *L. casei* CFCSs displayed significant effects on their interactions/infections with INT-407 cells. Specifically, metabolites in CFCS1 from LC-WT, CFCS1 restricted the adherence activities of both ST and EHEC on INT-407 cells by 1.8 and 0.9 log CFU/mL, respectively. CFCS1 also significantly reduced the invasive activities of ST and EHEC by 1.2 and 1.0 log CFU/mL separately in comparison with control infections. Whereas, CFCS2 from LC-CLA by interfering with ST and EHEC much intensively, depressed 2.4 log CFU/mL (99.66% reduction) ST and 1.8 log CFU/mL (98.53% reduction) EHEC adhesion, as well as 2.1 log CFU/mL (99.15%) ST and 2.5 log CFU/mL (99.70%) EHEC invasion on INT-407 cells.

Down-regulation on expression of bacterial virulence genes. The relative expression levels of multiple ST/EHEC virulence genes were found to be significantly down-regulated with CFCSs from both LC-WT and LC-CLA based on qPCR analysis, among which, the suppressive effects from CFCS2 were detected to be more intensive than CFCS1 (Figure 7). For ST, CFCS2 from LC-CLA notably down-regulated the expression of transcriptional regulator genes *hilA*, *hilC*, *hilD*, and *invF* by 3.2, 2.8, 3.9, and 5.1 folds respectively. Similarly, the expression levels of effector genes *invA*, *invG*, *invH*, and *prgK* were also significantly suppressed by CFCS2. Whereas insignificant fold-changes were detected in relative gene expression levels of *invC*, *prgH*, *prgI*, and *sipA*.

For EHEC, 8 virulence genes were investigated in this study, among which only effector gene *tir* kept conservative under the pressure of both CFCSs treatment. CFCS2 effectively down-regulated the expression levels of regulator gene *ler* as well as other effector genes including *eaeA*, *espA*, *espB*, and *espD* by 2.8, 3.6, 7.3, 6.9, and 7.1 folds respectively.

Anti-inflammatory effects of LC-CLA in vitro. Metabolites secreted by *L. casei* strains managed to induce anti-inflammatory effects on human macrophage cells by down-regulating pro-inflammatory cytokine genes and up-regulating anti-inflammatory cytokine genes (Figure 8). In details, CFCS1 from LC-WT suppressed the expression levels of IL-1 β , CXCL-8 (IL-8), IL-12, and TNF- α genes by 3.3, 3.0, 3.0, and 4.8 folds respectively, and at the same time, it raised the expression levels of IL-10 and TGF- β

genes by 4.4 and 2.5 folds separately. Whereas negligible difference was found on IL-6 and IL-23 gene expressions. On the other side, CFCS2 containing metabolites released from LC-CLA impressively amplified the anti-inflammatory activities, by which relative expression levels of pro-inflammatory cytokine IL-1 β , IL-8, IL-12, IL-23, and TNF- α genes were all significantly down-regulated by 7.7, 5.2, 6.0, 1.6, and 6.7 folds respectively; relative expression levels of anti-inflammatory cytokine IL-10 and TGF- β genes were significantly up-regulated by 8.0 and 5.9 folds.

Improvement of mice weight from enteric bacterial infections. The weight of each mice was monitored every day for the purpose of investigating if probiotics preventive administration could rescue mice from weight loss due to ST/EHEC infection (Figure 9). Within the entire 4-week rearing, a total of 12 mice in control group, 7 mice in group with LC-WT treatment, and 1 mouse in group with LC-CLA treatment were sacrificed due to their health abnormality induced by ST infection. These sacrificed individuals included 8 mice from control and 5 mice from LC-WT treatment found self-death due to ST challenge, but none from LC-CLA treatment, which provided us the ST survival rates as 60% in control group, 75% in LC-WT group, and 100% in LC-CLA group. The death of the mice was generally accompanied with extreme (>20%) weight loss to approximately 8-10 g.

In details, the 3-week-old mice started from an average of 10-12 g weight at 0 d and reached approximately 14-16 g for the first week's rearing, during which, either LC-WT or LC-CLA consumption contributed around 1-2 g more weight gain compared with mock probiotic administration. Once challenged with ST, the average weight gain

of mice in control group (14.64 g at the day of ST challenge) was found to suspend showing 14.65 g at 1st post-infection week, and it was even diminished to 14.36 g and 13.47 g at 2nd and 3rd post-infection weeks separately. However, LC-CLA administration assisted mice in continuous averaged weight gain, in spite of the negative effect induced by ST infection, from 16.21 g at the day of challenge to 16.88, 17.02, and 19.12 g at the 1st, 2nd, and 3rd post-infection weeks. LC-WT exhibited mild effects by maintaining the averaged mice body weight during the first two weeks of ST infection and raising around 1.5 g weight at the 3rd post-infection week.

At the other hand, we failed to observe any negative effects including average weight loss induced by EHEC infection. Whereas, the oral administration of LC-CLA, influencing much effectively than LC-WT which declined to induce any significant average weight gain in comparison with control over the same period, benefited the mice in their weight earnings by promoting 1.1 and 1.4 g averaged weight increasing at the 2nd and 3rd post-infection weeks.

Reduction on gut colonization of enteric bacterial pathogens. Either LC-WT or LC-CLA was orally administrated to mice in order to examine the preventive effects of probiotics on enteric bacterial infection. According to the colonization data collected from two individual trials, both *L. casei* pre-treatments significantly reduced the follow-up ST gut-intestinal infecting/colonizing levels, whereas LC-CLA fought back on ST much remarkably (Figure 10). To specify, 1.0, 0.9, and 1.2 log CFU/g less ST presence in cecum fluids with LC-WT prevention at 14, 21, and 28 d (1st, 2nd, and 3rd week post-infection) respectively, were found in comparison with control; LC-CLA

much intensively, reduced 2.0, 2.0, and 2.5 log CFU/g ST cecum-colonization at 14, 21, and 28 d.

For ST colonization on jejunum and ileum, LC-WT and LC-CLA pre-administration managed to lower 1.0 and 2.3 log CFU ST per gram jejunum fluids at 1st week post-infection, 0.9 and 2.5 log CFU/g on 2nd week post-infection, and 1.3 and 3.7 log CFU/g on 3rd week post-infection. Following a comparable way, LC-WT and LC-CLA pre-administration also abated 1.7 and 2.2 log CFU ST per gram ileum fluids at 1st week post-infection, 0.9 and 1.9 log CFU/g on 2nd week post-infection, and 1.2 and 3.4 log CFU/g on 3rd week post-infection.

The significant reduction on ST gut intestinal colonization was also presented by the difference of ST fecal shedding among groups. On the subsequent after ST challenge on ST-free mice (the 8th day), both groups with probiotic administration induced significant 0.8 to 1.1 log CFU ST less per gram feces, though the reductions attenuated to be unsubstantial at the 9th day. However, notably major effectiveness started to appear in LC-CLA group after 1st week post-infection, at which 1.3 log CFU/g less ST was recovered from mice feces. In the subsequent two weeks, 1.1 and 2.1 log CFU/g ST reduction on fecal shedding were observed, which is much significantly effective/preventive compared with LC-WT led to less than 0.6 log CFU/g ST reduction.

On the other hand, for EHEC gut intestinal colonization, LC-WT administration barely influenced the EHEC in a considerable level, whereas LC-CLA pre-treatment presented an influential outcome (Figure 11). In particular, LC-CLA administration was capable to significantly reduce 2.3, 1.6, and 0.9 log CFU/g EHEC cecum-colonization,

1.6, 1.8, and 2.7 log CFU/g EHEC jejunum-colonization, and 2.8, 1.8 and 2.1 log CFU/g EHEC ileum-colonization at the 1st, 2nd, and 3rd week post-infection time point. Meanwhile, consequential decreased EHEC fecal shedding was detected in LC-CLA treatment group as well, given that insignificant reductions (0.1 to 0.5 CFU EHEC less per gram feces) were found only during the first two days after EHEC challenge on EHEC-free mice (the 8th and 9th day). The LC-CLA administration substantially lowered 0.9, 1.9, and 2.2 CFU/g EHEC fecal shedding at the 1st, 2nd, and 3rd post-infection weeks in comparison with control.

Regulation of mice splenic inflammatory cytokine genes. The manipulation of splenic inflammatory cytokine gene expressions during 3-week ST infection as well as 1-week probiotic pre-administration was summarized in Table 7. Specifically, ST infection induced remarkably up-regulation on relative expression level of splenic pro-inflammatory cytokine genes, for example, 7.4-9.6 log folds raise of INF- γ gene, 6.1-7.9 log folds raise of IL-1 β gene, 3.5-5.7 log folds raise of TNF- α gene, 1.5-2.4 log folds raise of IL-6 gene, etc. The relative expression level of anti-inflammatory cytokine IL-10 gene was notably reduced by 2.0-3.2 log folds. Whereas, LC-CLA pre-treatment managed to mitigate the boost in inflammatory cytokine genes over-expression. For instance, LC-CLA significantly decreased 4.9-9.1, 3.4-6.6, and 1.5-3.5 log folds expressions of INF- γ , IL-1 β , and TNF- α genes in comparison with situations of ST infection. Simultaneously, LC-CLA also assisted in promoting the expression of IL-10 and TGF- β genes by 3.9-5.1 and 2.5-3.5 log folds. On the contrary, LC-WT induced mild anti-inflammatory effects on mice spleen against ST infection, in which

only INF- γ and IL-1 β genes were found to be consistently down-regulated at each time point of measurement (14th, 21st, and 28th days) at a statistically significant level ($p < 0.05$). As for IL-10 and TGF- β genes, LC-WT up-regulated their mRNA expression levels by 1.2-1.7 and 0.5-1.8 log folds compared with control, displaying a more moderate effect than LC-CLA.

Discussion

Probiotics, prebiotics, or a combination of the two as synbiotics have emerged as a promising alternative treatment for enteric bacterial infections (Hardy, Harris, Lyon, Beal, & Foey, 2013; Pandey, Naik, & Vakil, 2015; Mengfei Peng & Biswas, 2017; Mengfei Peng, Reichmann, et al., 2015; Vyas & Ranganathan, 2012). To improve and maintain host's gut health, the beneficial effects of probiotic depend largely upon the total quantity and type of functional metabolites they could produce. In recent studies, we found that several prebiotic-like components in cocoa and peanut facilitated *L. casei* in producing more linoleic acids and outcompeting major foodborne bacterial pathogens, including ST and EHEC (Mengfei Peng, Aryal, et al., 2015a; Mengfei Peng, Bitsko, et al., 2015; Serajus Salaheen, White, et al., 2014). On the basis of these evidences, we have overexpressed the *mcra* encoding LI in natural *L. casei* and verify the ability of LC-CLA in combating against enteric bacterial infection both *in vitro* and in murine model.

The myosin-cross-reactive antigens present across a wide range of taxa including *Lactobacillus*, not only take responsibility in linoleic acids construction and isomerization (Kishino et al., 2011; O'connell et al., 2013; B. Yang et al., 2014), but

also have been revealed to contribute in bacterial stress-tolerance, blood-survival, and host cell adherence (Y. Y. Chen, Liang, Curtis, & Gänzle, 2016; O'Flaherty & Klaenhammer, 2010; Volkov et al., 2010). In this study accordingly, the *mcra* overexpressed LC-CLA was found with prominently higher production of total linoleic acids, fitter growth pattern though not statistically significant, and remarkably improved intestinal colonization both *in vitro* and *in vivo* especially in mice cecum and jejunum. Though assisting in development of healthy gut microflora and maintenance of cardiovascular health, peanut, cocoa, or other prebiotic-like functional foods are not impeccable for application in long term or in specific populations due to their restrictions on the cost, inducing allergy, and limited bio-availability (Badrie, Bekele, Sikora, & Sikora, 2015; Feeney et al., 2016; Hasler, 2002). Therefore, the genetically engineered probiotic in our research, being self-sufficient, stands out in supply of increased bio-active byproducts devoid of any prebiotic.

As previously discussed by Peng, Reichmann and Biswas (2015), *Lactobacillus* by releasing antimicrobial components like organic acids, hydrogen peroxide, and poly-peptides, outcompete pathogenic bacteria in shorter period. Here in this study, LC-CLA exhibited even stronger effects against ST and EHEC than by LC-WT in mixture competitive exclusion, CFCS extensive growth elimination, as well as inducing bacterial cell injury. The outcomes are in accordance with findings on anti-pathogenic activities in CLA (Bhattacharya, Banu, Rahman, Causey, & Fernandes, 2006; Hontecillas et al., 2002; L.S. Meraz-Torres & H. Hernandez-Sanchez, 2012). However, we also surprisingly observed enhanced effects of LC-CLA in alteration ST/EHEC surface hydrophobicity, auto-aggregation, and biofilm formation. The over-produced

linoleic acids in LC-CLA might take responsibility in these alterations, since they were suggested to interact with cytoplasmic membrane of bacterial pathogens and further disrupt phospholipid or extracellular polysaccharides (Mengfei Peng & Biswas, 2017), both of which are crucial factors for bacterial physicochemical properties as well as biofilm development (Renner & Weibel, 2011; Vu, Chen, Crawford, & Ivanova, 2009).

Specific virulence genes of ST/EHEC involved in T3SS were significantly down-regulated by the secreted metabolites from LC-CLA. These genes include invasion regulator genes and effector genes especially *eaeA* that functions in EHEC A/E and *invH* encoding ST invasion lipoprotein. In fact, several research groups have reported the dose-dependent activities of PUFAs in regulation of *Salmonella* and *E. coli* (Cardenal-Muñoz & Ramos-Morales, 2011; S. Nakamura et al., 2012), however, the conclusion remains to be ambiguous and bearing little relativeness with bacterial infections (Mengfei Peng & Biswas, 2017). The repressed invasive genes and the disrupted bacterial physicochemical properties by LC-CLA, served as identical indicators, both further supported in the *in vitro* reduction of ST/EHEC-host cell adhesive and invasive activities. Through competitive occupying INT-407 cell surface receptor-like molecules (Bernet, Brassart, Neeser, & Servin, 1994; Matsuo, Miyoshi, Okada, & Satoh, 2012; Mengfei Peng, Reichmann, et al., 2015), together with enhanced linoleic acid regulatory effects on bacterial pathogenesis (Belury, 2002b; Hontecillas et al., 2002; Bo Yang et al., 2017), LC-CLA stands out with double inhibitory actions against enteric bacterial pathogens.

The preventive effectiveness of PUFAs against pathogenic bacterial colonization have been demonstrated by several *in vivo* studies including in murine

model (Garner et al., 2009; Snel, Born, & van der Meer, 2010). Whereas the current study, by applying novel probiotic strain with targeted genetical modification, systematically and in-depth investigated the double effects of both Lactobacillus and CLA on murine gut health. According to our results, 1-week consecutive consumption of LC-CLA (including 5-day ingestion through drinking water and 2-day orogastrically feeding) efficiently prevented/mitigated the following Salmonella infection. Although probiotic administration through water might generate variance of bio-availability in mice gut, it is worth mentioning that early-staged oral probiotic gavage was performed at only 6th and 7th days instead of through the entire 1st week to avoid potential induced injury in 3-week-old mouse esophagus. The bacterial fecal shedding serves as a key indicator about the gut intestinal colonization (S. M. Lee et al., 2013), correspondingly we observed reduced ST/EHEC in both fecal content and intestinal fluids. Though similar studies conducted based on EHEC were not systematic and completed, Salmonella colonization was claimed to be restricted by functional fatty acids oral supplements in vivo (Snel et al., 2010; Sunkara et al., 2011; Sunkara, Jiang, & Zhang, 2012; Willamil, Creus, Francisco Pérez, Mateu, & Martín-Orúe, 2011), in which the virulence gene factors of Salmonella were suggested to be manipulated (Hung et al., 2013; Y. Sun & O’Riordan, 2013).

On the other hand, probiotic itself was addressed to both directly through physical repellence and indirectly through host’s immune stimulation mediate colonization resistance against intestinal pathogens (Amalaradjou & Bhunia, 2012; Buffie & Pamer, 2013; Dicks & Botes, 2010; He, McLean, Guo, Lux, & Shi, 2014; McKenney & Pamer, 2015; Salminen et al., 2010). Fortunately, all these studies

mentioned above supported our in vivo findings in which either wild type or genetically engineered *L. casei* remarkably diminished ST/EHEC colonization in cecum, jejunum, and ileum, while LC-CLA displayed more intensive reductions considering the extraneous effects implemented by its over-promoted CLA production (Mengfei Peng & Biswas, 2017).

Finally, extensive anti-inflammatory effects of LC-CLA were presented both ex vivo on human macrophage cells and in vivo on murine splenic cells. In accordance with previous studies on linoleic acids (Akahoshi et al., 2004; Albers et al., 2003; Tricon et al., 2004), we also detected the reducing levels of pro-inflammatory cytokines including INF- γ , TNF- α , IL-1 β , and IL-12 in this study. Moreover, we identified the up-regulation of anti-inflammatory cytokine IL-10 and TGF- β genes as well, the two cytokines of which were believed to induce inhibition on T_h cells activation (L Gorelik, Constant, & Flavell, 2002; Leonid Gorelik & Flavell, 2002; Hsieh et al., 2012). The activated macrophage cells bearing bacterial pathogen challenges normally produce and release IL-12 for activation of T_h1 cells and further induces INF- γ , TNF- α , and IL-12 production (Bassaganya-Riera et al., 2003; Dong & Flavell, 2001; Kidd, 2003; Romagnani, 1999), which exactly explained the significantly elevated expressions of INF- γ , TNF- α , and IL-12 genes with ST infections. LC-CLA in secreting auxiliary amounts of CLA, ameliorated the ST infection-induced gut inflammatory responses by suppressing T_h1 cells through reducing IL-12 and pathogenic T_h17 cells through reducing IL-1 β (Acosta-Rodriguez, Napolitani, Lanzavecchia, & Sallusto, 2007; Cosmi, Maggi, Santarlasci, Liotta, & Annunziato, 2014; Monteleone, Pallone, & Monteleone, 2009). Besides, IL-6 was found to be only moderately influenced by LC-CLA since the

cytokine/myokine was revealed to be both pro-inflammatory and anti-inflammatory properties in inflammation and infection responses (Hunter & Jones, 2015; Scheller, Chalaris, Schmidt-Arras, & Rose-John, 2011; Yao et al., 2014). In the foremost place, the anti-inflammatory activities of linoleic acids have not been documented to impair any gut immunity against enteric bacterial pathogen infections (Mengfei Peng & Biswas, 2017; Turnock et al., 2001).

Findings from this study herald a new era wherein non-traditional therapeutics become the first line in defense against enteric bacterial pathogens in all age groups without causing morbidity to the gut microflora. LC-CLA with linoleate isomerase gene over-expression managed to colonize efficiently on mice gut intestine and secret larger amounts of linoleic acids. In this way by combating against ST and EHEC, the preventive probiotic strain competitively excluded their growth *in vitro*, altered their physicochemical properties as well as biofilm formation, reduced their colonization and infection on gut intestine *in vivo*, and attenuated the inflammatory process induced by enteric bacterial pathogens. The development and implement of such novel, cost-effective, and simple-to-use genetically engineered probiotic being independent from prebiotics or prebiotic-like functional food ingredients is promising in open the new avenue in prevention and treatment of GI infections and enteric diseases where antibiotics were failed to be prescribed without causing negative consequences.

Conclusions

1. LC-CLA overexpressed the *mcra* gene with linoleate isomerase activity and over-produced linoleic acids.

2. LC-CLA attached/colonized gut intestinal cells efficiently than LC-WT.
3. Enteric bacterial pathogens were competitively excluded much efficiently by LC-CLA.
4. The physicochemical properties, biofilm formation, host-pathogen interactions, and virulence gene expression of enteric bacterial pathogens were altered/disrupted by metabolites from LC-CLA especially linoleic acids.
5. The treatment/administration of LC-CLA prevented enteric pathogenic bacterial colonization and infection to a significant level as well as maintained the overall health condition in infected mice.
6. Metabolites particularly linoleic acids secreted from LC-CLA induced anti-inflammatory effects in their hosts.

Figure and Table list

Figure 4-1. Overexpression of *mcra* from wild type *L. casei* ATCC334 and chromosomal recombination constructing LC-CLA.

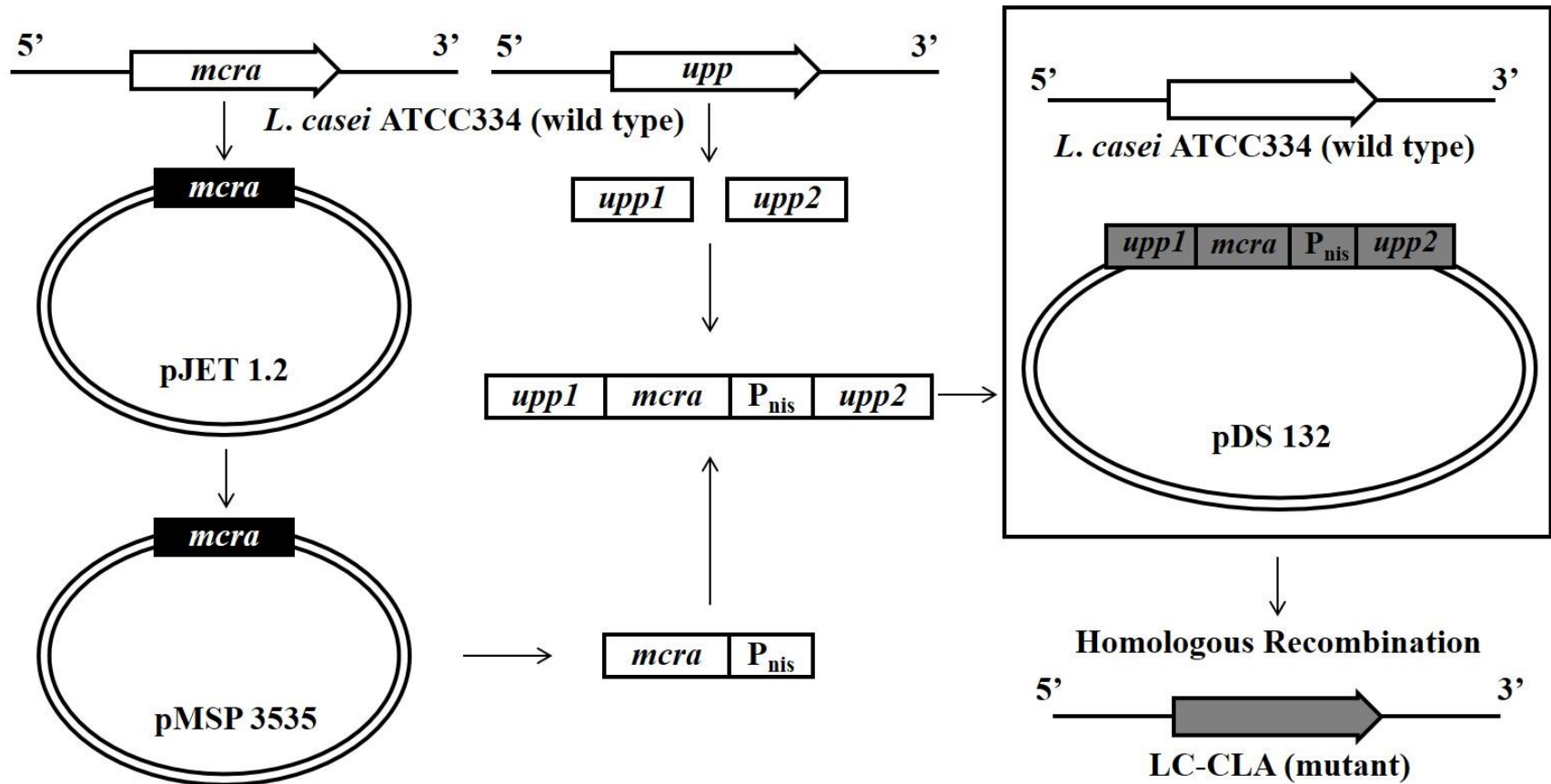


Figure 4-2. Phenotypic characterization of LC-CLA. The growth condition over 96 h (A), *ex vivo* adherence on human intestinal cells at 4 and 24 h (B), and *in vivo* colonization on mice cecum (C), jejunum (D), and ileum (E) were examined and compared between wild type *L. casei* and LC-CLA. Error bars indicate standard deviation from parallel trials. Letters ('a' and 'b') indicate significantly different on host cell adherence at $p < 0.05$.

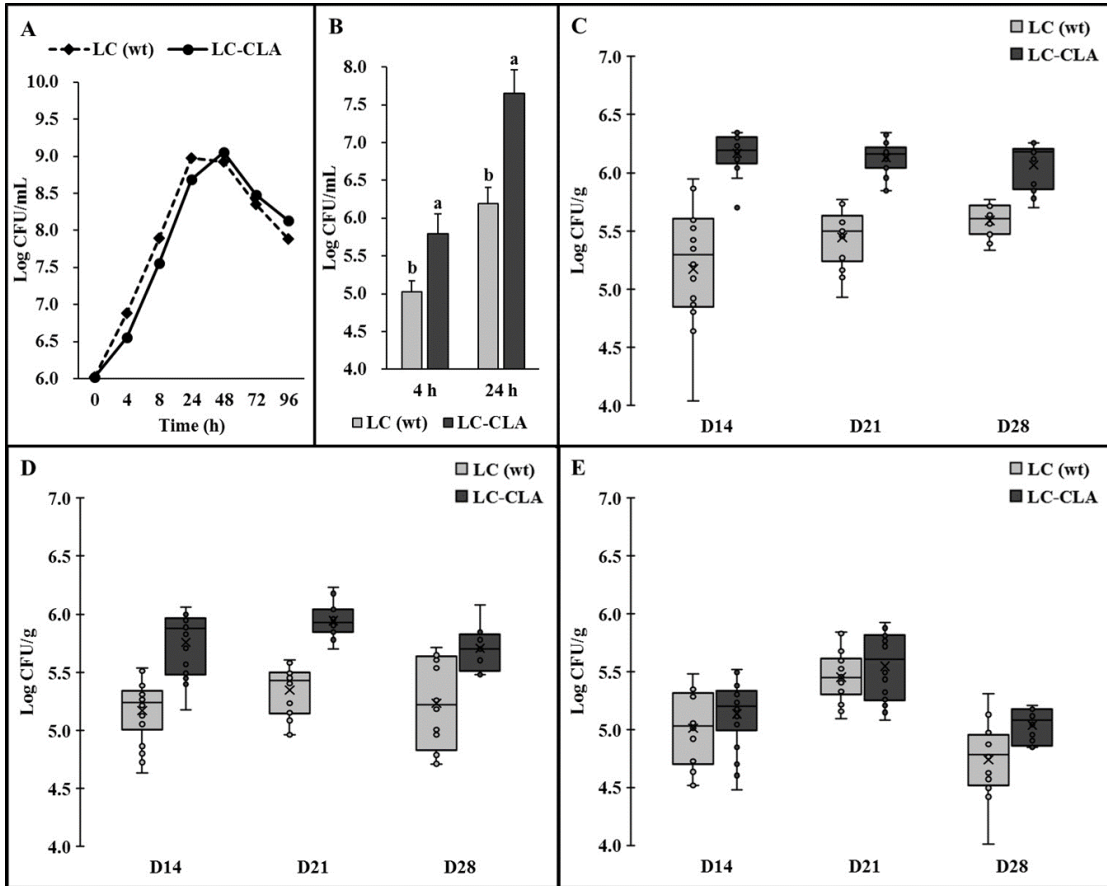


Figure 4-3. Competitive exclusion of enteric bacterial pathogens by LC-CLA. Comparative growth of *S. Typhimurium* (A) and EHEC (B) in single-culture or mix-culture with wild type *L. casei* or LC-CLA over 72 h was evaluated. Error bars indicate standard deviation from parallel trials.

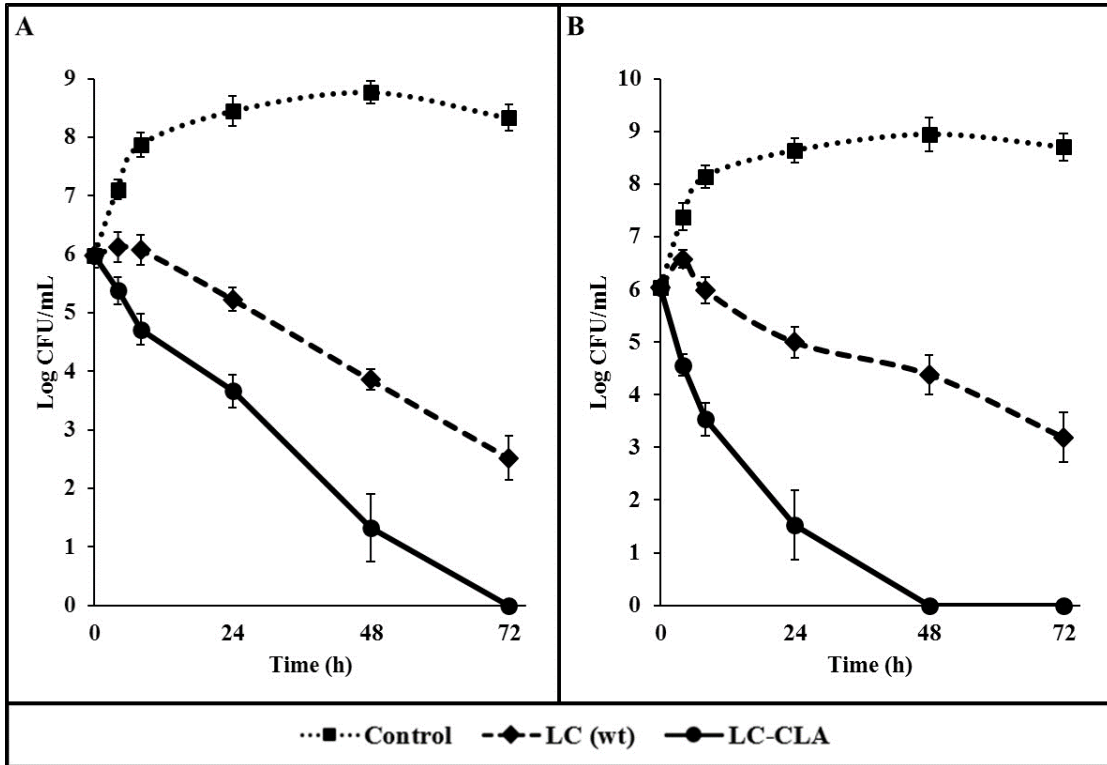


Figure 4-4. Antimicrobial activities of LC-CLA metabolites on enteric bacterial pathogens. Inhibitory effects of CFCs from wild type *L. casei* or LC-CLA were detected on growth of *S. Typhimurium* (A) and EHEC (B) over 72 h. Error bars indicate standard deviation from parallel trials. Different letters ('a' through 'c') at single time point within each strain are significantly different in growth at $p < 0.05$.

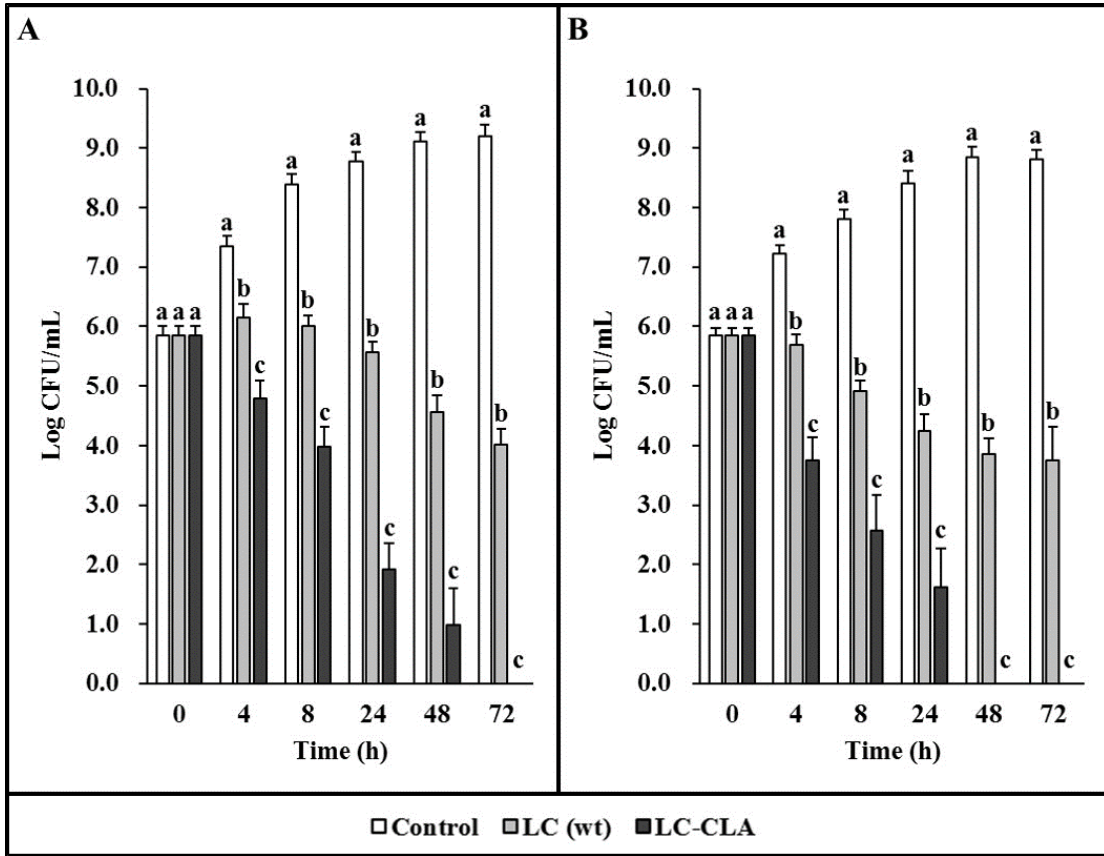


Figure 4-5. Reduction on enteric pathogenic bacterial biofilm formation by LC-CLA. Comparative biofilm formation of *S. Typhimurium* (A) and EHEC (B) under pressure of CFCS from either wild type *L. casei* or LC-CLA over 72 h was investigated. Error bars indicate standard deviation from parallel trials.

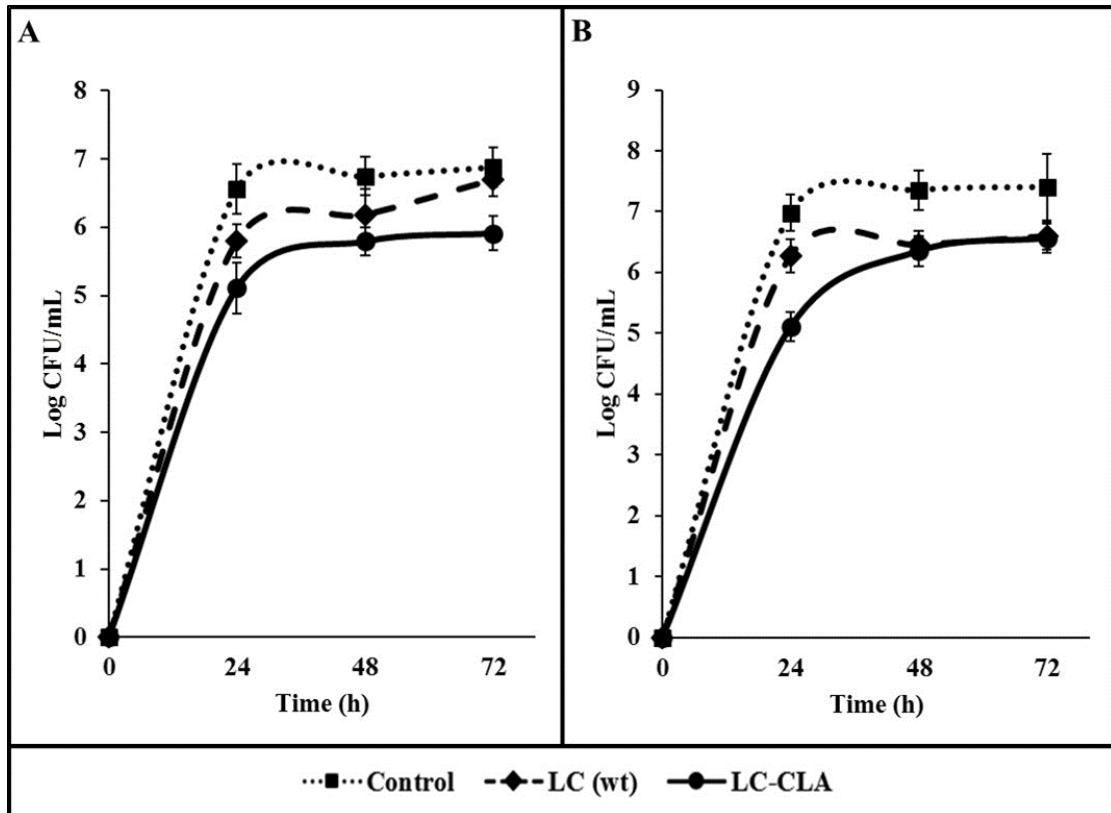


Figure 4-6. LC-CLA in interfering with enteric bacterial pathogen-cell interactions. Human intestinal cell adhesive and invasive activities of *S. Typhimurium* (A) and EHEC (B) with pre-treatment of either *L. casei* bacterial cells or CFCSs from *L. casei* were examined. A constant MOI=100 was applied in each sub-figure. Error bars indicate standard deviation from parallel trials. Different letters ‘A’ to ‘C’ or ‘a’ to ‘c’ within each bacterial pathogen are significantly different for cell adhesion or invasion at $p < 0.05$.

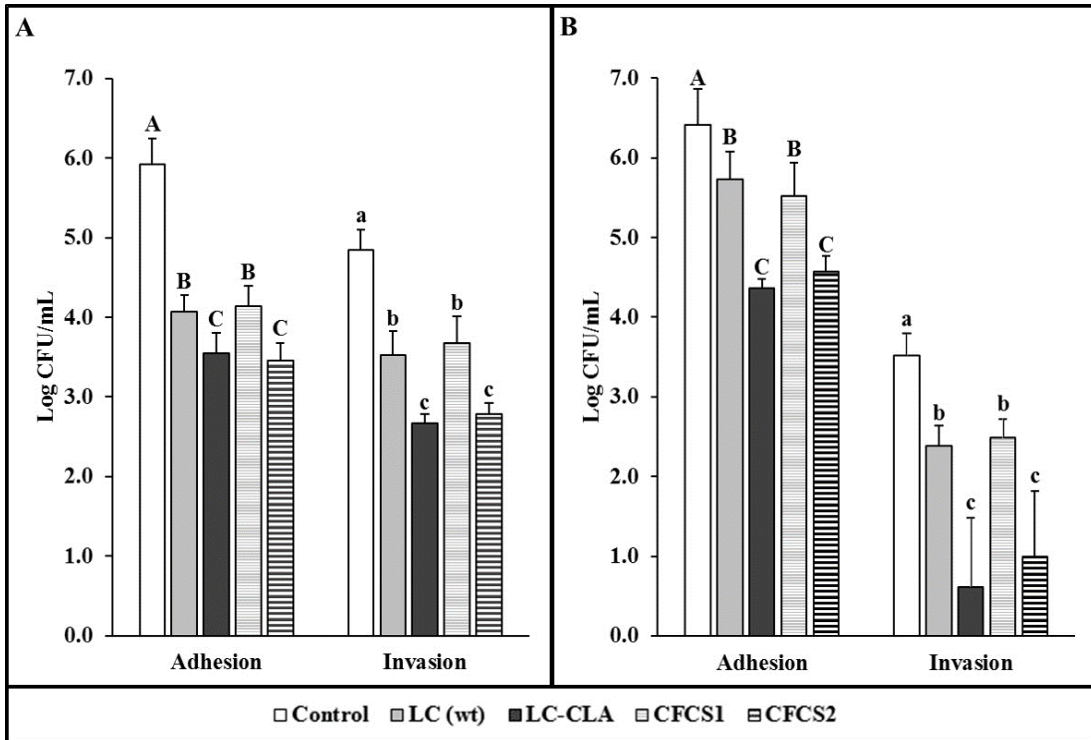


Figure 4-7. LC-CLA in suppression of enteric bacterial virulence genes. The relative expression of T3SS-related virulence genes from *S. Typhimurium* (A) and EHEC (B) under pressure of CFCSs from *L. casei* was investigated. The relative transcription levels are in the form of comparative fold change with control being 1.0. Error bars indicate standard deviation from parallel trials. Asterisks (*) indicate the significant difference in virulence gene expressions at $p < 0.05$.

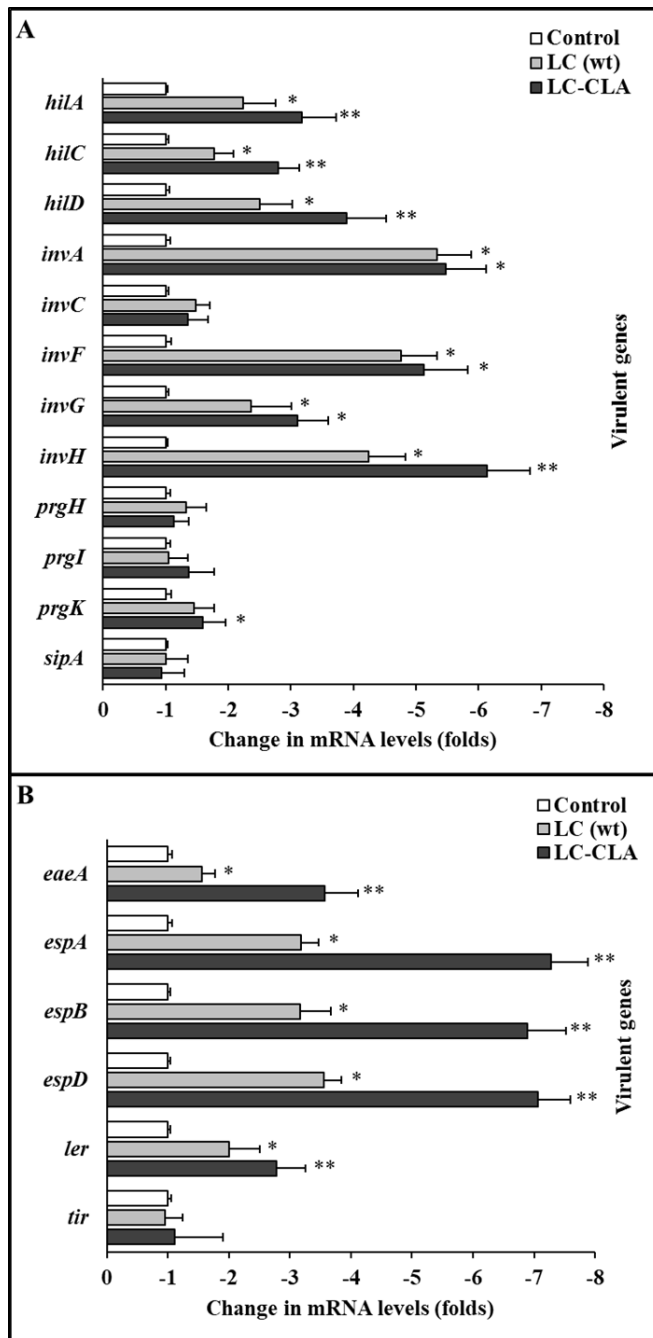


Figure 4-8. Anti-inflammatory effects of LC-CLA on human macrophage cells. The relative expression of macrophage (anti-)inflammatory cytokine genes with treatment of CFCSs from *L. casei* was investigated. The relative transcription levels are in the form of comparative fold change with control being 1.0. Error bars indicate standard deviation from parallel trials. Asterisks (*) indicate the significant difference in cytokine gene expressions at $p < 0.05$.

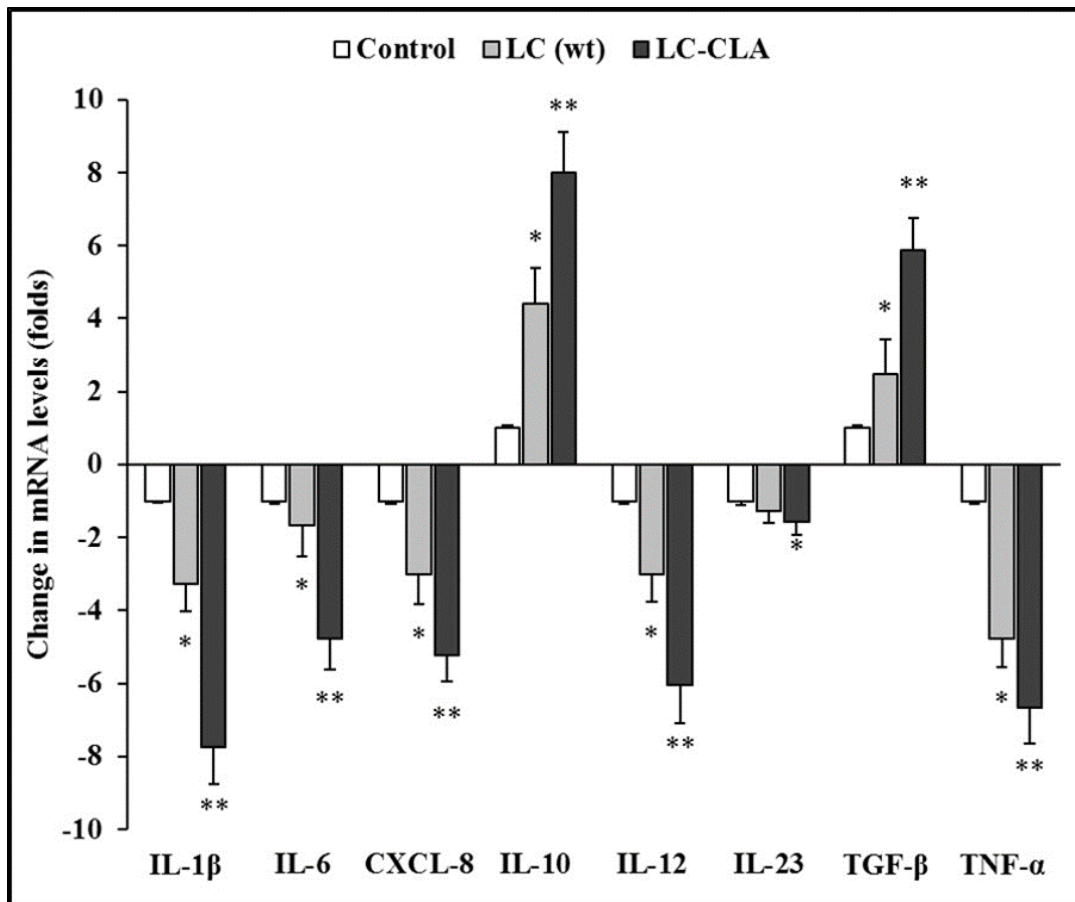


Figure 4-9. Preventive effects of LC-CLA against enteric pathogenic bacterial infections on weight loss. The weight of each mice in control groups (A and D), wild type *L. casei* administrated groups (B and E), and LC-CLA administrated groups (C and F) was measured and compared. The wild type *L. casei* and LC-CLA were administrated from D1 to D7. *S. Typhimurium* (A, B, and C) and EHEC (D, E, and F) were challenged at D7. Each dot indicates individual weight of one mice, and the horizontal bars indicate average value in each group.

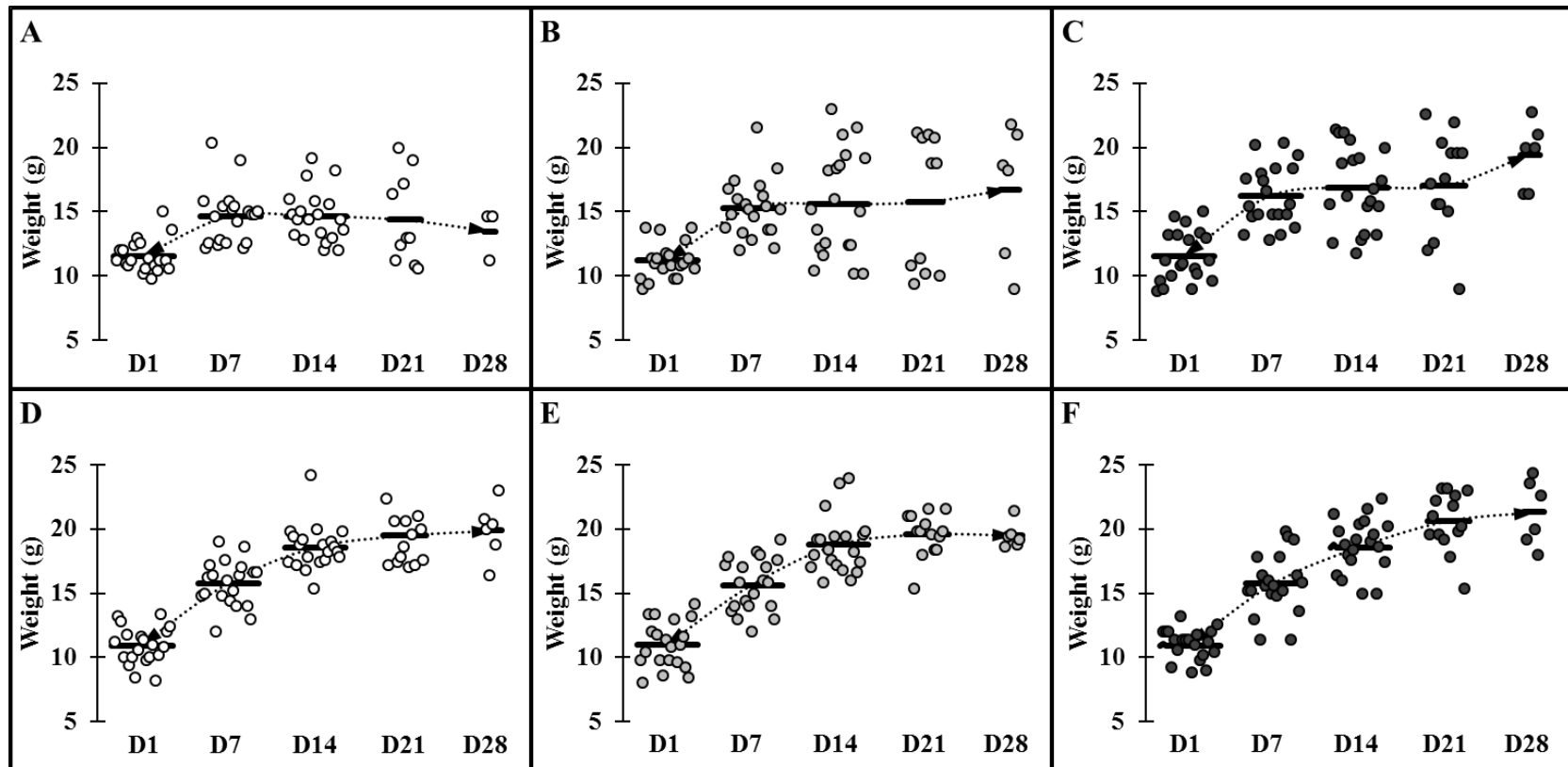


Figure 4-10. LC-CLA in reduction of *S. Typhimurium* colonization on mice gut. The *in vivo* colonization of *S. Typhimurium* on mice cecum (A), jejunum (B), and ileum (C) and the fecal shedding of *S. Typhimurium* (D) with or without *L. casei* preventive administration were examined. Error bars indicate standard deviation from parallel trials. Letters ('a' through 'c') indicate significantly different on *S. Typhimurium* gut colonization or fecal shedding at $p < 0.05$.

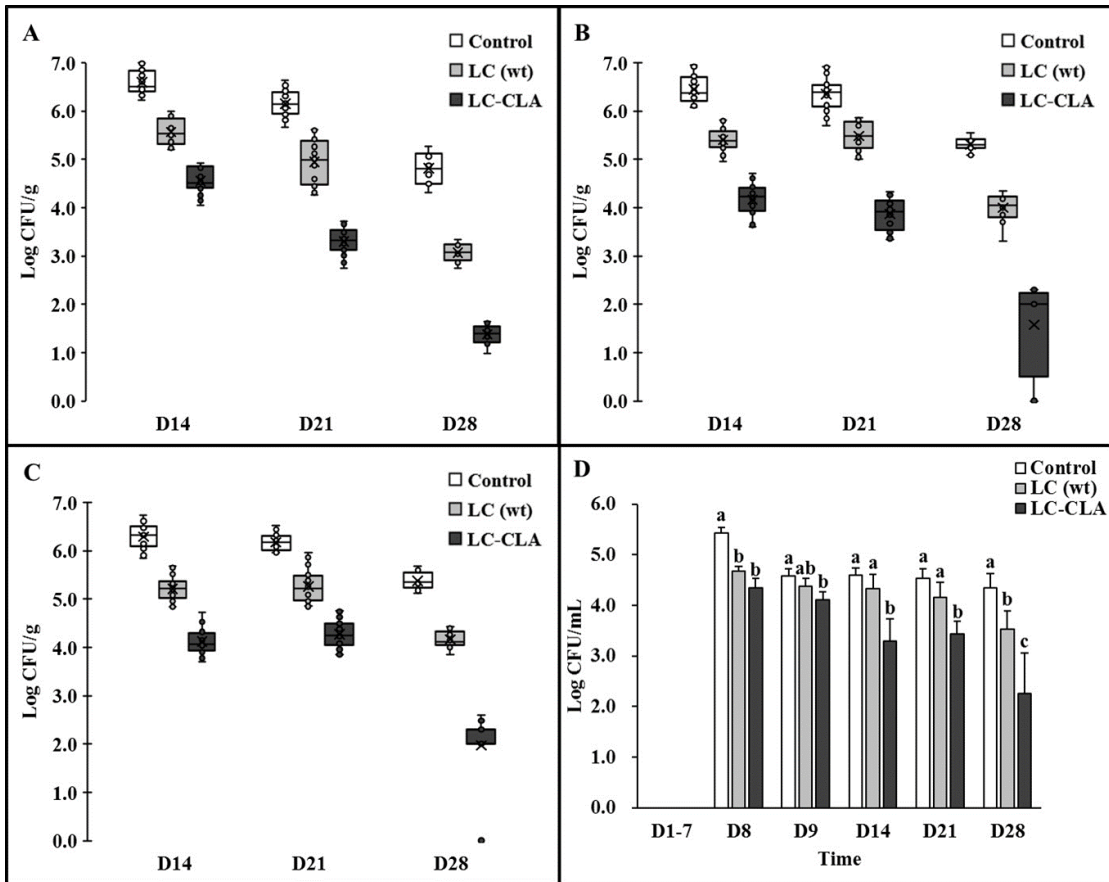


Figure 4-11. LC-CLA in reduction of EHEC colonization on mice gut. The *in vivo* colonization of EHEC on mice cecum (A), jejunum (B), and ileum (C) and the fecal shedding of EHEC (D) with or without *L. casei* preventive administration were examined. Error bars indicate standard deviation from parallel trials. Letters (‘a’ through ‘c’) indicate significantly different on EHEC gut colonization or fecal shedding at $p < 0.05$.

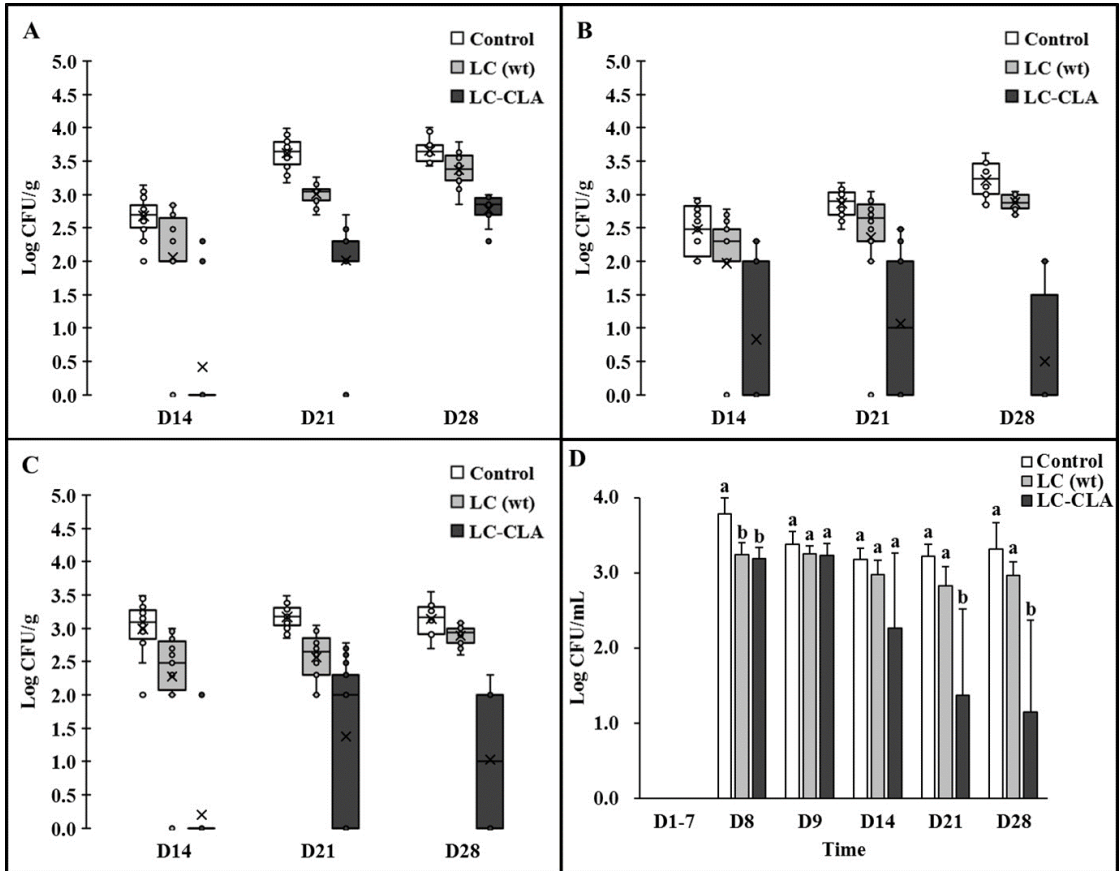


Table 4-1. Primers to be used for RT-qPCR analysis of EHEC and *S. Typhimurium*

Bacteria	Gene	Primer Sequence (5'-3')	Function
EHEC	<i>gapA</i>	F: ACTTCGACAAATATGCTGGC R: CGGGATGATGTTCTGGGAA	Housekeeping gene
	<i>eaeA</i>	F: CCCGAATTCGGCACAAGCATAAGC R: CCCGAATCCGTCTCGCCAGTATTCG	Attaching and effacing
	<i>espA</i>	F: GTTTTTTCAGGCTGCGATTCT R: AGTTTGGCTTTCGCATTCTT	Type III secretion protein
	<i>espB</i>	F: GCCGTTTTTGGAGAGCCAGAA R: AAAGAACCTAAGATCCCCA	Type III secretion protein
	<i>espD</i>	F: AAAAAGCAGCTCGAAGAACA R: CCAATGGCAACAACAGCCCCA	Type III secretion protein
	<i>ler</i>	F: ACTTCCAGCCTTCGTTTCAGA R: TTCTGGAACGCTTCTTTTCGT	Locus of Enterocyte Effacement regulator
	<i>tir</i>	F: GCTTGCAGTCCATTGATCCT R: GGGCTTCCGTGATATCTGA	Translocated intimin receptor
<i>S. Typhimurium</i>	50S ribosomal protein L5	F: GTAGTACGATGGCGAAACTGC R: CTCTCGACCCGAGGGACTT	Housekeeping gene
	<i>hilA</i>	F: TATCGCAGTATGCGCCCTTT R: CAAGAGAGAAGCGGGTTGGT	Transcriptional regulator
	<i>hilC</i>	F: AATGGTCACAGGCTGAGGTG R: ACATCGTCGCGACTTGTGAA	Transcriptional regulator
	<i>hilD</i>	F: CTCTGTGGGTACCGCCATTT R: TGCTTTCGGAGCGGTAAACT	Transcriptional regulator
	<i>invA</i>	F: CGCGCTTGATGAGCTTTACC R: CTCGTAATTCGCCGCCATTG	Invasion protein
	<i>invC</i>	F: GCTGACGCTTATCGCAACTG R: GGCGGTGCGACATCAATAAC	Type III secretion system ATPase
	<i>invF</i>	F: TCGCCAAACGTCACGTAGAA R: CATCCCGTGTATAACCCCCG	Transcriptional regulator
	<i>invG</i>	F: CGAATGACGCCAGCTGTTC R: TGCGTCAGGCGTCGTA AAA	Invasion protein
	<i>invH</i>	F: GGTGCCCTCCCTTCCT R: TGCGTTGGCCAGTTGCT	Invasion lipoprotein
	<i>orgA</i>	F: AGGCAGGGAGCCTTGCTT R: CCCTGATGCATTGCCAAAA	Oxygen-regulated invasion protein
	<i>orgB</i>	F: ACCATCCCAGAAACGCTTTTA R: TTGCCCTCAGGCTTATCG	Oxygen-regulated invasion protein
	<i>prgH</i>	F: TGAACGGCTGTGAGTTTCCA R: GCGCATCACTCTGACCTACCA	Type III secretion protein
	<i>prgI</i>	F: GGTCTATGAAAACGGACATTGTC R: CGCCGAACCAGAAAAAGC	Type III secretion protein
	<i>prgK</i>	F: GGGTGGAAATAGCGCAGATG R: TCAGCTCGCGGAGACGATA	Type III secretion lipoprotein
	<i>sipA</i>	F: CGTCTTCGCTCAGGAGAAT R: TGCCGGGCTCTTTCGTT	Cell invasion protein

Table 4-2. Primers for RT-qPCR analysis of U937 cells cytokine genes

Gene	Primer	Sequence (5'-3')	Function
18srRNA	Forward	ATCCCTGAAAAGTTCCAGCA	House keeping gene
	Reverse	CCCTCTTGGTGAGGTCAATG	
IL-1 β	Forward	GCCATGGACAAGCTGAGGAAG	Inflammatory cytokine gene
	Reverse	GTGCTGATGTACCAGTTGGG	
IL-6	Forward	GAACTCCTTCTCCACAAGCG	Pro-/Anti-inflammatory cytokine gene
	Reverse	TTTTCTGCCAGTGCCTCTTT	
IL-10	Forward	AGCAGAGTGAAGACTTTCTTTC	Anti-inflammatory cytokine gene
	Reverse	CATCTCAGACAAGGCTTGG	
IL-12	Forward	AATGTTCCCATGCCTTCACC	Pro-inflammatory cytokine gene
	Reverse	CAATCTCTTCAGAAGTGCAAGGG	
IL-23	Forward	GACACATGGATCTAAGAGAAGAG	Inflammatory cytokine gene
	Reverse	AACTGACTGTTGTCCCTGAG	
TGF- β	Forward	CTTGCTGTCCTCCTCTGCAC	Anti-inflammatory cytokine gene
	Reverse	TCACTGGGGTCAGCACAGAC	
TNF α	Forward	CAGAGGGAAGAGTTCCCCAG	Inflammatory cytokine gene
	Reverse	CCTTGGTCTGGTAGGAGACG	
CXCL-8	Forward	CTGCGCCAACACAGAAATTA	Inflammatory chemokine gene
	Reverse	ATTGCATCTGGCAACCCTAC	

Table 4-3. Group and number of mice (per group) and their bacterial treatment/challenge

Group (#)	Mice (n)	Probiotic Treatment (daily during 1st week)			Pathogen challenge (beginning of 2nd week)		
		PBS	LC (wt)	LC-CLA	PBS	ST	EHEC
A1	10	+	-	-	+	-	-
B1	10	-	+	-	+	-	-
C1	10	-	-	+	+	-	-
A2	10	+	-	-	-	+	-
B2	10	-	+	-	-	+	-
C2	10	-	-	+	-	+	-
A3	10	+	-	-	-	-	+
B3	10	-	+	-	-	-	+
C3	10	-	-	+	-	-	+

Table 4-4. Primers for RT-qPCR analysis of mice splenic cytokine genes

Gene	Primer	Sequence (5'-3')	Function
GADPH	Forward	TGTCCGTCGTGGATCTGAC	House keeping gene
	Reverse	CCTGCTTCACCACCTTCTTG	
IL-1 β	Forward	CAGGATGAGGACATGAGCAC	Inflammatory cytokine gene
	Reverse	CAGTTGTCTAATGGGAACGTCA	
IL-6	Forward	TGGAGTCACAGAAGGAGTGGCTAAG	Pro-/Anti-inflammatory cytokine gene
	Reverse	TCTGACCACAGTGAGGAATGTCAA	
IL-10	Forward	GGACAACATACTGCTAACCGAC	Anti-inflammatory cytokine gene
	Reverse	AAAATCACTCTTCACCTGCTCC	
IL-17	Forward	GAAGCTCAGTGCCGCCA	Pro-inflammatory cytokine gene
	Reverse	TTCATGTGGTGGTCCAGCTTT	
IL-22	Forward	ATACATCGTCAACCGCACCTTT	Pro-inflammatory cytokine gene
	Reverse	AGCCGGACATCTGTGTTGTTAT	
IFN γ	Forward	GAAGAAAGGTCAGCCAAGCGC	Pro-inflammatory cytokine gene
	Reverse	GCTTGATCACATCCATGCTCC	
TGF- β	Forward	GGAGAGCCCTGGATACCAAC	Anti-inflammatory cytokine gene
	Reverse	CAACCCAGGTCCTTCCTAAA	
TNF α	Forward	CCACCACGCTCTTCTGTCTAC	Inflammatory cytokine gene
	Reverse	AGGGTCTGGGCCATAGAACT	
CXCL-8	Forward	TTTCTGCAGCTCTCTGTGAGG	Inflammatory chemokine gene
	Reverse	CTGCTGTTGTTGTTGCTTCTC	

Table 4-5. Expression level of *mcra* and production of linoleic acids

Strain	Genotype	<i>mcra</i> mRNA expression	RTLA* per mL supernatant	RTLA* per bacterial cell
<i>L. casei</i> ATCC334	Wild type	1.00	1.00	1.00
LC-CLA	<i>mcra</i> over-expressed	7.15	4.48	21.06

* Relative Total Linoleic Acids based on HPLC-MS/MS analysis

Table 4-6. Physicochemical properties of ST and EHEC with CFCS treatments

Treatment	Hydrophobicity (%)		Auto-aggregation (%)		Injured bacterial cells (%)	
	ST	EHEC	ST	EHEC	ST	EHEC
Control	18.01±0.32 ^{a*}	14.56±0.83 ^a	14.72±0.41 ^a	6.79±0.91 ^a	19.80±1.79 ^c	16.98±4.18 ^c
LC (wt)	10.85±0.35 ^b	11.39±0.77 ^b	8.65±0.32 ^b	5.24±0.29 ^a	30.92±5.55 ^b	38.28±2.74 ^b
LC-CLA	6.32±0.43 ^c	4.35±0.65 ^c	5.11±0.41 ^c	3.02±0.55 ^b	42.84±2.64 ^a	50.64±4.15 ^a

* Means with different letters (a-c) in individual column are significantly different at p<0.05

Table 4-7. Relative expression of mice splenic inflammatory cytokine genes

Cytokine	D14 (log folds)				D21 (log folds)				D28 (log folds)			
	Control	Infection	LC (wt)	LC-CLA	Control	Infection	LC (wt)	LC-CLA	Control	Infection	LC (wt)	LC-CLA
IL-1β	0.00 \pm 0.02 ^{d*}	7.68 \pm 1.46 ^a	3.37 \pm 0.84 ^b	1.04 \pm 0.07 ^c	0.00 \pm 0.01 ^d	7.92 \pm 0.99 ^a	5.78 \pm 0.68 ^b	2.94 \pm 0.24 ^c	0.00 \pm 0.01 ^d	6.10 \pm 1.12 ^a	5.35 \pm 0.84 ^b	2.70 \pm 0.31 ^c
IL-6	0.00 \pm 0.01 ^c	1.55 \pm 0.47 ^{ab}	1.27 \pm 0.20 ^b	1.78 \pm 0.06 ^a	0.00 \pm 0.01 ^b	2.38 \pm 0.19 ^a	2.58 \pm 0.30 ^a	2.62 \pm 0.03 ^a	0.00 \pm 0.01 ^c	1.82 \pm 0.40 ^a	1.47 \pm 0.49 ^{ab}	1.24 \pm 0.08 ^b
CXCL-8	0.00 \pm 0.01 ^c	2.77 \pm 0.38 ^a	2.47 \pm 0.20 ^a	2.26 \pm 0.11 ^b	0.00 \pm 0.01 ^d	2.62 \pm 0.27 ^a	2.01 \pm 0.18 ^b	1.09 \pm 0.05 ^c	0.00 \pm 0.01 ^c	1.91 \pm 0.06 ^a	1.92 \pm 0.35 ^a	0.89 \pm 0.06 ^b
IL-10	0.00 \pm 0.01 ^c	-3.23 \pm 0.57 ^d	1.54 \pm 0.29 ^b	5.06 \pm 0.38 ^a	0.00 \pm 0.02 ^c	-2.50 \pm 0.62 ^d	1.20 \pm 0.16 ^b	4.86 \pm 0.51 ^a	0.00 \pm 0.01 ^c	-1.98 \pm 0.06 ^d	1.71 \pm 0.30 ^b	3.93 \pm 0.52 ^a
IL-17	0.00 \pm 0.04 ^b	1.29 \pm 0.09 ^a	1.10 \pm 0.05 ^a	1.18 \pm 0.08 ^a	0.00 \pm 0.01 ^c	3.49 \pm 0.50 ^a	2.58 \pm 0.42 ^b	2.41 \pm 0.09 ^b	0.00 \pm 0.02 ^c	4.91 \pm 0.95 ^a	4.71 \pm 0.67 ^a	3.33 \pm 0.52 ^b
IL-22	0.00 \pm 0.01 ^b	2.43 \pm 0.07 ^a	2.36 \pm 0.09 ^a	2.19 \pm 0.11 ^a	0.00 \pm 0.01 ^c	2.31 \pm 0.25 ^a	1.99 \pm 0.08 ^b	2.19 \pm 0.12 ^a	0.00 \pm 0.01 ^c	2.40 \pm 0.09 ^a	2.04 \pm 0.27 ^b	2.09 \pm 0.07 ^b
INF-γ	0.00 \pm 0.01 ^d	9.59 \pm 1.62 ^a	3.08 \pm 0.81 ^b	0.44 \pm 0.07 ^c	0.00 \pm 0.01 ^d	9.36 \pm 0.85 ^a	5.01 \pm 0.96 ^b	2.80 \pm 0.10 ^c	0.00 \pm 0.02 ^d	7.43 \pm 1.32 ^a	6.05 \pm 0.92 ^b	2.53 \pm 0.28 ^c
TGF-β	0.00 \pm 0.01 ^c	-1.67 \pm 0.14 ^d	1.71 \pm 0.10 ^b	3.12 \pm 0.21 ^a	0.00 \pm 0.01 ^c	-1.03 \pm 0.07 ^d	1.75 \pm 0.35 ^b	3.53 \pm 0.48 ^a	0.00 \pm 0.01 ^c	-1.59 \pm 0.37 ^d	0.49 \pm 0.09 ^b	2.53 \pm 0.07 ^a
TNF-α	0.00 \pm 0.01 ^c	3.99 \pm 0.30 ^a	3.83 \pm 0.32 ^a	1.89 \pm 0.06 ^b	0.00 \pm 0.03 ^c	3.50 \pm 0.18 ^a	3.70 \pm 0.25 ^a	1.98 \pm 0.27 ^b	0.00 \pm 0.01 ^d	5.73 \pm 1.33 ^a	4.43 \pm 0.81 ^b	2.27 \pm 0.82 ^c

* Means with different letters (a-c) in individual column are significantly different at p<0.05

Overall conclusions

1. Cocoa and peanut contains steroids, indoles, indazoles, and glycerophospholipid ingredients in support of probiotics, and they contain antimicrobial components like flavonoids, benzoic acids, and citrusin as well.
2. Cocoa and peanut flour stimulated the growth of multiple *Lactobacillus* strains. However, they induced only short time period-reduction on enteric bacterial pathogens.
3. Combining of cocoa/peanut and *L. casei* competitively excluded EHEC and *S. Typhimurium* in mixed-culture condition.
4. Metabolites from *L. casei* supplemented with cocoa/peanut altered the enteric pathogenic bacterial cell surface hydrophobicity, disrupted their host-pathogen interactions, and down-regulated their virulence gene expressions.
5. Antimicrobial properties of *L. casei* was attributed from multiple metabolites including hydrogen peroxide, organic acids, polypeptides, etc.
6. Cocoa/Peanut stimulated the production of linoleic acids by *Lactobacillus*.
7. LC-CLA overexpressed the *mcra* gene with linoleate isomerase activity, over-produced linoleic acids, and attached/colonized gut intestinal cells efficiently than LC-WT.
8. Metabolites from LC-CLA, especially linoleic acids, efficiently eliminated enteric bacterial pathogens, altered their physicochemical properties, reduced their biofilm formation, disrupted the host-pathogen interactions, and manipulated the virulence gene expressions.

9. The preventive administration of LC-CLA reduced enteric pathogenic bacterial colonization and infection by a significant level as well as maintained the overall health condition on infected mice.

10. Metabolites particularly linoleic acids secreted from LC-CLA induced anti-inflammatory effects on hosts.

Future directions

1. Histopathological analysis on mice kidney and cecum with enteric pathogenic bacterial infections and LC-CLA prevention.
2. Fatty acids composition analysis on mice liver and blood.
3. Gut microbiome profile in mice model with LC-CLA administration.
4. Preventive/Therapeutic studies of LC-CLA based on clinical trials.
5. Extensive cost-benefit survey on practical applicability of LC-CLA in food market or farm animal production.

Glossary

Probiotics	Live non-pathogenic microorganisms that are administered in order to improve gut intestinal microbial balance as well as protect the host from infective agents.
Prebiotics	Selectively functional ingredients that allow specific changes both in the composition and/or activity in the gut intestinal microflora that confer benefits upon host well-being and health.
Synbiotics	Mixtures of probiotics and prebiotics that beneficially affect the host by improving the survival and implantation of live microbial dietary supplements in the gut intestinal 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.
Functional food	Foods with existing or supplemental ingredients which provides additional functions on disease prevention and health promotion, e.g., prebiotic-like food components or specially formulated food nutrients.
Microbiota	The collective microbial including bacteria, virus, and archaea community which inhabits specific environments, e.g., mice cecal microbiota or human skin microbiota.
Microbiome	The collective microbial genomic contents which provide information on the total genetic capacity of their microbial community.

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