

## ABSTRACT

Title of Dissertation: ANTAGONISTIC MECHANISM OF METABOLITES FROM *LACTOBACILLUS CASEI* AGAINST FOODBORNE ENTEROHEMORRHAGIC *ESCHERICHIA COLI*

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Enterohemorrhagic *Escherichia coli* O157: H7 (EHEC), a foodborne enteropathogen, remains a significant public health concern since its discovery in 1982. With an incredibly low infectious dose (10-100 bacteria), this pathogen can cause self-limiting diarrhea, vomiting, and abdominal cramps. However, more complicated disease conditions such as bloody diarrhea or hemolytic colitis have been known to develop depending on the serotype involved in the infection, and on immune status and/or age of the patients. Due to its Shiga toxin (Stx) production ability, EHEC infection may lead to a kidney-related problem known as hemolytic uremic syndrome (HUS), which requires advanced medical care. Unlike other bacterial illnesses, therapeutic administration of antibiotics to treat EHEC infections is not recommended due to their controversial association with Stx production. As a result, only preventative/prophylactic and immune-supportive strategies are followed for EHEC infections. Using the antibacterial properties of probiotic bacteria and the metabolites they

produce are promising alternative strategies for preventing EHEC infections. We have targeted the probiotic bacteria *Lactobacillus casei* to determine the mechanism of this alternative strategy. In our study, we have executed microbiological, molecular, chromatographic, and metagenomic approaches to determine the antagonistic mechanisms of action of their metabolites, specifically conjugated linoleic acid (CLA) produced by *Lactobacillus casei*, against the growth and metabolism of EHEC. The metabolites of wild-type *L. casei* (LC<sub>wt</sub>) were augmented by supplementing it with a prebiotic-like dietary component, namely peanut flour (PF) (LC<sub>wt+PF</sub>), while another LC<sub>wt</sub> was also genetically engineered (LC<sub>CLA</sub>) to over convert CLA from linoleic acid (LA). These modifications showed effective results in controlling EHEC both *in vitro* and in *ex vivo* conditions. Total metabolites present in cell-free culture supernatant (CFCS) of LC<sub>wt</sub>, LC<sub>wt+PF</sub>, and LC<sub>CLA</sub> were able to control the growth of EHEC without negatively hampering the relative abundance of Firmicutes and Bacteroidetes present in rumen fluid (RF). Among these CFCSs, CFCS<sup>CLA</sup> exerted the most desirable outcome by eliminating EHEC. *In vitro* studies demonstrated that, a lower concentration of purified CLA worked synergistically with other metabolites of LC<sub>wt</sub> and augmented their inhibitory activity against EHEC. The orchestrated effect of metabolites has been observed to downregulate the virulence genes, disrupt the cell membrane, interfere with cell division, and damage their genomic DNA. The probable effect of these metabolites, specifically CLA, on Stx production and neutralization was also investigated by assessing host cell cytotoxicity. Total metabolites of *Lactobacillus* spp. as well as CLA itself, showed improvement in cell survivability when exposed to Stx. Our findings established a ground to explore the effect of specific metabolites obtained from probiotic bacteria in control and prevention of EHEC. The findings also showed a promising association of purified CLA in neutralizing Stx which can be further explored to use it in therapeutic purposes.

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*CASEI* AGAINST ENTEROHEMORRHAGIC *ESCHERICHIA COLI*

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## Preface

This dissertation is original, independent work by the author Arpita Aditya under the supervision of Dr. Debabrata Biswas.

## Acknowledgements

First and foremost, I wish to express my gratitude to my mentor, Dr. Debabrata Biswas. His appropriate guidance, inspiration, unlimited patience, and admonishment was the catalyzing enzyme in my graduate studies. Collaborating under him in designing research guided me in developing confidence, professionalism, and pragmatism.

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## Scholarly Articles

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

1. Aditya, A., Li, Y., and Biswas, D. (2022). Antagonistic Effects of Conjugated Linoleic Acids of *Lactobacillus casei* Against Foodborne Enterohemorrhagic *Escherichia coli*. *Journal of Food Protection* <https://doi.org/10.4315/JFP-21-414>.
2. Aditya, A., Peng, M., Young, A., and Biswas, D. (2020). Antagonistic Mechanism of Metabolites Produced by *Lactobacillus casei* on Lysis of Enterohemorrhagic *Escherichia coli*. *Front. Microbiol.* 11. <https://doi.org/10.3389/fmicb.2020.574422>.

Under review:

1. Arpita Aditya, Shaik O. Rahaman, and Debabrata Biswas. Impact of *Lactobacillus*-originated metabolites on enterohemorrhagic *E. coli* in rumen fluid. (Under review with *FEMS Microbiology Ecology*).
2. Arpita Aditya, Zajeba Tabashsum, Zabdiel Alvarado-Martinez, Chuan Wei Tung, Grace Suh, Phuong Nguyen, and Debabrata Biswas. Diarrheogenic *E. coli* in dairy farm ecosystems and their antibiotic resistance pattern. (Under review with *Journal of Dairy Science*).

Manuscript preparation in progress:

1. Arpita Aditya, Zajeba Tabashsum, Zabdiel Alvarado Martinez, and Debabrata Biswas. Investigating effects of *Lactobacillus casei* metabolites on production and neutralization of Shiga toxin from enterohemorrhagic *Escherichia coli*.

## Table of Contents

	Page number
Preface	i
Acknowledgements	ii
Scholarly Articles	iii
Table of Contents	iv
Chapter 1: Literature review	1-16
1.1 Foodborne diseases and their worldwide severity	1
1.2 <i>Escherichia coli</i> : The good, the bad and the ugly	1
1.2.1 The beneficial role of <i>E. coli</i>	2
1.2.2 <i>E. coli</i> as a foodborne infectious agent	3
1.3 Enterohemorrhagic <i>E. coli</i> (EHEC) as a serious foodborne pathogen	3
1.3.1 Common sources of EHEC	4
1.3.2 Clinical manifestation of EHEC	4
1.3.3 Outbreaks of EHEC and its economic impact	5
1.4 Molecular mechanism of EHEC pathogenesis.	6
1.4.1 Entry and initial attachment of <i>E. coli</i> O157: H7	7
1.4.2 Regulation of virulence genes and bypassing of host defense.	7
1.4.3 Effect of EHEC invasion on host	8
1.5 Treatment of EHEC.	8
1.5.1 Risks of some common therapeutics to treat EHEC infection.	9
1.6 Alternative strategies to control and prevent EHEC infections	10
1.6.1 Phage therapy	10
1.6.2 Nutraceuticals	11
1.6.2.1 Use of nutraceuticals to prevent EHEC infections	11
1.6.2.2 Synbiotic approach	12
1.6.2.2.1 Prebiotics	12
1.6.2.2.2 Probiotics and its general health benefits	12
1.6.2.2.3 Metabolites produced by lactic acid bacteria (LAB)	13
1.7 Microorganisms originated linoleic acid and derivatives	14
1.7.1 Potential anticarcinogenic mechanism of CLA	15
Chapter 2: Diarrheagenic <i>E. coli</i> in dairy farm ecosystems and their antibiotic resistance pattern	17-37
Introduction	17
Materials and methods	18
Results	20
Discussion	22
Conclusions	25
List of tables	26
List of figures, captions, and legends	32
Overall Hypothesis and Specific Aims	38
Chapter 3: Impact of <i>L. casei</i> -originated metabolites on EHEC in ex vivo condition	39-57
Introduction	39
Materials and methods	40
Results	42
Discussion	46
Conclusions	48

List of figures, captions, and legends	49
Chapter 4: Identify effective concentration of specific bioactive isomers of linoleic acid in metabolites produced by <i>L. casei</i>	58-73
Introduction	58
Materials and methods	59
Results	62
Discussion	63
List of tables	67
List of figures, captions, and legends	68
Chapter 5: Mechanism of metabolites produced by <i>L. casei</i> on lysis of EHEC	74-91
Introduction	74
Materials and methods	75
Results	78
Discussion	80
List of tables	85
List of figures, figure legends, and captions	86
Chapter 6: Investigating effects of <i>Lactobacillus casei</i> metabolites on production and neutralization of Shiga toxin from enterohemorrhagic <i>Escherichia coli</i>	92-103
Introduction	92
Materials and methods	94
Results	96
Discussion	98
List of figures, figure legends, and captions	100
Summary and Future Directions	104
Bibliography	105-143

## Chapter 1: Literature Review

*1.1 Foodborne diseases and their worldwide severity.* Foodborne infections, intoxication, and diseases associated with it are one of the burning causes of morbidity and mortality in both economically advanced and relatively impoverished countries. The occurrence of foodborne disease cases is more frequent in low-income countries which may be primarily linked to the flimsy enforcement of regulatory standards in food preparation, food storage, and use of potable water to clean and process foods. Besides, the climatic pattern of many geographic locations favors the propagation of pathogens, pests, and naturally occurring toxins which also add to the overall load. Moreover, integrative agricultural and intensive animal husbandry practices may contribute to the increased prevalence of foodborne pathogens and parasites from farm-to-fork (WHO, 2015). On the other hand, affluent societies seem to be more concerned about food safety, but they are also exposed to the same risk. With the increasing globalization of food production, manufacturing, and marketing the risk of transmission of foodborne pathogens is equal for rich and poor countries (Käferstein et al., 1997). The ever-expanding globalization and interdependence of the economy lead to the mass movement of people with their values and habits across international borders. For instance, the contemporary outbreak of coronavirus (SARS-CoV-2) was first reported in Wuhan City, China in December 2019. As of 18 July 2022, it killed about 6.3 million people all over the world and countless people were diagnosed as positive in almost all of the countries of the world. Considering the severity CDC recommended stay home to control the virus from spreading (CDC, 2020; WHO, 2020; BBC News, 2020, Coronavirus Death Toll and Trends - Worldometer, 2022).

Contaminated food can slow down socioeconomic development by spreading disease, malnutrition, hampering health care facilities, loss of productivity, and weakening national trade and tourism. According to the World Health Organization (WHO), about 600 million cases of foodborne diseases are reported per year worldwide. Among them about 420,000 people accept untimely death; 30% of these deaths occur among children under 5 years. Innocuous sicknesses caused by contaminated food can cause a loss of 33 million years of healthy lives each year (WHO, 2015). The more frequent occurrence of foodborne illnesses triggered by bacterial pathogens may be linked to their wide range of growth temperatures (<10°C to 45°C) with an optimum of 37°C (human body temperature). Besides, their ability to form heat-stable toxins (e.g. *Staphylococcus aureus*, *Clostridium botulinum*) and endospores (e.g. *Bacillus* spp. and *Clostridium* spp.) contribute to their potent pathogenicity (Bintsis, 2017). *Bacillus cereus*, *Brucella* spp., *Campylobacter jejuni*, *Clostridium botulinum*, *C. perfringens*, *Cronobacter sakazakii*, *Mycobacterium bovis*, Shiga-toxin producing *Escherichia coli*, *Listeria monocytogenes*, invasive non-typhoidal *Salmonella enterica*, *Shigella* spp., *Staphylococcus aureus*, *Vibrio* spp., *Yersinia enterocolitica* are some of the examples of disease-causing foodborne bacteria (Bintsis, 2017; Ortega et al., 2010). Apart from these, many parasites mainly protozoans and helminths are transmitted through food. Some notorious protozoan parasites in the USA are *Toxoplasma gondii*, *Cryptosporidium* spp., *Cyclospora cayetanensis*, *Giardia intestinalis*; among the helminths (commonly named as roundworms) *Trichinella* spp. and *Anisakis* spp. are more common (“CDC - Parasites - Food,” n.d.). *Viruses such as human norovirus, hepatitis A and E virus,*

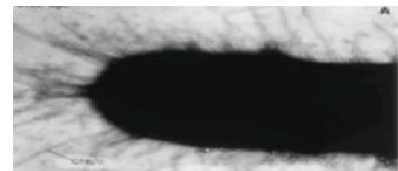
*aichi virus, rotavirus, parvovirus, etc. also add to the burden of food and waterborne diseases (D'Souza, 2015). Last but not the least, chemical toxins e.g. arsenic, aflatoxin, cassava cyanide, dioxins, peanut allergen are also a major source of foodborne diseases (Bintsis, 2017).*

Since the microorganism or its toxin gets in the human body through the gastrointestinal (GI) tract, the usual symptoms of food-related sickness include nausea, vomiting, stomach cramps, diarrhea, fever, headache, etc. Although the illness is recovered by itself, in extreme cases prolonged (more than 3 days) or bloody diarrhea, frequent vomiting, dehydration, inadequate urination, and high fever ( $> 102^{\circ}$  F) are observed (“Key Facts About Food Poisoning | Food Safety | CDC,” 2019). The symptoms and duration may become more severe even fatal depending on the type of pathogen involved and the individual’s immune status (CDC, 2019). Complex sequelae may be followed by simple symptoms of foodborne illness. E.g. Guillain-Barré syndrome, hemolytic uremic syndrome (HUS), arthritis, and paralysis can be responsible for *Campylobacter*, Shiga toxin-producing *E. coli*, *Salmonella* and *C. botulinum* respectively (Ford et al.; Lindsay, 1997).

Food mediated illnesses can be controlled by practicing some simple steps, e.g. washing hands before and after food preparation and eating, cleaning the utensils properly, storing foods at the proper temperature, and separating cooked and raw food to prevent cross-contamination (“Basic Food Safety | Food Safety | CDC,” 2019).

Food associated illnesses are more than simple sickness. Therefore, we should think deeply about the search for novel antimicrobials and more efficient ways to treat foodborne pathogens.

*1.2 Escherichia coli: The good, the bad and the ugly.* In recent times, *Escherichia coli* is one of the well-known bacteria all over the world. This bacterium was first discovered in the human colon by a German pediatrician and bacteriologist Theodore Escherich (1857-1911). Considering its colon origin T. Escherich named this bacterium as



**Fig 1.1:** *Escherichia coli* O157:H7

*Bacterium coli commune*, later it was reclassified and renamed as *Escherichia coli* in 1919 after the discoverer (Shulman et al., 2007). *E. coli* is a gram-negative, aero-anaerobic, rod-shaped, non-spore forming, motile bacterium with a dimension of 0.25-1  $\mu\text{m}$  width, 2  $\mu\text{m}$  in length and an average volume of 0.6–0.7  $\mu\text{m}^3$  (El-Hajj and Newman, 2015; Tenaillon et al., 2010). *E. coli* is a common intestinal bacterial flora of warm-blooded animals; so, 37°C and a neutral pH is the best condition for their growth. Apart from that *E. coli* has been detected in environments ranging from 4-49°C; can survive refrigeration and freezing temperature. They can withstand extremely acidic gastric pH 1.5- 3.0 and water activity ( $A_w$ ) 0.90 (Lim et al., 2007; Tenaillon et al., 2010; Waterman and Small, 1996).

*E. coli* and many other gram-negative organisms express three major antigens on their surface, they are known as O, K, and H antigens (Scheutz et al., 2004). The O-antigen or O-polysaccharide is composed of repeating oligosaccharide units (O-units) which form the part

of lipopolysaccharide (LPS) of the outer membrane. The O-antigen is one of the most variable structures because of the sugar composition, their arrangement and linkages are the basis of the serotyping of many gram negative-bacteria (Chen et al., 2015; Kauffmann, 1947). The protein subunit of flagella which is named as flagellin bears the H-antigen and capsular polysaccharide or K antigen are the other two criteria of serotyping (Wang et al., 2003). To date, 173 O-antigens, 80 K-antigens, and 56 H-antigens have been identified. The combination of these antigens makes different serotypes. Theoretically, a possible number of *E. coli* serotypes present in nature are 50,000-100,000 or even more (Orskov and Orskov, 1992). Among them, only 200 serotypes have been identified so far. The O: H serotyping is the widely followed method of *E. coli* typing and seems to be irreplaceable by any other existing technique (Scheutz et al., 2004). Phylogenetically these large number of *E. coli* can be narrowed down to four groups A, B1, B2, and D where A and B1 contains mostly the commensal strains whereas the other two includes the virulent *E. coli* strains (Clermont et al., 2000; Herzer et al., 1990).

*1.2.1 The beneficial role of E. coli.* The commensal *E. coli* strains are part of the normal flora of humans and other warm-blooded animals e.g. sheep, chicken, cattle, pigs, goats, cats, dogs, gulls, etc. It benefits the host by producing vitamin K<sub>2</sub> (menaquinone) as a metabolite (Bentley and Meganathan, 1982). It also helps to nutritionally outcompete the pathogens which ultimately helps the host to defeat the pathogenic invasion. *E. coli* strain Nissle 1917 (serotype O6:K5: H1) is one of the well-documented probiotics which inhibits the growth of other *E. coli* and *Salmonella* spp. by secreting microcins H47 and M (Grozdanov et al., 2004; Patzer et al., 2003). This trait is employed to efficiently improve many human intestinal disorders such as Crohn disease, necrotizing enterocolitis, pouchitis, and irritable bowel syndrome (Olier et al., 2012; Schultz, 2008; Yan and Polk, 2010). Since the discovery during World War I by Alfred Nissle from a soldier, it is being sold as probiotic for over a century (Barth et al., 2009; Blum-Oehler et al., 2003).

The presence of *E. coli* in potable water indicates the heightened presence of pathogenic fecal contamination many of which are *Salmonella* spp. or the hepatitis A virus. The United States Environmental Protection Agency (US EPA) has recommended the *E. coli* test for maintaining the drinking water quality (Odonkor and Ampofo, 2013). The triumphant beginning of biotechnology was started with the work in *E. coli*, using plasmids and restriction enzymes. This research paved the way for commercial production of insulin and a human growth hormone somatostatin using *E. coli* (Baeshen et al., 2014; Russo, 2003). Genetically engineered *E. coli* are also being used in bioremediation and biofuel production (Howard et al., 2013). Besides, *E. coli* is the most studied organism in life science and microbiological research. It is one of the earliest organisms whose whole genome had been completely sequenced (Blattner et al., 1997). This organism also helped to understand the bacterial gene transfer mechanism via conjugation and initial knowledge about phage genetics (Willetts, 1993).

1.2.2 *E. coli* as a foodborne infectious agent. *E. coli* got attention as a potential human pathogen during the outbreaks of Oregon and Michigan of the USA in 1982. The infected people were suffering from watery diarrhea and severe abdominal cramp followed by bloody diarrhea with little or no fever. The infection started from eating sandwiches containing undercooked meat at the same chain of fast-food restaurants. A rare *E. coli* serotype O157:H7 was diagnosed in the fecal specimen of infected individuals (Riley et al., 1983). Since then, *E. coli* O157:H7 is being reported as a common etiological agent of diarrhea in European and North American countries (Boyce et al., 1995). A low number of this pathogen such as 10-100 has the potential to cause disease in humans. Pathogenic *E. coli* strains are classified into 6 pathotypes or virotypes. Since they commonly cause diarrhea, they are also known as diarrheagenic *E. coli*.

Among the diarrheagenic *E. coli*, enteroinvasive *E. coli* (EIEC) causes symptoms very similar to *Shigella* spp. but less severe. (Vieira et al., 2007). Both *Shigella* spp. and EIEC causes dysentery, fever, and cramps using the same mode of action. Routine microbiological techniques cannot distinguish between them which can be a reason for a comparatively small number of reported cases of this virotype. But gene-based quantitative PCR assay is applied for this purpose (Ud-Din and Wahid, 2015). Travelers to developing countries very often suffer from traveler's diarrhea. The responsible etiological agent is enterotoxigenic *E. coli* (ETEC) which also causes fatal diarrhea in infants of low-income countries. On average about 73,041 deaths and about 233 million diarrheal cases result from this virotype (Pires et al., 2015). ETEC produces both heat-labile (LT1, LT2) and heat-stable (STa, STb) toxins; through which they cause disease. Antibiotics such as ciprofloxacin, rifaximin, or azithromycin are often recommended to the travelers since it is effective against ETEC (DuPont, 2009a). Another virotype that affect infants and toddlers of both rich and low-income countries with persistent diarrhea is enteropathogenic *E. coli* (EPEC) (Ochoa et al., 2008). Its complex pathogenesis is characterized by firm attachment and protein injection to the intestinal mucosa which leads to pedestal formation and loss of intestinal microvilli which causes malabsorption (DuPont, 2009b). Enteroaggregative *E. coli* (EAEC) expresses many possible virulence factors like enterotoxins and cytotoxins which favor them to cause childhood diarrhea, traveler's diarrhea, and diarrhea in AIDS patients through yet unelucidated pathogenesis mechanism (Pawlowski et al., 2009; Selendy, 2019). The most notorious of the diarrheagenic *E. coli* is enterohemorrhagic *E. coli* (EHEC) due to their ability to produce shiga-toxin. The toxin causes damage to colonic microvasculature which might result in bloody diarrhea and injury to renal endothelial cells leading to hemolytic uremic syndrome (HUS) (Page and Liles, 2013). This pathogen is a frequent cause of *E. coli* outbreaks with an estimated 2.8 million diarrheal cases annually (Majowicz et al., 2014; Selendy, 2019).

1.3 Enterohemorrhagic *E. coli* (EHEC) as a serious foodborne pathogen. EHEC is a member of shiga toxin-producing *E. coli* (STEC) (Table:1). Typically, shiga toxin (STx-1 and STx-2) consists of one A (active) subunit and five B (binding) subunits which exert an irreversible cytopathogenic effect on renal cells (Vero cells) (Paton and Paton, 1998). In addition to STx,

EHEC harbors a large virulence plasmid and type III secretion system (T3SS) to inject effector proteins into the target host cell (Levine, 1987). Among the different serotypes of STEC, O157: H7 is the most frequently reported foodborne pathogen in Europe, Japan, and North American countries. O26, O45, O91, O103, O104, O111, O128, and O145 serotypes are also reported occasionally (Cramer, 2014; Mellmann et al.).

**Table 1:** Classification of STEC (Vanaja et al., 2013)

	Groups	Serotypes
STEC	EHEC 1	O157:H7, O55:H7
	EHEC 2	O111:H8, O111:H-, O26:H11, and O111:H11
	STEC 1	O113:H21, OX3:H21, and O91:H21
	STEC 2	O103:H2, O103:H6, and O45:H2

*1.3.1 Common sources of EHEC.* The cattle harbor various types of *E. coli* in their intestine which colonize asymptotically in the GI tract and rectoanal junction (RAJ) mucosa (Lim et al., 2007; Naylor et al., 2003). However, there could some rare events of diarrheal disease in young calves with *E. coli* O157: H7 (Lim et al., 2010). Other small ruminants and birds e.g. sheep, goat, deer, pig, horse, rabbit, dog, cat, chicken, and turkey also harbor this pathogen. On an average 8.3% of dairy and beef cattle harbor *E. coli* O157: H7 and add them to the environment through fecal shedding (Solomon et al., 2002). This pathogen can survive as high as 70°C and as low as -20°C for 60-100 days in manure (Kudva et al., 1998; Wang et al., 1996). Therefore, even strictly followed manure composting guidelines might result in the application of *E. coli* O157: H7 containing manure as fertilizer to produce crops which may ultimately find their way in infecting humans through fresh produce (WHO, 2018). Cattle feces mixed with rain or flood water can contaminate the natural water bodies and thus could be another way of pathogen spread (Wang et al., 1996). Irrigating contaminated water also contributes to their transmission (Hilborn et al., 1999). *E. coli* O157: H7 cells can penetrate leaf stomata and cut edges and trapped 20-100 µm below the leaf surface which can protect them from chlorine and other surface-disinfecting agents (Seo and Frank, 1999).

Generally, *E. coli* O157: H7 transmits via contaminated water and food such as juice, uncooked or poorly cooked meat, vegetables, etc. However, it can also be transmitted via person-to-person, cross-contamination during food preparation, occupational hazards, close contact with pets, and other animals, etc. (Rangel et al., 2005). Some common foods that were related to *E. coli* O157: H7 outbreaks previously are undercooked burger patties, salami, unpasteurized apple cider, yogurt, and cheese made from raw milk (Lim et al., 2010).

*1.3.2 Clinical manifestation of EHEC.* After EHEC ingestion, an individual may experience abdominal cramps and watery diarrhea within 2-3 days and recover without any complex treatment. Almost half of the infected individuals (both children and adults) develop bloody diarrhea in another 2-4 days later which is usually accompanied by nausea and vomiting (Cramer, 2014). Within a week most patients recover without developing further complications while about 10-15% of patients develop serious complications like HUS (Bell and Gochenaur, 2006; Tarr et al., 2005). The clinical onset of HUS happens within 24-36

hours when some patients fail to recover from bloody diarrhea (Cramer, 2014). HUS patients are diagnosed with an elevated level of lactate dehydrogenase (LDH), creatinine, leukocytes, C-reactive proteins, and low level of platelets which are the indication of edema, hemolysis and, kidney failure. A higher level of LDH is often referred to as tissue damage while a high level of leukocytes and C-reactive protein suggest systemic inflammation (Cramer, 2014; Zoufaly et al., 2013). Elevated white blood cells, vomiting, bloody diarrhea, and older age are the risk factors of EHEC-infected patients for developing HUS (Zoufaly et al., 2013). EHEC infection is responsible for acute renal failure which ends in kidney transplantation in children predominantly in populations eating unpasteurized dairy products and beef (Rivero et al., 2010; Tarr et al., 2005). During the *E. coli* outbreak in Germany (2011), the patients experienced intestinal and neurological complications such as peritonitis, epileptic seizures, oculomotor disturbances, myoclonus, aphasia, oculomotor disturbances, cognitive impairment, etc. (Magnus et al., 2012).

*1.3.3 Outbreaks of EHEC and its economic impact.* EHEC is an originator of extreme economic loss both for the food-producing industries and mass people in terms of money associated with treatment, product recalls, and lawsuits. In 2000, the United States Department of Agriculture (USDA) estimated \$1 billion as the annual cost of EHEC related illnesses. In 2009, a \$478 million loss was estimated due to EHEC O157 in the US (Frenzen et al., 2005; Vanaja et al., 2013). The total cost of each outbreak also includes legal expenses and losses due to product recalls; e.g. the EHEC outbreak from apple juice ended in \$1.5 million federal fines, \$6.5 million loss from a product recall, and \$12 million in compensations (Pennington, 2010). A chronological description of major EHEC related outbreaks in the last 10 years is presented in Table: 2. On average in the US, foodborne diseases are responsible for 76 million sicknesses, 325 thousand hospitalizations, and 5 thousand deaths each year. Among these, *E. coli* O157: H7 alone causes about 73,000 infections, 2,000 hospitalizations, and 60 deaths (Mead et al., 1999). In several independent studies conducted in the US during 1985-1990 found *E. coli* O157: H7 to be the third or fourth most common bacterial stool pathogen (Griffin PM, 1995).

**Table: 2** Outbreaks caused by various strains of EHEC

Year	Associated reason	<i>E. coli</i> Serotype	Hospitalization/ Reported cases	HUS	Death	Economic Impact
2021	Packaged Salads	O157:H7	4	0	1	No product recall
	Baby spinach	O157:H7	4	0	0	"
	Cake mix	O121	7	0	0	"
	Unknown	O157:H7	11		1	"
2020	Unknown	O157: H7	15	1	1	N/A
	Leafy greens	O157: H8	20	4	0	No product recall
	Clove sprouts	O103	3	0	0	Product recall
2019	Fresh Express Sunflower Crisp Chopped Salad Kits	O157: H7	4/10	1	0	No product recall
	Romaine Lettuce	O157: H7	85/167	15	0	Product recall
	Ground Bison	O103, O121	18/33	0	0	"
	Flour	O26	3/21	0	0	"
	Ground Beef	O103	29/209	2	0	"
2018	Romaine Lettuce	O157: H7	25/62	2	0	"
	Ground Beef	O26	6/18	0	0	"
	Romaine Lettuce	O157: H7	96/210	27	0	No product recall
2017	Leafy Greens	O157: H7	9/25	2	1	"
	Soy Nut Butter	O157: H7	12/1	9	0	Product recall
2016	Beef Products	O157:H7	7/11	1	0	"
	Flour	O121, O26	17/63	1	0	"
	Alfalfa Sprouts	O157	2/11	0	0	"
2015	Chicken Salad	O157: H7	5/19	2	0	"
	Chipotle Mexican Grill Restaurants	O26	21/55	0	0	N/A
2014	Raw Clover Sprouts	O121	8/19	0	0	No product recall
	Ground Beef	O157: H7	7/12	0	0	Product recall
2013	Ready-to-Eat Salads	O157: H7	7/1	2	0	"
	Farm Rich Brand Frozen Food Products	O121	9/1	2	0	"
2012	Organic Spinach and Spring Mix Blend	O157: H7	13/33	2	0	"
	Unidentified	O145	4/18	0	1	N/A
	Raw Clover Sprouts	O26	7/29	0	0	N/A
2011	Romaine Lettuce	O157: H7	33/49	3	0	N/A
	Travel to Germany	O104: H4				
	Lebanon Bologna	O157: H7	3/13	0	0	N/A
	In-Shell Hazelnuts	O157: H7				
2010	Cheese	O157: H7	15/38	1	0	Product recall
	Romaine Lettuce	O145	12/30	3	0	N/A
	Beef	O157: H7	9/1	1	0	N/A

(Source: Reports of Selected *E. coli* Outbreak Investigations | *E. coli* | CDC, 2022)

*1.4 Molecular mechanism of EHEC pathogenesis.* The pathogenesis of EHEC has been studied intensely because of its diversified pathogenic strategy that also helps to explain other bacterial infections and global clinical significance. However, the majority of our understandings are based on *in vitro* studies on O157: H7 serotype of EHEC. This may be a

limitation to explain the actual pathogenesis of other EHEC serotypes and can be a useful field of future research (Vanaja et al., 2013).

*1.4.1 Entry and initial attachment of E. coli O157: H7.* Like all other foodborne pathogens, *E. coli* O157: H7 is transmitted to humans via fecal-oral route i.e. through the consumption of contaminated food and water. Because of its extremely low infectious dose (10-100 organisms), it can efficiently pass through stomach acid (pH 1.5–3.0) to the intestine (Griffin and Tauxe, 1991; Large et al., 2005; Tilden et al., 1996). The low pH induces the stationary-phase acid resistance (AR) system to protect the bacteria in low pH (Gorden and Small, 1993). Acid resistance of EHEC is regulated by three AR systems (AR1, AR2, and AR3), RNA polymerase associated protein SspA, the chaperone HdeB, DNA-binding protein Dps, and small non-coding DsrA RNA have been shown to have a role in acid resistance (Hansen et al., 2005; Kern et al., 2007; Vanaja et al., 2013).

After passing through the stomach; essentially, EHEC adheres to the epithelium layer of the large intestine to colonize in the host and compete with surrounding normal microbiota (Torres et al., 2005). EHEC encodes a variety of fimbrial and non-fimbrial proteins known as adhesins to facilitate initial interaction to the host cell mucosa. The most studied adhesin of EHEC is a ~94 kDa outer membrane protein encoded by *eae* gene called intimin (Beebakhee et al., 1992; Jerse et al., 1990; Yu and Kaper, 1992). By binding to translocated intimin receptor (Tir) intimin forms idiosyncratic attaching and effacing (AE) lesions on the intestinal epithelial cell surface. Upon binding to the host cell receptors  $\beta_1$  chain integrin and nucleolin, intimin eliminates intestinal microvilli helping the pathogen to come in intimate contact with the host cell. Tir after being secreted through the type III secretion system (T3SS), plays a major role in actin localization beneath the bound bacteria which is known as a pedestal formation. Intimin can function without Tir but with a significantly reduced efficiency (Kenny et al., 1997; McKee and O'Brien, 1995; Moon et al., 1983; Sinclair et al., 2006; Tzipori et al., 1995).

Other adhesins such as long polar fimbriae (LPF), hemorrhagic coli pilus (HCP), F9 fimbriae, curli fimbriae, Efa, Flagella, T3SS secreted protein EspA, T2SS proteins AdfO, YodA, and stcE, non-fimbrial proteins OmpA, H7 flagella, etc. also play a significant role in initial interaction (Bardiau et al., 2010; Cergole-Novella et al., 2007; LA RAGIONE et al., 2000; Torres et al., 2005). Interestingly, Stx2 also plays a role in EHEC adherence by increasing nucleolin surface localization (Liu et al., 2010; Robinson et al., 2006).

*1.4.2 Regulation of virulence genes and bypassing of host defense.* The major virulence components of EHEC; shiga toxin, large pO157 plasmid, and the central virulence element locus of enterocyte effacement (LEE) are regulated by multiple environmental signals and genes through highly complex pathways to successfully colonize and infect the host. LEE is composed of 41 open reading frames arranged in five polycistronic operons (*LEE1-LEE5*). Expression of LEE is controlled by regulators encoded in both *LEE* and elsewhere on the

EHEC genome, and extracellular factors as well (Croxen and Finlay, 2010; Mellies et al., 2007).

*LEE* encoded elements H-NS, Ler, GrlA, and GrlR plays role *LEE* genes regulations. H-NS is a suppressor of *LEE* expression which is disrupted by Ler leading to increased transcription of *LEE2-LEE5*. Upon binding to Ler promoter GrlA promotes *Ler* expression whereas GrlR inhibits *LEE* expression by interfering with GrlA activity (Iyoda et al., 2006; Jiménez-Colmenero et al., 2001; Mellies et al., 2007). Non-*LEE* encoded proteins such as Pch, IHF, RcsCDB, and GrvA helps in *LEE* expression by increasing Ler production. GadE, GadF, EtrA, EivF, Hha, CadA, and SdiA act as inhibitors of *LEE* expression (Iyoda and Watanabe, 2004; Tobe et al., 2005; Vanaja et al., 2009; Vazquez-Juarez et al., 2008; Zhang et al., 2004). Some environmental factors such as glucose, short-chain fatty acids, calcium, sodium bicarbonate, ammonium chloride, and iron are also critical to *LEE* expression (Abe et al., 1997; Herold et al., 2009). The pathways are known to control *LEE* expression also regulate other virulence factors e.g. Ler increase the expression of secreted protease StcE from pO157 plasmid and LPF of EHEC. The quorum-sensing system that recognizes bacterial autoinducer 3 (AI-3) and host catecholamine also regulate shiga toxin and flagella production in a complex manner (Jeon and Itoh, 2007; Torres et al., 2007).

In general, AE pathogens including EHEC can evade the host innate immune system response which gives them an advantage in initiating infection through multiple mechanisms. T3SS proteins like EspB, EspF, EspH, and EspJ through their concerted activity block the closure of the phagocytic cup thereby inhibit phagocytosis (Iizumi et al., 2007; Wong et al., 2012). Some non-*LEE* encoded (Nle) T3SS effector proteins (e.g. NleE, NleB, NleC, NleD, and NleH) of EHEC can interfere with the stimulation of pro-inflammatory response by blocking multiple steps simultaneously (Nadler et al., 2010, 2010; Newton et al., 2010; Vossenkämper et al., 2010; Zhang et al., 2012). Interfering with NF- $\kappa$ B, EHEC can further disrupt the proinflammatory cytokine response which plays a significant role in clearing the infectious agent (Kawai and Akira, 2007).

*1.4.3 Effect of EHEC invasion on host.* After 3-5 days of the incubation period, EHEC usually causes watery diarrhea. However, it may progress into bloody diarrhea, indicating hemorrhagic colitis (Griffin and Tauxe, 1991). Diarrhea could result from the loss of microvilli which also increases intestinal permeability and ion release (Ewe, 1988). T3SS effector proteins such as EspF and Map disrupt the intestinal tight junction allowing the lumen contents to enter the adluminal compartment of the epithelium resulting in diarrhea (Guttman and Finlay, 2008; Vanaja et al., 2013).

*1.5 Treatment of EHEC.* Illnesses due to EHEC infection require supportive therapy to improve the symptoms and prevent the related sequelae, i.e., HUS. Since EHEC can cause renal failure, the conventional oral rehydration is insufficient for disease management. Intravenous rehydration is helpful in nephroprotection for which the patients are better to be

admitted to hospitals for monitoring the high sodium infusions carefully (Ake et al., 2005; Tarr et al., 2005).

Rigorous care and monitoring are required for HUS patients to avoid fluid overload in the kidney. Erythrocyte transfusion can be given to HUS patients who develop anemia. Kidney dialysis is needed if the patients have significant volume overload, high serum concentration, oliguria, persistent acidosis, hyperkalemia, etc. (Pennington, 2010; Tarr et al., 2005). A monoclonal antibody, eculizumab was used to treat patients with severe symptoms during STEC O104: H4 outbreak (2011) in Germany. It interferes with the hemolytic cascade by inhibiting the cleavage of C5 complement component. FDA has approved eculizumab for treating atypical HUS (Gruppo and Rother, 2009; Loos et al., 2012; Noris et al., 2012).

*1.5.1 Risks of some common therapeutics to treat EHEC infection.* Conventionally antibiotics are a common and effective way of treating bacterial infections. But the occurrence of antibiotic-resistant bacteria and the broad-spectrum effect of many antibiotics are becoming more evident to the scientific community. Although controversies exist regarding the effectiveness of antibiotics in treating EHEC infections, it is not recommended as therapeutic for reasons; firstly, the possibility of elimination of normal flora by antibiotics and the overgrowth of antibiotic-resistant EHEC. Secondly, antibiotics induce the expression of the shiga-toxin (Stx) gene which worsens the patient's condition (Panos et al., 2006).

Just after the discovery of *E. coli* as a pathogen (1984-1987), it was susceptible to all available antibiotics. However, after that time resistant strains of EHEC against streptomycin, sulfisoxazole, tetracycline, and ampicillin were reported in the USA, Spain, and Japan (Ito et al., 1997; Kim et al., 1994; Mora et al., 2005). Some antibiotics e.g. cefdinir, cefaclor, fosfomicin, norfloxacin, nalidixic acid, kanamycin, minomycin, doxycycline can exert their activity against EHEC (Ito et al., 1997). But using them in treating EHEC is still controversial. Some *in vitro* studies have shown that roxithromycin, rokitamycin, clindamycin, minocycline, doxycycline, and macrolide decrease the amount of Stx, but they did not show significant bactericidal effect (Murakami et al., 2000). On the contrary, there are ample *in vitro* studies proving the association of antibiotic and Stx release. The sub-inhibitory and sub-lethal concentration of polymyxin B, trimethoprim, sulphamethoxazole, ciprofloxacin, cefixime, tetracycline, quinolone, azithromycin, and gentamycin increased the Stx release with bacterial cell wall destruction (Grif et al., 1998; Ito et al., 1997; Karch et al., 1986; Karmali et al., 1985; Murakami et al., 2000; Yoh et al., 1999). *In vivo* studies also showed inconclusive results. Studies conducted in mice found that the application of fosfomicin, quinolone, minocycline, kanamycin, and norfloxacin during EHEC infection induce lower mortality and a less amount of fecal Stx (Sawamura et al., 1999; Yoshimura et al., 1999). However, treating EHEC infected mice with trimethoprim, sulphamethoxazole resulted in increased Stx toxin in blood and a 95% mortality of mice (Kurioka et al., 1999).

Clinically observed EHEC infection cases in hospitals mostly find a correlation between antibiotic use and the release of Stx from the bacteria (Wong et al., 2012). However, some studies also concluded about the beneficial role of antibiotics even no correlation as

well. Similarly, antimotility agents and narcotics have been reported to increase HUS development. Anti-inflammatory non-steroidal drugs can lower the renal blood flow and suggested to be avoided (Bell et al., 1997; Cimolai et al., 1992; Murray and Brater, 1993).

So, it is difficult to make a concrete conclusion about the helpful role of synthetic therapeutics to treat EHEC infected patients because of the inability of large randomized, double-blind, placebo-controlled human trials (Panos et al., 2006).

*1.6 Alternative strategies to control and prevent EHEC infections.* Due to the adverse broad-spectrum side effects of synthetic antibiotics and other therapeutics and the emergence of multi-drug resistant bacteria, the need for novel alternative controlling methods has recently gained momentum. Many of the novel approaches are being explored focusing on controlling EHEC disease progression at our contemporary time, such as bacteriophage therapy, development of a vaccine, use of natural antimicrobials, and use of prebiotics, probiotics, and symbiotic.

*1.6.1 Phage therapy.* Bacteriophages or phages are the simplest biological entities living on bacteria. There are  $\geq 10^{30}$  phage particles in the atmosphere which outnumbers any other biological bodies (Chibani-Chennoufi et al., 2004). Because of very tiny size (24-200 nm), phages can penetrate the skin and any tissue which helps them to reach chronically infected deeper layers of skin. They are also capable of crossing the blood-brain barrier which is the most desirable property of any therapy to treat central nervous system infections. Moreover, they are strictly specific for prokaryotic cells and have no known affinity to eukaryotic cells (Rios et al., 2016).

Before the era of large-scale production of antibiotics, phages showed the enormous potential to treat bacterial infections centering mostly the then Soviet scientists' research works. The first attempt of applying bacteriophage in a human was reported by d'Herelle in 1931 to treat staphylococcal skin furuncles (d'Herelle, 1931). There were a good number of reports regarding the application of phages in human infection during the 1919-1940s (Abedon et al., 2011). At that time, phage research did not increase across the globe may be because of non-English literature, and the discovery of antibiotics in North America. Besides, the non-randomized, uncontrolled trial reports failed to draw the mass attention of western researchers (Wittebole et al., 2014). Later in the 1980s, the increasing circulation of Soviet and Polish research works helped to rediscover phage therapy and then coupled with the genomics and broad ecology-based research techniques it gained a considerable momentum which paved the way of application of phages continuing to this day (Abedon et al., 2011).

A cocktail of phage consists of SP15, SP21, and SP22 reduced *E. coli* O157: H7 cells in *in vitro* and *in vivo* experiment (Tanji et al., 2005). In one FDA approved phase I clinical trial, the phage cocktail was proved to be safe and highly effective against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* in venous leg ulcers (MT (ASCP) et

al., 2013). Another study reported that bacteriophage vB\_Eco4M-7 can effectively infect EHEC.

Apart from clinical applications, phages have a vast potential to control foodborne pathogens. Application of phages in fermented foods (like cheese, yogurt, etc.), to preserve meat, fruits and vegetables are well documented in the literature (Leverentz et al., 2001; Rios et al., 2016; Viazis et al., 2011). Furthermore, phages can contribute to reducing the pathogenic microbial load and horizontal bacterial transmissions, like *E. coli*, *Salmonella* spp., *P. aeruginosa*, *C. difficile*, *Campylobacter* spp. coming from food-producing animals and birds (Andreatti Filho et al., 2007; Carrillo et al., 2005; Huff et al., 2002; McVay et al., 2007; Ramesh et al., 1999; Wills et al., 2005). Phage preparations are applied in feed and administered to the animal before slaughter (Johnson et al., 2008). Application of phages in wastewater, as well as potable water treatment systems, is being explored in the hope of preventing the transmission of multidrug-resistant pathogens, improved water, and public health quality (Tamaki et al., 2012). However, using bacteriophage as an alternative to antibiotics is not feasible because of its high specificity and relatively less control over its life cycle pattern.

*1.6.2 Nutraceuticals.* Nutraceuticals or bioceuticals, prebiotics, functional foods, diet supplements, etc., generally refer to a similar concept with some nuances. By and large, the nutrient contents of all kinds of foods provide physiological benefits to ameliorate host health and immunity. Foods can be designed to add more of the existing or new beneficial ingredients to gain maximum benefits (Hasler, 2002). During the 1980s the concept of functional food was first instigated in Japan to improve the health of elderly people which are now recognized as Foods for Specified Health Use (FOSHU) (Arai, 1996). FOSHU help to maintain healthy stability in the intestine, blood pressure, and blood cholesterol level (Henry, 2010). Although functional foods do not possess such established identity in the US, they are popularly consumed believing their superior nutritional and therapeutic impact in healthier life (Hasler, 2002). Some well-known nutraceuticals are *Echinacea*, ginseng, green tea, lutein, glucosamine, omega-3 fatty acids, folic acid, biotin, cod liver oil, etc. (Nasri et al., 2014). The worldwide market for physiologically beneficial foods is continuously expanding and worth \$250 billion of the food industry (Hardy, 2000; Nasri et al., 2014).

*1.6.2.1 Use of nutraceuticals to prevent EHEC infections.* Dietary components can work in favor of preventing many foodborne pathogens through diverse mechanisms. More than 30,000 antimicrobial components have been identified from about 1350 edible plant parts so far (Tajkarimi et al., 2010). Numerous *in vitro* studies have reported the growth in reducing the potential of many of our regular dietary items against EHEC. Comestibles such as peanut, blackberry, chokeberry, pimento, green tea, essential oils extracted from rosemary, bay, clove, etc., spices such as oregano, thyme, garlic, cinnamon, turmeric, cumin, etc. have well-established antimicrobial activity (Aditya et al., 2019; Arora and Kaur, 1999; Kim et al., 2016; Lins, 2018; Peng et al., 2015a; Praditya et al., 2019). The presence of some critical

ingredients in these dietary items confers their antimicrobial potential. Effect of terpenoids, sesquiterpenes, diterpene attached to different simple and complex hydrocarbon, glucoside, saponin, tannins, alkaloids, flavonoids, phenolic and non-phenolic compounds (Balasundram et al., 2006; Praditya et al., 2019; Tajkarimi et al., 2010). Exactly which of these compounds of diet are responsible for the antimicrobial activity and their mechanism of action is still under investigation. But it is vaguely believed that the concerted activity of these compounds on EHEC from various aspects are responsible for the observed growth inhibition. One study has reported that biotin-rich diet help to prevent EHEC infections by inhibiting the adherence of EHEC to the host cell epithelium by repressing LEE gene expression (Yang et al., 2015).

Nutraceuticals are a promising strategy to improve host health and immunity which in term helps to prevent EHEC infections. However, it still it is not a universal option to use as a therapeutic method because of personal food choices, individual digestive capacity, cost, and availability of the food.

*1.6.2.2 Synbiotic approach.* Our gut microorganisms can enzymatically utilize many ‘apparently indigestible’ food components for their growth and metabolism which in term also become beneficial for the host. This concept has been termed as synbiotic where prebiotics and probiotics act synergistically to provide a health benefit to the host (Davani-Davari et al., 2019; Gibson and Roberfroid, 1995; Gurry, 2017). With this strategy, host gut microbiome composition can be modified to gain a powerful therapeutic potential.

*1.6.2.2.1 Prebiotics.* Food compounds that bolster the growth and activity of beneficial microorganisms such as bacteria, fungi, etc. are called prebiotics. Prebiotic components should be resistant to host stomach acid and cannot be digested and assimilated by the host digestive enzymes. These traits of prebiotics create a discerning opportunity for the host gut flora by selectively stimulating or restricting their growth (Gibson et al., 2010). Prebiotic components encompass fructans, galactooligosaccharides, starch, and glucose-derived oligosaccharides known as resistant starch, pectin, flavonols, polyphenols, etc. Common resident microbial flora of human gut such as lactic acid bacteria (LAB), Bifidobacteria, *Ruminococcus bromii*, *Eubacterium rectale*, *Bacteroides thetaiotaomicron* can degrade those compounds enzymatically to produce many useful metabolites for the host and support their multiplication as well (Costabile et al., 2012; Fuentes-Zaragoza et al., 2011; Gullón et al., 2013; Louis et al., 2016; Tzounis et al., 2011; Yoo et al., 2012; Ze et al., 2012). Many of our diets contain prebiotics such as asparagus, blueberry, chokeberry, onion, garlic, peanut, wheat, honey, banana, soybean, milk, beans, microalgae, seaweeds and many more (Aditya et al., 2019; Ali and Nizar, 2018; Peng et al., 2015a; Salaheen et al., 2016).

*1.6.2.2.2 Probiotics and its general health benefits.* ‘Probiotic’ originated from a Greek word meaning ‘for life.’ A German scientist Werner Kollath (1953) first introduced this word to describe ‘active substances that are essential for the healthy development of life’ (Gasbarrini

et al., 2016). In 1965, Lilley and Stillwell used this word to define ‘substances secreted by one organism which stimulate the growth of another.’ A few years later, Sperti (1971) used this word to indicate tissue extracts that enhanced microbial growth. Later, Parker (1974) defined probiotics as ‘Organisms and substances which contribute to intestinal microbial balance.’ This wide definition would include antibiotics as well. So, Fuller (1989) redefined the probiotic concept emphasizing on probiotic to be a live organism as ‘A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance’ (Fuller, 1992). Almost all living beings harbor an enormous number of probiotics as part of their gut microbiota. The gut colonization starts with birth and shaped by factors such as- mode of delivery, diet, environmental exposure, and antimicrobial treatment (Davis, 1996; Dominguez-Bello et al., 2010). The source, growth conditions, and species of probiotics influence the probiotic value (Annuk et al., 2003). There are lots of probiotic bacteria; namely *Lactobacillus* spp., *Bifidobacterium* spp., *Escherichia coli* Nissle 1917, *Saccharomyces* spp., *Lactococcus* spp., *Streptococcus* spp., *Enterococcus* spp., *Bacillus* spp., *Carnobacterium* spp., *Melissococcus* spp., *Oenococcus* spp., *Pediococcus* spp., *Tetragenococcus* spp., *Vagococcus* spp., *Weissella* spp., *Aerococcus* spp., *Microbacterium* spp., and *Propionibacterium* spp. (Djadouni and Kihal, 2012; Fijan, 2014).

Fermented foods are a major source of probiotics. Foods such as yogurt, kefir, sauerkraut, tempeh, pickled vegetables, kimchi, miso, kombucha, traditional buttermilk, natto, cheese, etc. are excellent sources of LAB. Oral administration of an adequate amount of LABs confers health benefits to the host (Reid and McCormick, 2002). They help to digest fibers or prebiotics of regular food, stimulate the immune system, prevent the growth of pathogenic bacteria, help to produce biotin and vitamin k, hormones, and most importantly influence the host behavior. They also contribute to maintain a lower cholesterol level and prevent bacteria and/or virus-associated diarrhea (Charernjiratragul et al., 2010; Fuller, 1989a).

*1.6.2.2.3 Metabolites produced by lactic acid bacteria (LAB).* Probiotics alone or in the presence of prebiotics produce a variety of cell-associated and extracellular molecules and metabolites as their normal physiological process. The formation of these molecules is dependent on the substrate, population density, and kinetics (Delgado et al., 2007; Jones et al., 2008). LAB have been reported to produce metabolites such as flavonoids, glycosyl compounds, steroids, indole, indazole, benzoic acids, gluco-phospholipids, catechol, hydrocinnamic acid, salicylic acid, phenyllactic acid, hydrocoumaric acid, vanillic acid, azelaic acid, hydroferulic acid, hydrocaffeic acid, ferulic acid, caffeic acid, lactic, acetic, formic acids, linoleic acid, 2,3-butadione, reuterin, acetaldehyde, hydrogen peroxide, hydroxyl radical, peptides or proteins such as the bacteriocins and numerous derivatives (Broberg et al., 2007; Cleveland et al., 2001; Peng et al., 2015b; Schnürer and Magnusson, 2005). These metabolites of probiotics have been shown to possess inhibitory activity towards the multiplication of many enteropathogens including EHEC (Charernjiratragul et al., 2010; Jones et al., 2008; Mezaini et al., 2009; Ren et al., 2018)

*1.7 Microorganisms originated linoleic acid and derivatives.* One of the two essential fatty acids for the human is linoleic acid (LA), an omega-6 fatty acid (another one is  $\alpha$ -linolenic acid, an omega-3 fatty acid), i.e. this must be obtained through diet (Burr and Burr, 1930). Conjugated linoleic acids (CLA) are derivatives of linoleic acid which are geometric and positional isomers of octadecadienoic acid (LA) where two double bonds are conjugated in a cis or trans configuration without methylene interruption (Carvalho et al., 2010; Taha, 2020). CLA is present in foods like cheese, milk fat, ruminant meat, oils, etc. (Carvalho et al., 2010; Nagao et al.). So far 28 CLA have been discovered in nature; major isomers are c9t11-CLA (rumenic acid), t7c9-CLA, c11t13-CLA, c8,t10-CLA which also possess promising bioactive properties (Banni, 2002; Whelan and Fritsche, 2013). Apart from the dietary sources, CLA can be obtained from microbial biosynthesis which widens the horizon of nutraceuticals. Many probiotics can produce CLA such as *Bifidobacterium breve*, *B. pseudocatenulatum*, *Lactobacillus acidophilus*, *L. brevis*, *L. casei*, *P. acnes*, *Corynebacterium*, *Enterococcus faecalis*, *Streptococcus brevis* and many more (Kishino et al., 2002; Ogawa et al., 2005).

Two pathways have been suggested to describe the biosynthesis of CLA; through LA biohydrogenation in the rumen and  $\Delta^9$ -desaturation of trans-vaccenic acid (TVA) (C18:1 t11) in the mammary gland (Salsinha et al., 2018). In the rumen, the various fatty acids are released via lipolysis of dietary fatty acids. The polyunsaturated fatty acid (PUFA) i.e. LA is converted to its isomers mainly c9, t11-CLA by microorganism originated linoleic acid isomerase. These products are further hydrogenated by hydrogenase to a saturated end product which is usually stearic acid (C<sub>18:0</sub>) (Farmani et al., 2010; Fukuda et al., 2006; Jenkins, 1993; Jenkins et al., 2008; Lourenço et al., 2010; Turpeinen et al., 2002).

The intermediate and end molecules provide the enormous beneficial potential to the host. Besides being used as an energy source, CLA as part of membrane phospholipids can maintain membrane fluidity. They can take part in various cell signaling pathways by being stimulated and modified by other cellular factors (Whelan and Fritsche, 2013).

*1.7.1 Potential anticarcinogenic mechanism of CLA.* In experimental models (both *in vivo* and *in vitro*), CLA slowed down the neoplasia in the skin, mammary, prostate, and forestomach cancers. Events that occur post-initiation and during promotion was studied the anti-cancerous mechanism of CLA. In the premalignant stage, the imbalances between dysregulated differentiation, cell proliferation, and apoptosis lead to benign tumor formation. At this stage, CLA has been reported to reduce cell growth *in vitro* (Belury, 2002). *In vivo* studies using CLA as dietary supplement inhibited the proliferation of terminal end bud and lobuloalveolar bud structure where the mammary gland cancer initiates (Thompson et al., 1997). Another study reported reduced expression of cyclins A and D which are responsible for regulating the conversion of G<sub>1</sub> to S phase in the cell cycle (Figure: 2). They also observed reduced incorporation of bromodeoxyuridine (BrdU) (Ip et al., 2001).

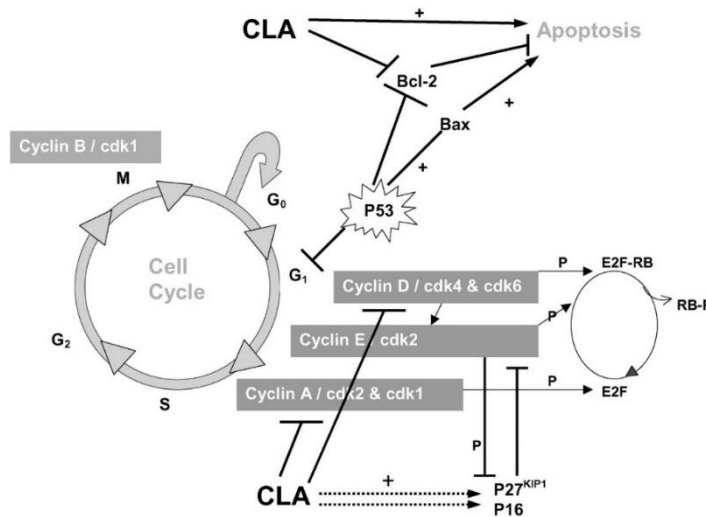


Figure: 2 A schematic diagram of the involvement of conjugated linoleic acid (CLA) in modulating cell cycle and apoptosis. Adopted from Belury, 2002.

Dietary CLA also stimulate p16 and p27 proteins which indicates that CLA reduce cell proliferation by blocking DNA synthesis and cell cycle proteins that regulate this process (Futakuchi et al., 2002; Ip et al., 2001). CLA also plays a stimulatory role in programmed cell death by reducing Bcl-2 protein. Bcl-2 and Bcl-x<sub>L</sub> proteins of *Bcl-2* gene family suppress apoptosis whereas Bax and Bak proteins promote apoptosis. Although CLA has a moderate effect on Bax, it essentially stimulates apoptosis by reducing the suppressor of apoptosis (Belury, 2002; Ip et al., 2001). However, the effect of CLA on the critical signaling steps regulating both cell proliferation and apoptosis e.g cyclin-dependent kinase (CDK) or p53 needs further study (Ip et al., 2001).

## Chapter 2: Diarrheagenic *E. coli* in dairy farm ecosystems and their antibiotic resistance pattern

### Introduction

Food is inextricably linked to both animals and humans, providing them energy for their regular activities. However, food can be a source of diseases and even death for a variety of reasons. Human foodborne illnesses are getting more attention than ever due to active food trade among countries of the world. Currently, meeting the dietary demands of the market and local population is not always possible due to many factors including variability of the average temperature, seasonality (average length of the summer and winter), geography, and average rainfall (i.e., available water sources) of the location, as well as costs associated with hiring of skilled and unskilled manpower, availability of equipment, machineries, feed, etc. (Käferstein et al. 1997). In addition to this, the ever-expanding globalization and interdependence of the economy has led to the mass migration of people with their values and habits across international borders, which increases the demand for particular goods that need to be imported. Given all these considerations, food exchange between geographic areas is essential, but is tied to the rise in foodborne illnesses, as the food processing practices might differ across countries (WHO, 2018).

One of the major causes of food mediated illnesses is the presence of biological contaminants such as, bacteria, fungi, viruses, and parasites, making food unsafe for consumption. Infection with bacterial foodborne pathogens remains one of the major contributors to illness, hospitalization, and death (Fatima and Aziz, 2021). Even though controlling bacterial foodborne illness is relatively easy by washing and adequately cooking food, it is still a problem worldwide for various reasons. Preference for eating fresh greens, differences in food handling practices and cooking style, pathogen load in the food, and variability in the immune status of consumers contribute altogether to the number of cases. Although the number of foodborne illnesses is higher in the developed countries, it is severely underreported in most of the developing countries (WHO, 2018).

One of the most notorious bacterial agents of foodborne illness is *Escherichia coli* (*E. coli*) which in turn counts with a diverse range of strains that are naturally present in the gastrointestinal tract and recto-anal junction (RAJ) mucosa of consumption ruminants such as cattle, sheep, and goats (Lim et al., 2007; Naylor et al., 2003). Most *E. coli* strains are harmless and form part of the normal flora of the lower intestine in warm-blooded animals, including humans. However, some *E. coli* strains have acquired specific combinations of virulence traits via bacteriophages, plasmids, pathogenicity islands, and transposons, which have become an integral part of their genome throughout the evolutionary process. Only a few successful combinations of virulence traits persisted under natural selection to become specific “virotypes” of *E. coli*. There are six *E. coli* virotypes capable to infect humans and cause intestinal illness which are also collectively referred to as diarrheagenic *E. coli*. They are classified based on their virulence pattern and named as enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) (Kaper et al., 2004; Nataro and Kaper, 1998). Unfortunately, these *E. coli* virotypes are also part of the normal flora of ruminants but are pathogenic to humans when transmitted via the enteral route (Doyle, 1991). Some of the *E. coli* virotypes, such as ETEC, cause sickness in young calves and with signs commonly including depression, dehydration, diarrhea, lack of appetite, and vomiting (Kersting; Lim et al., 2007). Though adult cattle can remain asymptomatic even if they are exposed to  $10^{10}$  CFU of EHEC cells (Stein and Katz, 2017), dairy and beef cattle have been shown to release *E. coli* virotypes into the environment through fecal shedding

(Solomon et al., 2002). These pathogens can survive temperatures as high as 70°C and as low as -20°C for 60-100 days in manure (Kudva et al., 1998; Wang et al., 1996). Therefore, even strictly followed manure composting guidelines might result in the application of pathogenic *E. coli* containing manure as fertilizer to produce crops, which may ultimately find their way in infecting humans through fresh produce (WHO, 2018). Cattle feces (Figure 1) mixed with rain or flood water can contaminate natural water bodies used in irrigation and thus could be another way the pathogen is spread (Wang et al., 1996). Irrigating contaminated water also contributes to their transmission (Hilborn et al., 1999). Moreover, *E. coli* cells can penetrate leaf stomata and cut edges, trapping them 20-100 µm below the leaf surface, which protects them from chlorine and other surface-disinfecting agents (Seo and Frank, 1999). Generally, *E. coli* virotypes transmit via contaminated water and food such as juice, uncooked or poorly cooked meat, and vegetables. Some common foods that were related to *E. coli* outbreaks previously are undercooked burger patties, salami, unpasteurized apple cider, yogurt, and cheese made from raw milk (Lim et al., 2010). Besides, *E. coli* contamination of dairy farm surroundings can result in spoiling of milk and its by-products (Vahedi et al., 2013).

In this study, environmental samples were collected from various sources of conventional dairy farms to estimate the prevalence and antibiotic resistance of *E. coli* virotypes. The microbial ecology of cattle grazing land is a potential source of pathogen transmission, since it is a common ground shared among farm workers, cattle, pet animals, visitors, etc., in addition to the insects, wild birds, and small wild animals that can also act as vehicles of *E. coli* transmission and have free access to the farm. Therefore, we also analyzed the microbiome composition of the grazing land soil over the winter and summer seasons to understand the potential association of climate on overall pattern of pathogen survival and transmission.

### Materials and methods

*Study Population, Sample Collection, and Transportation.* A longitudinal study was conducted to determine the prevalence of pathogenic *E. coli* (referred to as *pE.coli* in the rest of the study) on dairy farms located in Maryland, USA (39.0458° N, 76.6413° W) during the summer seasons (May to September) between the years 2019 and 2021, with sample collection being interrupted in 2020 due to the COVID-19 pandemic. Five dairy farms (two research institution facilities and three commercial farms) were chosen for this study and visited twice each year within a week interval, serving as biological replicates, with multiple samples being collected from several sites (between 5-10) serving as technical replicates for each sample group. A total of 846 samples belonging to various categories (Table 1) were collected during the summer season. Comparatively dry and semi-solid samples (feed, feces, bedding, soil, and compost) were collected in Whirl Pack bags (Nasco), whereas liquid samples (cattle drinking water and lagoon water) were collected in sterile 50 mL tubes (VWR™). For further processing and analysis in the laboratory, all samples were transported to in a cooler containing frozen gel packs for preservation (Peng et al., 2021).

*Sample Processing, Enrichment, and Culturing on Selective Medium.* The collected farm samples were processed following the previously published procedure from our research group with appropriate adjustments (Peng et al., 2016). Briefly, each sample was initially processed by adding and thoroughly mixing 25 mL of PBS (pH 7.4) to 1 g of each individual sample. The resulting solutions were used to inoculate Luria-Bertani (LB) broth enriched with 5% sheep blood (Ward's Science, USA) to achieve a final ratio of 1:9 v/v of sample per

media, which was later incubated overnight at 37°C under aerobic conditions to allow for proper enrichment. After incubation, the enriched broth was streaked on sorbitol MacConkey (SMAC) agar, which was used as a selective and differential media to identify presumptive *pE. coli* colonies based on their colony characteristics and morphology. The presumptive *pE. coli* colonies (colorless) were re-streaked on SMAC agar for an additional passage to ensure proper isolation and later were streaked onto LB agar consecutively. Presumptive colonies from the LB agar were stocked in LB broth containing 25% glycerol for storage at -80°C (Nutrition, 2020, 2021).

*Confirmation of E. coli and Identification of Virotypes by Gene-Specific PCR.* Presumptive positive *pE. coli* isolates were confirmed and virotyped by polymerase chain reaction (PCR) using gene and virotype-specific primers (Table 2), according to previously published studies, with some modifications (Hanning et al., 2010; Peng et al., 2016). Briefly, the presumptive *pE. coli* stocks previously stored at -80 °C were sub-cultured on LB agar and incubated at 37°C overnight. One well separated colony was aseptically collected and suspended in 50 µL PBS, which was thoroughly mixed to make a bacterial suspension that was heat lysed at 95°C for 15 min to extract DNA. This heat lysed bacterial suspension was centrifuged to pellet bacterial debris, while the supernatant containing the DNA was used as the DNA template for subsequent PCR reactions. Isolate confirmation was performed by using primers that targeted and amplified the *uid* gene (listed in Table 2), which has been previously used to confirm *E. coli* at the molecular level (Maheux et al., 2009). The PCR-confirmed *pE. coli* samples were further tested to identify their virotypes. To identify the virotypes, 7 sets of primers (listed in Table 2) were used to analyze the virulence genes associated with specific virotype of diarrheogenic *E. coli* (Tobias and Vutukuru, 2012). All PCR reactions were performed in a final volume of 20 µL containing 10 µL of 2× GoTaq® Green Master Mix (Promega), 0.5 µL of each set of primer (forward and reverse) (working concentration 1 µM), and 2 µL of template DNA, as per the recommendation of the manufacturer. All assays were conducted under the same cycling parameters, and the reactions were performed using the Master cycler (BioRad) under the following conditions: 1 cycle of 95°C for 5 min, followed by 40 cycles of 95°C for 30s, 46.8°C for 30s and 72°C for 1 min, and a final extension of 72°C for 5 min. PCR products were analyzed by visualization in 1.5% (w/v) agarose gel electrophoresis (Sigma-Aldrich) that ran for 50 min at 80 V.

*Antibiogram of pE. coli virotypes.* Antibiotic resistance patterns of *pE. coli* virotypes were determined by standard agar dilution method following the protocol recommended by the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2019). A group of antibiotics were chosen based on their clinical importance (Table 3). Antibiotic containing agar plates were prepared using five different concentrations of the selected antibiotics in molten Muller-Hinton (MH) agar (Becton Dickinson & Co), as described before. (Peng et al., 2016). For performing the antibiogram, the *pE. coli* isolates that were previously confirmed and virotyped were initially cultured on MH agar and incubated at 37°C overnight. Following aseptic technique, isolated colonies were picked from each positive sample and suspended in 0.85% normal saline and fixed to an optical density 600 nm (OD<sub>600</sub>) between 0.08 and 0.1 (equivalent to 0.5 McFarland standard). A portion of this suspension (0.1 mL) was added to 0.9 mL of saline solution and thoroughly mixed to achieve a bacterial suspension of ~10<sup>7</sup> CFU/mL. Then, 2 µL of each *E. coli* suspension was inoculated on antibiotic containing MH agar to achieve a final bacterial load of ~10<sup>5</sup> CFU/mL and incubated overnight at 37°C. The lowest concentration of antibiotic that completely inhibited visible bacterial growth was

considered as the minimum inhibitory concentration (MIC). Antibiotic resistance of the isolates was interpreted according to CLSI (2019) breakpoints and can be found in Table 3.

*Soil Microbiome Analysis.* To evaluate the effect of seasonal variation on the bacterial microbiome composition of grazing land, soil samples were collected during the winter (December and January; categorized as “Winter”) within the span of two years. Another set of samples were collected from the same locations during the summer (May to September, categorized as “Summer”). Approximately 300 mg of soil were used to extract total bacterial genomic DNA according to previously published studies (Peng et al., 2021; Tabashsum et al., 2020) by using the PureLink Microbiome DNA Purification Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer protocol. DNA extracted from replicate samples belonging to the same soil site were each combined according to their identification to form individual DNA pools. With the Nextera XT DNA Library Preparation Kit and Nextera XT Index Kit (Illumina), the 16S library was prepared and later also pooled together into an aliquot of equimolar concentration, according to the manufacturer's instructions. Illumina MiSeq was used to obtain high-quality, full-length reads of the V3 and V4 regions utilizing paired-end (2×300-bp) and the MiSeq v3 600-cycle kit (Illumina). MiSeq Reporter program (MSR) or BaseSpace were used to produce FASTQ Workflow and classify organisms using the Greengenes database (<http://greengenes.lbl.gov/>). A total 176451.35 pass-filter reads were analyzed for the 16S rRNA composition (entries classified with no genus or species were filtered as well). All the raw sequences were submitted to GenBank SRA under BioProject PRJNA849987.

*Statistical Analysis.* Comparisons of prevalence of *pE.coli* and their virotypes in various sample category and their antimicrobial resistance profiles were carried out using MS Excel. Fisher's exact tests and the Cochran-Mantel Haenszel (CMH) test was used to control for sample category. Fisher's exact test was also applied to compare the prevalence of *pE.coli* of each sample category. Student's t-test was applied to detect the significance in the abundance of different bacterial phylum and genus between seasons.

## Results

*Prevalence of pE.coli in Dairy Farm Environment and Their Virotypes.* The presence of *pE.coli* was found in all the sample categories, however it significantly ( $p < 0.05$ ) varied based on the sample sources. The overall *pE.coli* prevalence was 8.39% (71/846) with the highest prevalence in lagoon slurry 21.57% (11/51), while the lowest prevalence was 2.99% in compost (4/134). Interestingly, other sources which come in close contact with the cattle, such as water (6.36%), feed (7.14%), feces (10.71%), bedding (9.09%), and grazing land soil (10.08%), also had notable prevalence of *pE.coli*. Comparing among farms, the prevalence of *pE.coli* was significantly higher ( $p < 0.05$ ) in research institutional dairy farms (87.32%) as compared to commercial dairy farms (12.67%) (data not shown separately). The prevalence of *pE.coli* in different environmental samples collected from various sources of dairy farms is presented in Figure 2.

*Virotype Specific Distribution of pE.coli.* In this study, five virotypes of *pE.coli* associated with human enteric illness i.e., diarrheagenic *E. coli* were targeted. A total of 97 isolates were positive for at least 5 main different *pE.coli* virotypes, namely EPEC, STEC, EIEC, EAEC, and ETEC. Among them, a widespread distribution of ETEC (51.54%) was discovered

throughout various dairy farm environments, while the lowest prevalence was observed for EIEC (2.06%). The prevalence of other virotypes were 21.64%, 13.30%, and 11.34% for STEC, EPEC, and EAEC, respectively.

The predominance of specific virotypes within a sample group was linked to the nature of the different sources of dairy farms. Across various sources that we considered in this study, the highest prevalence of all virotypes were detected in feed (23.71%) with the majority of these virotypes being found in isolates from feed samples collected directly from the cattle barn, while only one feed isolate being identified as EPEC, which was collected from storage (out of 35 samples of that category). Other environmental sources such as grazing land soil (18.55%), lagoon slurry (16.49%), feces (14.43%), bedding (13.40%), cattle drinking water (9.27%), and compost (4.12%) also harbored the p*E. coli* virotypes. The distribution of p*E. coli* virotypes among various samples are presented in Table 3.

*Antibiotic Resistance Pattern.* From the isolates collected and identified in this study, 95.87% of the p*E. coli* virotypes were resistant to at least one antibiotic, while only 4.12% were sensitive to all tested antibiotics. Among the resistant virotypes 5.37% were resistant to single antibiotic and the rest were resistant to multiple antibiotics. All the isolated virotypes from lagoon slurry and compost were resistant to multiple antibiotics. No sensitive virotype was detected from feces or bedding materials. The highest percentage of sensitive isolates were found in water (11.11%) and grazing land soil (11.11%) while feed contained the lowest (4.34%) sensitive virotypes.

Among the tested antibiotics, resistance to gentamycin (12.37%) was the lowest while resistance to ciprofloxacin (68.04%) was the highest. Other antibiotics that isolates were found to be resistant to were azithromycin (65.97%), amoxicillin (63.91%), trimethoprim/sulfamethoxazole (62.88%), tetracycline (57.73%), ampicillin (56.70%), and ceftriaxone (50.51%). The overall prevalence of antibiotic resistant p*E. coli* virotypes is presented in Table 5.

*Seasonality Influences the Grazing Land Soil Bacterial Microbiome Composition.* The effect of seasonal variability on the soil bacterial microbiome was more noticeable ( $p < 0.05$ ) on certain major bacterial phyla than others. During the summer season, the relative abundance of Actinobacteria (15.97%), Chloroflexi (6.81%), and Gemmatimonadetes (2.64%) were significantly higher ( $p < 0.05$ ) than in the winter season. However, the abundance of another major bacterial phylum, Verrucomicrobia (2.43%), were recorded to be significantly ( $p < 0.05$ ) suppressed during the summer season (Figure 3 A). A numerically higher abundance ( $p > 0.05$ ) of Proteobacteria (26.70%) and Acidobacteria (13.32%) was recorded in the summer than in winter (Figure 3 A). The atmospheric factors of winter season favored a numerically higher abundance ( $p > 0.05$ ) of Bacteroidetes (12.68%), Firmicutes (13.24%), Planctomycetes (4.94%), and Parcubacteria (2.33%) as compared to the summer (Figure 3 A). Proteobacteria was the constantly predominant bacterial phylum in all farms regardless of the season. While comparing relative abundance of specific phyla among farms within their respective season, the proportion of Proteobacteria was found to decrease as the abundance of Firmicutes increased (Figure 3 B and C).

The ambient seasonal variation had very distinguishing impact on the abundance of bacterial genera. During the summer, the most predominant ( $p < 0.05$ ) bacterial genera were *Gemmatimonas* (2.66%), *Sphingomonas* (4.66%), *Gp16* (1.85%), *Aridibacter* (1.63%),

*Nocardioides* (1.90%), *Flavisolibacter* (1.39%) and *Arthrobacter* (1.20%). On the other hand, during winter the abundance ( $p < 0.05$ ) of the genera *Parcubacteria\_genera\_incertae\_sedis* (2.33%), *Saccharibacteria\_genera\_incertae\_sedis* (2.88%), and *Campylobacter* (0.03%) were found to be significantly predominant (Figure 4 A). However, no statistically significant difference was found in the prevalence of bacterial genus among farms within a season (Figure 4 B and C). At the species level, a statistically higher ( $p < 0.05$ ) number of species were documented during summer season (3027.4) as compared to the winter season (2134.2) (Figure 5 A). However, no significant difference ( $p > 0.05$ ) was documented in the diversity within community when calculating alpha-indices (Shannon and Simpson index) (Figure 5 B And C).

## Discussion

Since the discovery of *E. coli* as a foodborne pathogen in 1982, it remains one of the most notorious biological agent of public health concern (Rangel et al., 2005). In general, ruminants including dairy and beef cattle are the major reservoir of various *E. coli* virotypes (Stein and Katz, 2017) which has been well documented throughout the results of this study. The overall prevalence of p*E.coli* in various sources of typical dairy farm environment was 8.39% ( $p < 0.05$ ). Notable presence of p*E.coli* was documented at various sites of a farm setting, such as cattle drinking water, feed, feces, bedding, and grazing land soil, which come into frequent contact with the cattle. There were no statistically significant observations that showed a difference in the prevalence of p*E.coli* among these sources ( $p > 0.05$ ), which likely indicates that any of these farm components can act as a potential source that mediates p*E.coli* transmission. Studies showed that, other than being foodborne and waterborne (Stein and Katz, 2017), this pathogen may be transmitted through other sources, such as direct person-to-person contact via farm workers (Rangel et al., 2005; Spika et al., 1986), or through direct contact with farm animals from farms, agricultural fairs and petting zoos (Goode et al., 2009).

Although direct access of cattle is usually restricted, this study found the highest and lowest prevalence ( $p < 0.05$ ) of p*E.coli* in lagoon (21.57%) and compost heap (2.99%), respectively. Both lagoon water treatment and composting are commonly used methods to treat farm animal manure in order to safely dispose of them before releasing into the environment (Van Epps and Blaney, 2016). Solid and liquid manure can both be produced by flushing a dairy barn with water and passing it through a solid-liquid separation system. After separation, the solids are stored in piles for composting while the liquids are collected in a lagoon (Pandey et al., 2018). Lagoons and composting process convert the manure into more useable product such as biogas for fuel and nutrient rich fertilizer valuable for crop production (Pandey et al., 2016; Van Epps and Blaney, 2016). Though the effectiveness of these manure processing methods on the microbial population are poorly understood (Agga et al., 2022; Pandey et al., 2018), we observed a significantly ( $p < 0.05$ ) lower prevalence of p*E.coli* in compost as compared to lagoon. A reason for this difference could be credited to fact that the upper liquid layer of the lagoon that was collected receives some aeration and are maintained at ambient climatic conditions (Lovanh et al., 2009), making that environment a more favorable one for the survival and proliferation of more p*E.coli*, which showed in the prevalence found in the collected lagoon slurry samples.

We documented a significantly higher prevalence of p*E.coli* in research institutional farm which was 87.32% (62/71). This observation corroborates the fact that individual farm practice specifically choosing cattle diet and use of veterinary chemicals to ensure cattle good

health and maximizing production are some of the controlling factors of *pE.coli* prevalence (Patoliya et al., 2022). Pesticides are often used in dry feed and pasturage which leave various recalcitrant residues in the environment. So far, limited number of studies focused on the presence of pesticidal residues in feed (Bruinenberg et al., 2022). Synthetic chemicals that are commonly used for tick and fly treatment and cattle drinking water in farms such as deltamethrin, tebuconazole, calcium hypochlorite, etc., find their way in feed which eventually might interfere with the microbial community (Walorczyk, 2008, USDA, 2006).

According to IFSAC (2021), leafy greens, beef, and dairy are the top three food categories responsible for more than three-quarters of *E. coli* O157-related infections (IFSC, 2021), which is data that agrees with the findings of the current study. Generally, leafy greens such as spinach, cilantro and lettuce, are eaten raw or with minimal processing, hence possessing high chances of transmitting illness-causing foodborne bacteria to consumers, if they are already contaminated. Application of lagoon effluent and compost as a natural fertilizers that can also modifying the soil microbial community for bettering crop production are traditional commonly used practices for sustainable agriculture (Agga et al., 2022; Peng et al., 2021). Animal waste is a valuable source of many nutrients such as amino acids, sugars, nucleic acids, vitamins, nitrogen, phosphorous, potassium and some micronutrients critical for plant growth (Berry et al., 2003; Zhang et al., 2019). Apart from supplying phytonutrients, improperly prepared compost or application of comparatively less composted portions of the compost heap can contaminate the surface of consumer crops and fruits. Factors such as compost type, storage condition, and the initial microbial load influence the survival of pathogens in the lagoon effluent and compost heap. Significant survival of *E. coli* O157:H7 has been previously documented for very long periods of time (>168 days) in compost, especially when it is kept at lower temperature and under greenhouse conditions (Chen et al., 2018).

Among the *E. coli* virotypes, STEC receives most of the attention due to its Shiga toxin production ability and very low infectious does ( as low as 10 cells) (Etcheverría and Padola, 2013). It is also frequently reported as the cause of the majority of *E. coli* mediated foodborne outbreaks, many of which have lead to subsequent product recalls (Aditya et al., 2020; CDC, 2019). Moreover, antibiotics are not prescribed to treat STEC mediated illness because they can induce Shiga toxin production from the pathogen, which worsen the patient's condition (Mühlen and Dersch, 2020). In this study, 21.64% of the *pE.coli* were classified as STEC and they were ubiquitously distributed in places that typically come into direct contact with cattle. The study did not find any STEC in the lagoon slurry or the compost, which ensures the effectiveness of these manure handling practice in controlling the particular propagation of STEC.

It is important to note that very limited literature was found documenting the prevalence of ETEC in the dairy farm environment. Nonetheless, it should come as no surprise that prevalence for ETEC (51.54%) was the highest among the isolated *pE. coli* virotypes, and were also ubiquitously distributed among all sample categories. ETEC is the major causative agent of coli-septicemia and enteric colibacillosis in newborn calves (Acres, 1985; Van Metre et al., 2008). Nearly half of all few day-old calves harbor ETEC (KROGH, 1983). The feces of the ETEC-harboring cattle are the major source of transmission. Each ETEC-infected cattle, including the newborn calves, shed several folds more bacteria than those initially acquired, and may continue for several months even after recovery (KROGH, 1983; Moon and Bunn, 1993). ETEC infection is very common in communities with poor sanitation which is mostly seen in less developed countries, posing a particular risk to

travelers in these countries (traveler's diarrhea, TD) and infants (Evans and Evans, 1996; Gaastra and Svennerholm, 1996). However, though this study found a high prevalence of ETEC in the Maryland dairy farm environment, illnesses caused by this virotype is comparatively lower in humans, which can be attributed to the high host-specificity of ETEC between animal and human strains (Blanco et al., 1995; Qadri et al., 2005). ETEC serotypes in humans produce enterotoxins (STa/STb or LT-1) which has colonization factors CFA/I to CFA/IV, whereas the bovine ETEC STa harbor bacterial surface K99 or F41 colonization factor (Blanco et al., 1995). Besides, the pathogenicity of ETEC serotypes has been observed to be altered over time and geographic area (Stoll et al., 1983). More extensive study focused on the outer membrane polysaccharide (O antigen) and flagellar (H) antigen is needed to determine the prevalence of animal and human specific ETEC-serotypes.

Although other virotypes, i.e., EPEC, EAEC, and EIEC are not frequently reported as a disease-causing agent in the USA; considerable prevalence was detected among the sample categories. EPEC is a major cause of infant malnutrition and mortality in developing countries (Deborah Chen and Frankel, 2005; Kliegman et al., 2020). In developed countries, it causes self-limiting sporadic diarrhea which is often traced back to pediatric wards and daycare centers (Martín-Rodríguez et al., 2022). Besides causing several episodes of diarrhea, EAEC infection in particular can result in chronic digestive disorders such as irritable bowel syndrome (IBS) (Kaur et al., 2010), while EIEC and *Shigella* spp. have some shared genotypic and phenotypic characteristics that make their infections difficult to differentiate (Pasqua et al., 2017). Their presence was only detected in compost and grazing land soil which was also the estimated lowest prevalence among the specific virotypes of this study.

Despite restrictions on the use of antibiotics as a growth enhancer in cattle in the USA, we discovered a high incidence of resistant virotypes (95.87%,  $p < 0.05$ ) to a variety of antibiotics often used in human therapy (Editors, 2017). Among the resistant isolates, only 5.15% were resistant to single antibiotic whereas 90.72% were multi drug resistant (MDR). Isolates collected from lagoon and compost samples were detected to be 100% MDR which agrees with the studies published by other research groups (Fatoba et al., 2022; Yoshizawa et al., 2020). Considering individual antibiotics, resistance to gentamycin was the lowest across the sample types whereas resistance to ciprofloxacin, ampicillin, tetracycline, azithromycin, trimethoprim/sulfamethoxazole, amoxicillin, were comparatively higher ( $p < 0.05$ ) than other antibiotics used in this study, which is in agreement with previous studies (Sawant et al., 2007). These findings serve to emphasize that fact that to work against the dissemination of antibiotic resistant *pE. coli* proper farm management is warranted (Rasheed et al., 2014)

In this study, the grazing land soil was the only sample category where all targeted *E. coli* virotypes were identified. Soil is the most important natural element which plays critical role for the wellbeing of all ecosystems (Boschiroli et al., 2016). Soil of grazing land is heterogenous in nature and receives inputs of bacteria and other microorganisms from the cattle excrement. Depending on the individual farm practice these fecal wastes are collected for composting or left for natural decomposition (Manyi-Loh et al., 2016). As a result, the grazing land soil becomes a reservoir of all type of microorganisms both good and bad. Variation of the ambient climatic conditions and physical factors of soil including temperature, moisture, pH, oxygen availability, nutrients favor the prevalence of certain groups of microbes, while many groups remain dormant (Boschiroli et al., 2016). Since ruminant feces are the primary source of *pE.coli* the microbial composition was evaluated through metagenomic analysis of the soil during summer and winter.

The most predominant documented phylum in this experiment regardless of seasonal variation was Proteobacteria ( $p > 0.05$ ) with their relative abundance being numerically

higher in summer. Proteobacteria consists of many Gram-negative enteropathogens including *pE.coli*. Firmicutes, which are another phyla that includes many beneficial bacterial genera such as *Lactobacillus* spp. were relatively lower in summer ( $p > 0.05$ ). When comparing the relative abundance of bacterial phyla among farms within seasons, there was an inversely proportional relationship noted between the abundance of Proteobacteria and Firmicutes, namely, their relative proportion keeps each other under certain control (Figure 3 B and C).

When comparing at genus level, the abundance of most predominant genera was unaffected by seasonal variation, with some exceptions. A higher abundance of *Nitrolancea*, a nitrogen fixing soil bacteria (Speick et al., 2020), was observed in summer (2.80%), while their presence in winter was nearly negligible (0.04%) ( $p < 0.05$ ). Abundance of *Nitrolancea* is an indication of higher nitrogen fixation which is directly related to higher sunlight and temperature (Kelly and Becker, 1975). Some other notable genera of common soil bacteria, such as *Gemmatimonas*, *Aridibacter*, and *Gp16* were higher in summer. When comparing the abundance of major genera among farms within season, much variation was observed. This indicates that the relative abundance of a specific genus in a specific farm setting is largely influenced by the farm practices that have been put in place. One of the genera that were consistently present in both seasons in all farms, was *Sphingomonas*, which is an opportunistic pathogen. Although they are commonly found in soil, drinking water, and plants, they mostly cause nosocomial infection. Which can be linked to transmission of this genus from farm environment to hospitals via human interaction.

A relatively higher abundance of the genus *Escherichia* was found in winter as compared to summer ( $p > 0.05$ ), which is slightly inconsistent with our usual understanding. However, certain experimental limitation can plausibly explain this fact. Culture-based analysis was not performed for soil samples that were collected and processed for extraction of total bacterial total genomic DNA, therefore metagenomic analysis could be accounting for live, dead, and/or dormant bacteria. Considering the presence of countless commensal *E. coli* serotypes in nature, this experiment could also not control or differentiate for the inclusion of non-pathogenic and/or *pE.coli*. Other physical and climatic reasons such as less rain flushes and limited grazing of cattle during the winter could be some of the compelling reasons that might explain why during winter, relative abundance of *Escherichia* was higher (Boschioli et al., 2016). We found a significantly higher total number of species in summer. It indicates wetter soil conditions support more common soil bacterial species while a drier soil suppress them (Bickel and Or, 2021). However, the overall diversity (calculated by Shannon and Simpson index) was unaffected between the seasons.

## Conclusions

The findings of this study demonstrate potential risk of antibiotic resistant *pE.coli* transmission from various elements within a dairy farm environment. Application of cattle manure-based fertilizer (compost) in the backyard and community garden could be posing a potential food safety risk. Some strategies, such as avoiding direct contact between compost and plant leaves or fruits, as well as implementing better composting processes and lagoon cleaning systems, may be crucial practices for reducing the contamination load of cattle and dairy products in the future. Finally, the soil of grazing land remains the reservoir of all sorts of pathogens even in winter season which warrants proper precautions for outdoor recreational activities and food preparation.

List of tables

Table 1. Samples collected from various sources in dairy farm environment

Sample Category	Sample Details	No. of Samples
Water	Water collected from trough	110
Lagoon slurry <sup>a</sup>	Liquid refuse of dairy barn stored in an earthen basin for anaerobic treatment. Samples collected from the upper liquid layer of lagoons.	51
Feed	Grass, hay, and pellet from the grazing land and barn	175
	Dry feed collected from the storage <sup>b</sup>	35
Feces	Fresh cattle droppings (manure)	112
Bedding	Composed of hay, sand, pellet collected from the barn	110
Soil (grazing land)	Collected from cattle grazing land, area containing fresh feces was avoided	119
Compost	Collected from different depths of compost heaps, ranging from surface to five inches	134
	Total	846

*a, b.* Because all farms did not have this category, the overall number of samples of the respective category was reduced.

Table 2. Primers used in identification and virotyping of *E. coli* in this study.

Genes	Primer names	Sequences (5'-3')	Product Sizes (bp)	References
<i>uid</i> <sup>a</sup>	<i>uid-1</i>	ATGGAATTCGCCGATTTTGC	187	(Maheux et al., 2009)
	<i>uid-2</i>	ATTGTTTGCCTCCCTGCTGC		
<i>stx</i> <sup>c</sup>	<i>stx-VT1</i>	GAGCGAAATAATTTATATGTG	518	(Botkin et al., 2012)
	<i>stx-VT2</i>	TGATGATGGCAATTCAGTAT		
<i>est</i> <sup>e</sup>	<i>est-AL1</i>	TTAATAGCACCCGGTACAAGCAGG	147	"
	<i>est-AL2</i>	CCTGACTCTTCAAAAAGAGAAAATTAC		
<i>elt</i> <sup>e</sup>	<i>elt-LT1</i>	TCTCTATGTGCATACGGAGC	322	"
	<i>elt-LT2</i>	CCATACTGATTGCCGCAAT		
<i>ipa</i> <sup>d</sup>	<i>ipa-H1</i>	G TTCCTTGACCGCCTTTCCGATACCGTC	619	"
	<i>ipa-H2</i>	GCCGGTCAGCCACCCTCTGAGAGTAC		
<i>agg</i> <sup>f</sup>	<i>agg-R1</i>	GTATACACAAAAGAAGGAAGC	254	"
	<i>agg-R2</i>	ACAGAATCGTCAGCATCAGC		
<i>bfp</i> <sup>b</sup>	<i>bfp-1</i>	GGAAGTCAAATTCATGGGGGTAT	300	"
	<i>bfp-2</i>	GGAATCAGACGCAGACTGGTAGT		
<i>eae</i> <sup>b,c</sup>	<i>eae-SK1</i>	CCCGAATTCGGCACAAGCATAAGC	881	"
	<i>eae-SK2</i>	CCCGGATCCGTCTCGCCAGTATTCG		

*a.* Identification of p*E. coli*

*b.* EPEC

*c.* STEC

*d.* EIEC

*e.* ETEC

*f.* EAEC

Table 3. Distribution of different *pE. coli* virotypes\* among various sample categories

Sample Category	EPEC	STEC	EIEC	EAEC	ETEC
Water	1	3	0	1	4
Lagoon slurry	4	0	0	1	11
Feed	2	5	0	1	15
Feces	1	3	0	2	8
Bedding	3	3	0	4	3
Soil (grazing land)	1	7	1	2	7
Compost	1	0	1	0	2
Total	13 <sup>Ba3*</sup>	21 <sup>Ba2*</sup>	2 <sup>Cb5\$</sup>	11 <sup>Ba4*</sup>	50 <sup>Aa1#</sup>

\*Different uppercase and lowercase letters, numbers, and special characters indicate statistically significant difference ( $p < 0.05$ ) among the prevalence of the *E. coli* virotypes.

Table 4. Antibiotics, antibiotic groups, and resistance breakpoints used in antimicrobial susceptibility tests for p*E. coli*<sup>a</sup>

Antimicrobial class	Antimicrobial agent	Resistant breakpoint (µg/mL)
Aminoglycosides	Gentamicin	≥16
	Streptomycin	≥ 64
β-Lactam	Amoxicillin	≥ 32
Cephems	Ceftriaxone	≥ 4
Folate pathway inhibitors	Trimethoprim/sulfamethoxazole	≥ 4/76
Macrolides	Azithromycin	≥ 32
Penicillin	Ampicillin	≥ 32
Phenicol	Chloramphenicol	≥ 32
Quinolones	Ciprofloxacin	≥ 0.12
Tetracyclines	Tetracycline	≥ 16

<sup>a</sup> Antimicrobial susceptibility test was performed using agar dilution method according to guideline established by CLSI. *E. coli* ATCC 25922 were used as the quality control organism.

Table 5. Overall antimicrobial resistant profile of p*E.coli* virotypes

Sample Category*	Resistant	Sensitive
Water	88.88 <sup>A</sup> (8/9)	11.11 <sup>A</sup> (1/9)
Lagoon slurry	100 <sup>A</sup> (16/16)	0 <sup>A</sup> (0/16)
Feed	95.65 <sup>A</sup> (22/23)	4.347 <sup>A</sup> (1/23)
Feces	100 <sup>A</sup> (14/14)	0 <sup>A</sup> (0/14)
Bedding	100 <sup>A</sup> (13/13)	0 <sup>A</sup> (0/13)
Soil	88.88 <sup>A</sup> (16/18)	11.11 <sup>A</sup> (2/18)
Compost	100 <sup>A</sup> (4/4)	0 <sup>A</sup> (0/4)

\* No statistical significance ( $p > 0.05$ ) was observed in the prevalence of resistant and sensitive isolates among the sample categories

Table 6. Antimicrobial resistance profile of *pE. coli* virotypes

Antibiotic Group	Antibiotic	Resistant (%)	Sensitive (%)
Aminoglycosides	Gentamicin	12.37 <sup>B</sup> (12/97)	87.62 <sup>A</sup> (85/97)
	Streptomycin	30.92 <sup>B</sup> (30/97)	69.07 <sup>A</sup> (67/97)
$\beta$ -Lactam	Amoxicillin	63.91 <sup>A</sup> (62/97)	36.08 <sup>B</sup> (35/97)
Cephems	Ceftriaxone	50.51 <sup>A</sup> (49/97)	49.48 <sup>B</sup> (48/97)
Folate pathway inhibitors	Trimethoprim/Sulfamethoxazole	62.88 <sup>A</sup> (61/97)	37.11 <sup>B</sup> (36/97)
Macrolides	Azithromycin	65.97 <sup>A</sup> (64/97)	34.02 <sup>B</sup> (33/97)
Penicillins	Ampicillin	56.70 <sup>A</sup> (55/97)	43.29 <sup>B</sup> (42/97)
Phenicol	Chloramphenicol	24.74 <sup>B</sup> (24/97)	75.25 <sup>A</sup> (73/97)
Quinolones	Ciprofloxacin	68.04 <sup>A</sup> (66/97)	31.95 <sup>B</sup> (31/97)
Tetracyclines	Tetracycline	57.73 <sup>A</sup> (56/97)	42.26 <sup>B</sup> (41/97)

<sup>A, B</sup> statistically significant difference ( $p < 0.05$ ) between the prevalence resistant and sensitive isolates of the respective antibiotic.

List of figures, captions, and legends

Figure 1.

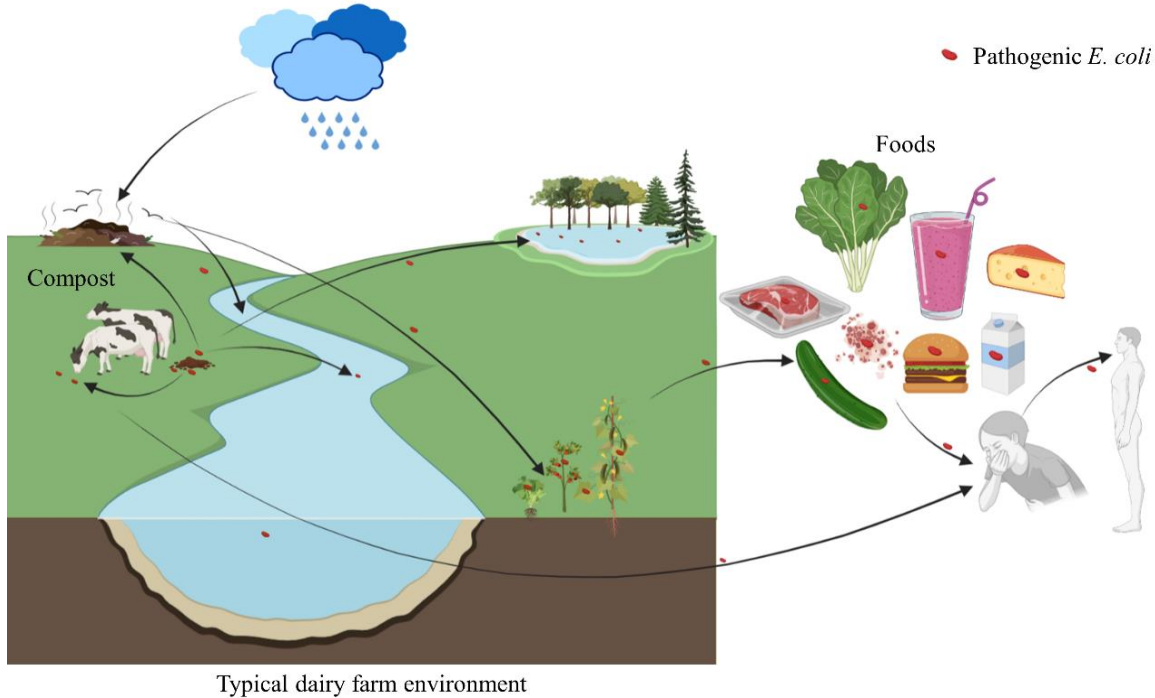


Figure 1. Schematic representation of the possible transmission patterns of p*E. coli* from various sources of dairy farm environment. Ruminants are the major source of p*E. coli* virotypes which are released into the environment through their feces. The pathogens can spread and survive in various places of the farm environment such as compost, grazing soil, being washed with rainwater, water bodies, etc., and can later be transmitted to other warm blooded hosts including humans. Common pathways of transmission include contaminated irrigation water, contaminated fresh produce, meat, and dairy products, direct person-to-person contact, etc. (figure generated through BioRender.com).

Figure 2.

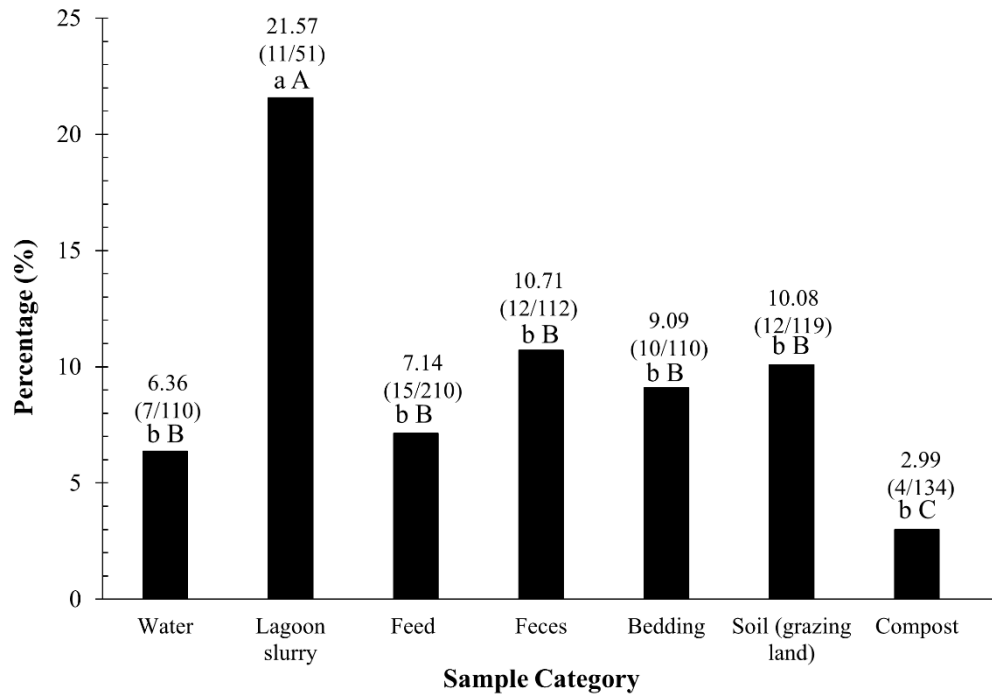


Figure 2. Distribution of *pE. coli* ( $n = 846$ ) across different sample category representing different potential source of *pE.coli* in a dairy farm. Percentages with different uppercase and lowercase letters indicate statistically significant difference ( $p < 0.05$ ) among different sample categories.

Figure 3.

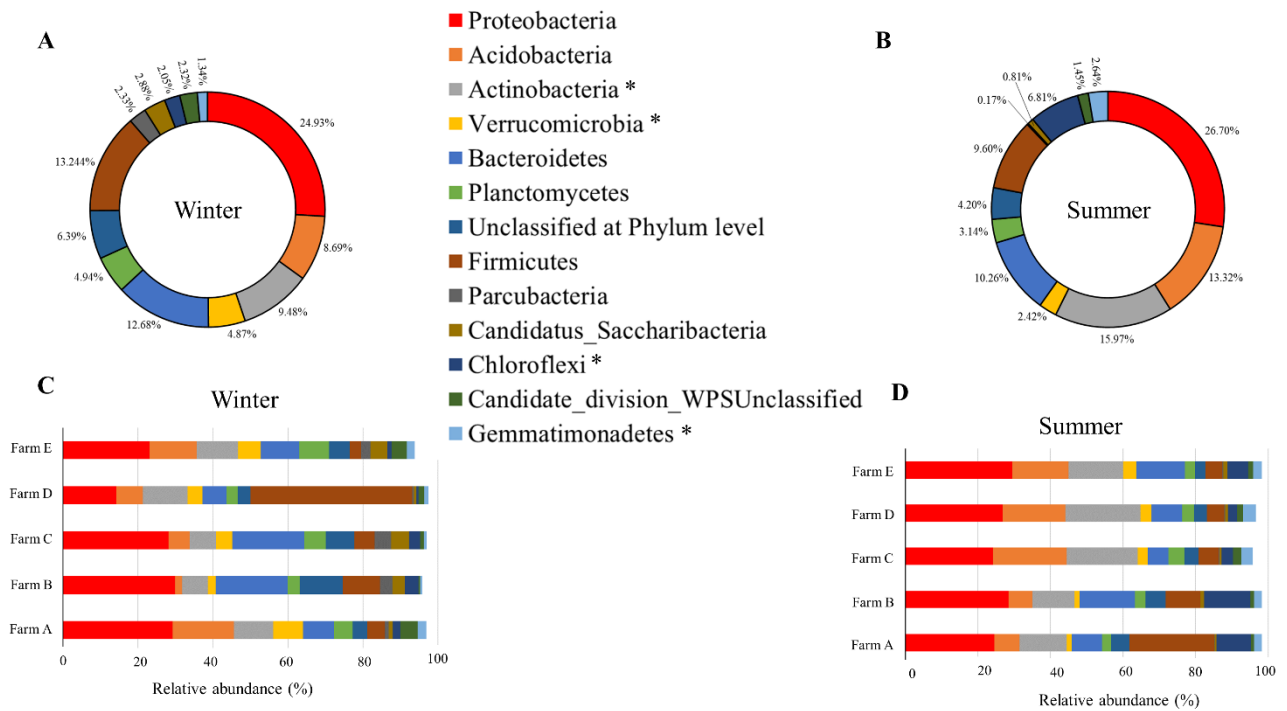
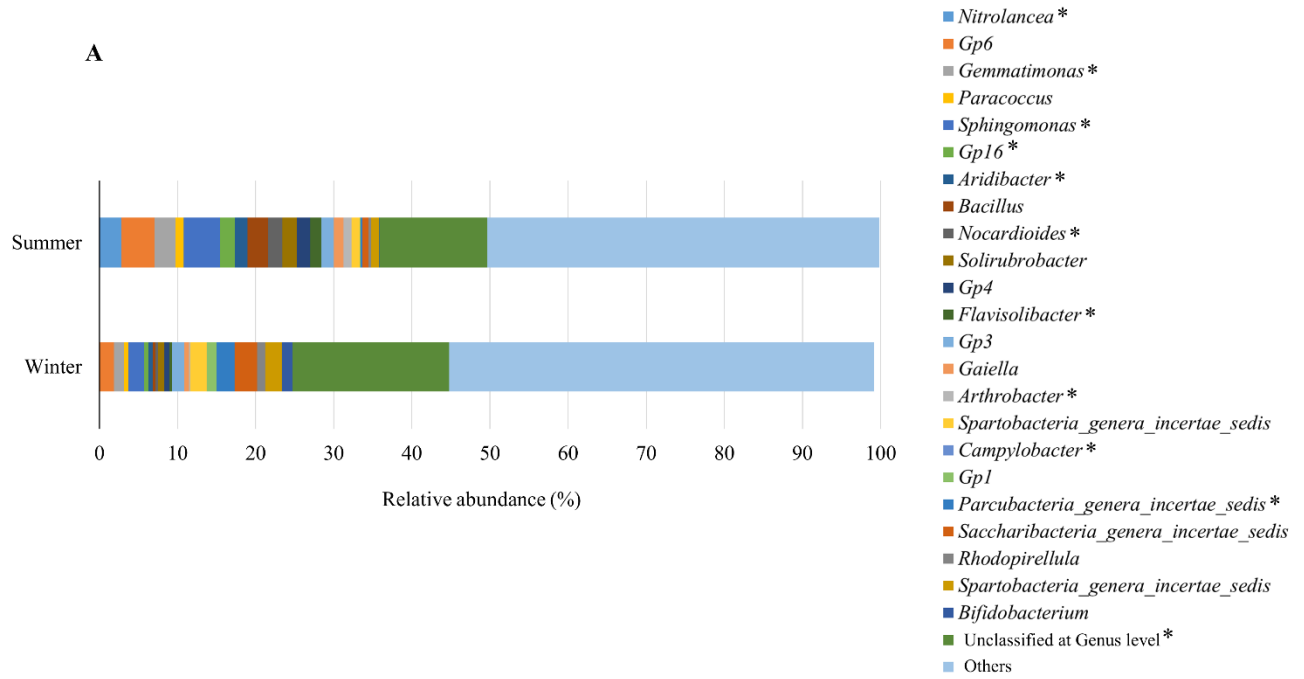


Figure 3. The relative abundance of major bacterial phylum between seasons ( $n = 40$ ) (A and B) and among farms within season (C and D). The asterisks (\*) beside the phylum name indicate statistically significant difference ( $p < 0.05$ ) in the abundance of that phylum between seasons (winter and summer) .

Figure 4.



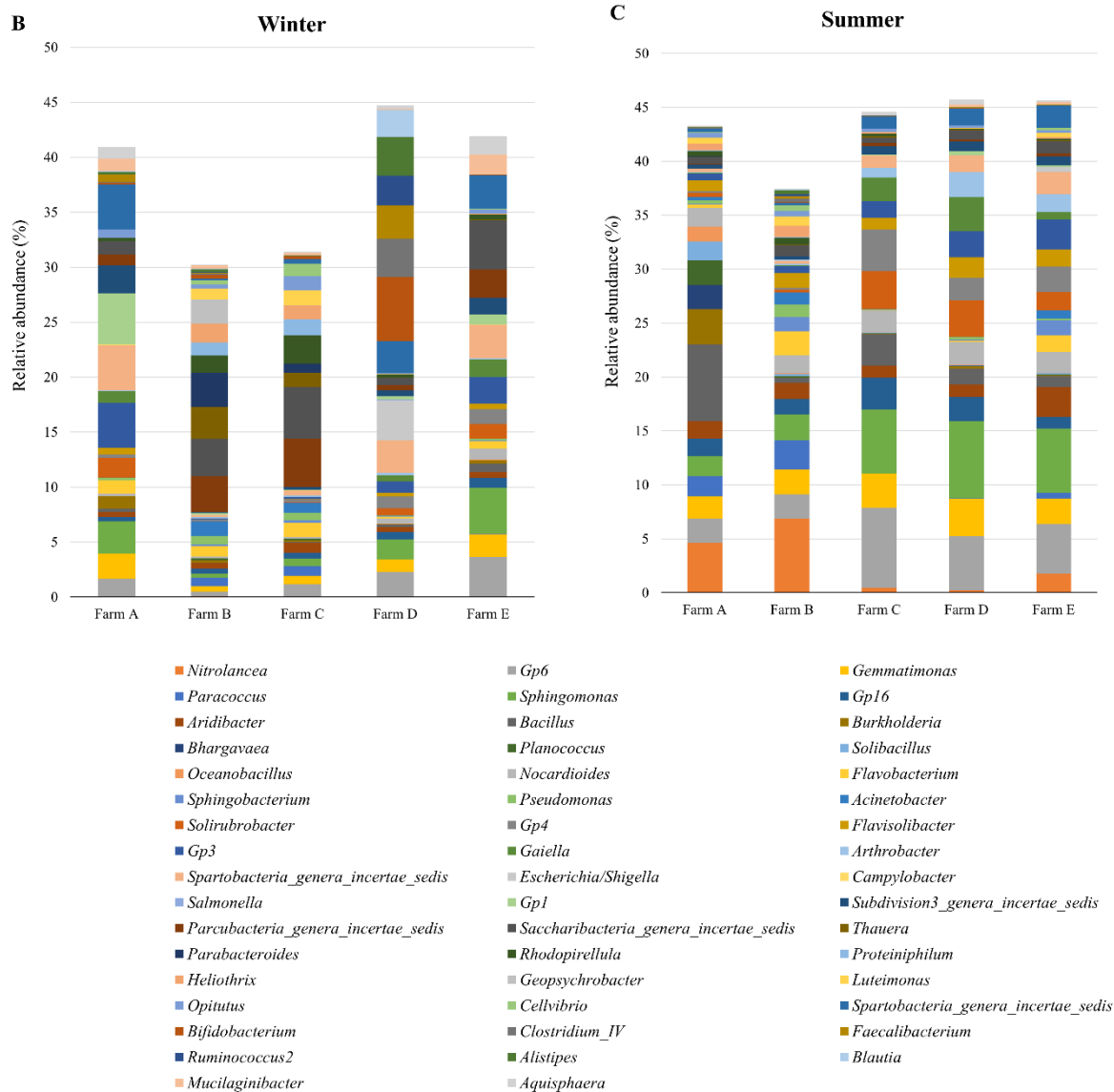


Figure 4. Effect of seasonality on the relative abundance of soil bacteria at genus level between seasons (A) and among farms within season (C and D). The asterisks (\*) beside the genus name indicate statistically significant difference ( $p < 0.05$ ) in the abundance of that genus between seasons (winter and summer).

Figure 5.

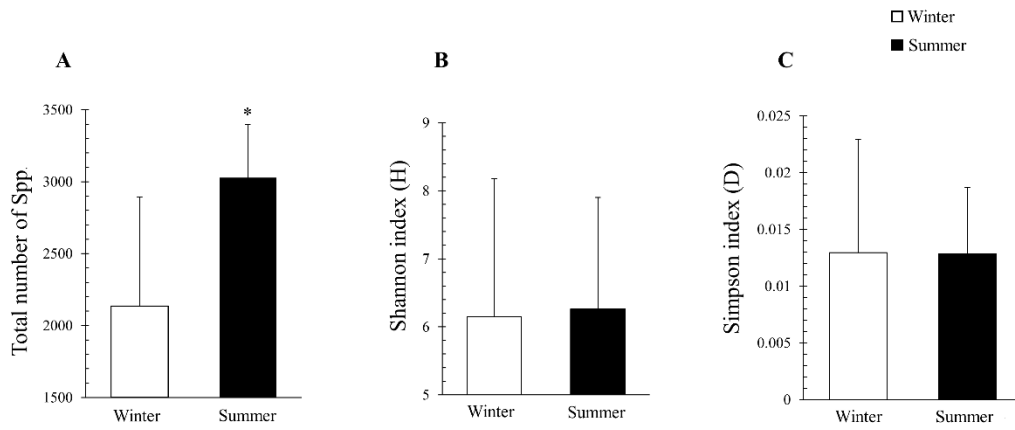


Figure 5. Calculation of soil microbial diversity at species level in terms of total number of species (A), Shannon index (H) (B), and Simpson index (D) (C). The asterisk (\*) indicates statistically significant difference ( $p < 0.05$ ) between seasons (winter an

## Overall Hypothesis and Specific Aims

Metabolites obtained from various probiotic bacteria (e.g. *Lactobacillus* spp.) have been largely reported to exert inhibitory activity against pathogen growth and their toxin production (Peng et al., 2018; Withey et al., 2015). But their mechanism of action is still unelucidated. To address this yet undiscovered field my overall hypothesis was:

“Antagonistic mechanisms of action of metabolites, specifically conjugated linoleic acid (CLA) produced by *Lactobacillus casei*, are involved in interfering growth and metabolism on *E. coli* O157: H7 (EHEC).”

To investigate the hypothesis, the following aims were fulfilled,

Aim 1: Impact of *L. casei*-originated metabolites on EHEC in *ex vivo* condition

Aim 2: Identify effective concentration of specific bioactive isomers of linoleic acid in metabolites produced by *L. casei*

Aim 3: Mechanism of metabolites produced by *L. casei* on lysis of EHEC

Aim 4: Investigate metabolites produced by *L. casei* in production and neutralization of Shiga toxin of EHEC using Gb<sub>3</sub> receptor containing mammalian cells

### Introduction

*Escherichia coli* is a very diverse bacterial species where most of its serotypes are harmless inhabitants of environment including soil, and water, as well as gastrointestinal (GI) tracts of animals and humans (Croxen et al., 2013). Only few serotypes of *E. coli* are pathogenic that cause diseases in humans and animals (LeJeune et al., 2001). Several pathogenic serotypes of *E. coli*, apparently cause simple ‘stomach upset or diarrhea’ but few may lead to complications such as bloody diarrhea, sepsis, meningitis, urinary tract infections (UTI), and kidney failure and that vastly depend on age, and immunity of the host (Kaper et al., 2004; Mellata, 2013; Nordstrom et al., 2013). Among these severe pathogenic serotypes of *E. coli*, enterohemorrhagic *E. coli* (EHEC) is one of the emerging enteric bacterial pathogens which is also a major public health concern since its discovery in 1982 during an outbreak in the USA (Schlundt, 2017). EHEC-mediated human diseases have gained special attention because of its ability to produce Shiga toxin (Stx) and its association with very difficultly curable complication of kidney which is known as hemolytic uremic syndrome (HUS) (Karch, 2001). Stx is a potent cytotoxin which binds with Gb3 (glycolipid globotriaosylceramide) receptor of kidney cells and lyse the cells, and ultimately leading to kidney failure under some circumstances (Chan and Ng, 2016). This condition is referred to as HUS which can’t be treated/cured by administration of antibiotics since there is evidence that antibiotics may worsen the situation (Pacheco and Sperandio, 2012; Riley et al., 1983; Tarr and Freedman, 2022). Moreover, EHEC has an incredibly low infectious dose (10-100 bacteria) (Nguyen and Sperandio, 2012) and is being frequently reported to be the causative agent of foodborne outbreak in the USA that comes with a huge economic impact due to hospitalization cost, long term care, loss of productivity, product recall, and lawsuits (Batz et al., 2012).

Typically, the foregut of domestic and wild ruminants such as cattle, sheep, goats, pigs, wild deer, moose, etc. offer an ideal habitat for EHEC. Several critical parameters of rumen environment such as temperature (38°-42°C), pH (6.2-7), redox potential, volatile fatty acids (VFA), presence of other microorganisms including bacteriophages create an atmosphere where EHEC can thrive (de Vaux et al., 2002) and transiently (3-4 weeks) discharged to the environment via feces (LeJeune et al., 2001). Although EHEC is excreted in the cattle feces for a brief period of time, it is persistently isolated from almost all sources on farms (Rice et al., 1999). The molecular stability of EHEC (as well as other pathogenic serotypes) in the farm environment contributes to the possibility of being transmitted to humans through different routes; such as direct contact with cattle saliva and feces, cross contamination of produce (vegetables and leafy greens) and in slaughterhouse (LeJeune et al., 2001; López-Catalina et al., 2021). According to the recent (2018) outbreak reports, major foods that are frequently associated to EHEC mediated outbreak includes leafy greens (61.4%; such as lettuce, sprouts, spinach, etc.), beef and other meat products (32%) (CDC, 2019; The Interagency Food Safety Analytics Collaboration (IFSAC), December 2020). The rumen originated EHEC can be transmitted to humans through cross-contaminated beef and milk. It can also attach to the plant leaves and tissue from the contaminated irrigation water (Bertin et al., 2011; Jeong et al., 2011). Furthermore, EHEC can survive in the animal waste-based compost (such as dairy manure and poultry litter) for a long time (> 168 days) based on compost type, storage, and the abundant of the pathogen. Compost is commonly used to fertile the soil as organic nitrogen source as well as

other plant nutrients (Sharma et al. 2019). The pathogen containing compost may contaminate water, soil, produce, and fruits with EHEC when applied (Chen et al., 2018).

Despite of the rigorous application of safe composting methodology and stringent cleaning strategies during post-harvest, food processing, and preparation steps; it is impossible to eliminate the presence of EHEC. Hence, minimizing the pathogenic *E. coli* i.e., EHEC load at preharvest level i.e., in the ruminant GI tract by dietary intervention is a promising strategy to prevent significant portion of foodborne illnesses and safer environment (Bertin et al., 2011; Jeong et al., 2011). Approaches such as bacteriophage treatment, addition of antagonistic microbes with feed have been reported to be effective in reducing colonization in rumen and fecal shedding of EHEC (Gaggia et al., 2010). To attain these, a variety of components that have already been tested include plant-derived antimicrobials (e.g., carvacrol, eugenol, thymol, etc.) (Ananda Baskaran and Venkitanarayanan, 2014), chitosan microparticles (Jeong et al., 2011), probiotic bacteria (i.e., *L. reuteri*) (Bertin et al., 2011). In this study, we aimed to stimulate the existing probiotic population (specifically *Lactobacillus* spp.) in a complex environment i.e., rumen by supplementing live *Lactobacillus casei*, peanut flour and their metabolites collected in MRS broth and evaluated their effect on EHEC and other predominant bacterial flora.

### Materials and methods

*Bacterial strains and their growth conditions.* Shiga toxin-producing enterohemorrhagic *Escherichia coli* O157:H7 EDL933 (EDL933) (ATCC700927) was cultured overnight at 37°C on Luria-Bertani (LB) agar (Becton, Dickinson, and Co.) under aerobic conditions. As representatives of probiotic bacteria, *Lactobacillus casei* (LC<sub>wt</sub>) (ATCC334) and an engineered *L. casei* (LC<sub>CLA</sub>) capable of overexpressing linoleate isomerase (by overexpressing myosin cross-reactive antigen gene, *mcra*) (Peng et al., 2018) were cultured on de Man-Rogosa-Sharpe (MRS) agar (Merck KGaA) at 37°C in presence of 5% CO<sub>2</sub>.

*Collection and usage of rumen fluid to simulate the environment in the bovine intestine.* Ruminant fluids (RFs), which contain containing solid and liquid parts, were collected anaerobically from cannulated permanently nonlactating cow consuming timothy hay in the morning hour before feeding (Judd and Kohn, 2018). RFs were collected four times in 50-mL centrifuge tubes at one-week interval from cannulated permanently nonlactating cows housed at the campus farm, Department of Animal and Avian Sciences, University of Maryland, College Park, MD. The collected RFs were vortexed vigorously to homogenize and used it immediately as a simulation of bovine rumen system in *ex vivo* condition (Kim et al., 2019; Rivas et al., 2010).

*Treatments of rumen fluids.* Probiotic strains (LC<sub>wt</sub> and LC<sub>CLA</sub>) as well as their extra-cellular metabolites were evaluated to observe their direct effect on EDL933 growth and pathogenic properties in simulated bovine rumen system. Besides using LC<sub>wt</sub> by itself (inoculum size: ~10<sup>6</sup> CFU/mL), it was armorized with 0.5% (w/v) peanut white kernel flour (PF; prepared according to Aditya et al., 2020 (LC<sub>wt+PF</sub>)). Again, LC<sub>wt</sub> was engineered to overexpress linoleate isomerase (LC<sub>CLA</sub>; inoculum size: ~10<sup>6</sup> CFU/mL) which boosted 21-fold more conversion of linoleic acid isomers (i.e., conjugated linoleic acid, CLA) per cell than the LC<sub>wt</sub> (Peng et al., 2018). Hereafter,

these treatments i.e., LC<sub>wt</sub> and LC<sub>CLA</sub> are mentioned as ‘probiotic’ and LC<sub>wt+PF</sub> as ‘synbiotic.’ We also assayed the effect of the presence of 25% (v/v) total extracellular metabolites present in the cell free culture supernatant (CFCS) collected in MRS broth from *L. casei* in different cultural conditions i.e., from LC<sub>wt</sub>, LC<sub>wt+PF</sub>, and LC<sub>CLA</sub> labeled as CFCS<sup>wt</sup>, CFCS<sup>wt+PF</sup>, and CFCS<sup>CLA</sup>, respectively (Aditya et al., 2020). Hereinafter, metabolites containing CFCSs are referred to as the ‘postbiotic’.

*Effect of probiotics, synbiotics, and postbiotics on the growth of EDL933 in rumen fluid.* Homogenized RFs were transferred to a 50-mL Falcon™ tissue culture flask (Thermo Fisher Scientific Inc.) to analyze the growth pattern of EDL933. The final volume of each treatment was 6 mL. For the probiotic and synbiotic group, the probiotic inoculum (LC<sub>wt</sub> and LC<sub>CLA</sub>) was prepared by fixing the optical density at 0.2 at 600 nm (OD<sub>600</sub>) in the phosphate buffered saline (PBS, pH 7.4) using a spectrophotometer (PerkinElmer) to achieve an inoculum size of ~10<sup>6</sup> CFU/mL. Same volume of PBS (100 µL) was added to the RFs to be considered as a control. To evaluate the synbiotic effect, 0.5% (w/v) PF was added in combination of LC<sub>wt</sub>. For the postbiotic group, 25% (v/v) CFCS collected at distinct cultural conditions (i.e., CFCS<sup>wt</sup>, CFCS<sup>wt+PF</sup>, and CFCS<sup>CLA</sup>) added to the RFs separately. For this part of our study, samples containing same volume of MRS broth (1.5 mL) was counted as control.

Approximately 10<sup>3</sup> CFU/mL of EDL933 (60 µL) was added to each treatment by fixing the OD<sub>600</sub> at 0.1 followed by a 100-fold dilution in PBS. To monitor the rumen microbial community's response to additional PBS and MRS broth as a control, one set of samples was included which did not contain any treatments or control adjustments, hereafter mentioned as ‘O-rf’ (primary control). All rumen fluid containing tubes, i.e., controls and treatments, were incubated at 37°C in presence of 5% CO<sub>2</sub> to evaluate the growth pattern of EDL933 and *Lactobacillus* spp. The assays were performed in four separate trials with technical duplicates. To enumerate the numbers of EDL933 and *Lactobacillus* spp. until 72 h of treatment, a portion of the samples were retrieved at 0, 12, 24, 48, and 72 h which were serially diluted and plated on Sorbitol MacConkey (SMAC) agar and MRS agar to count EDL933 and *Lactobacillus* spp. respectively. The number of colorless colonies on SMAC agar were counted to determine the growth of pathogenic *E. coli*. The pH of each treatment condition at each time point were also measured.

*Genomic DNA extraction and metagenomic study by analyzing 16s ribosomal RNA gene (16s rRNA).* Genomic DNA was extracted from the untreated (O-rf) and treated (probiotic, synbiotic, and postbiotic group) RF at 0, 24, and 48 h of incubation using QIAamp® Fast DNA Stool Mini Kit (QIAGEN) according to the protocol provided by the manufacturer with appropriate adjustments (Peng et al., 2020a). The concentration of the extracted DNA was measured by nanodrop spectrophotometer (Thermo Fisher Scientific Inc.). Influence of the treatments (probiotic, synbiotic, and postbiotic) on experimentally added *Lactobacillus* spp., EDL933 and overall bacterial composition in the RF was evaluated as part of metagenomic research by targeting variable regions (V3 and V4) of conserved 16S rRNA gene. This part of experiment was performed according to the method previously described by our research group with relevant modification (Peng et al., 2020a; Tabashsum et al., 2020).

In the beginning, extracted genomic DNA of the same treatment condition were pooled together from three separate trials and the concentration of all the samples were equalized at 5 ng/μL for microbiome study. 16S library was prepared and pooled into equimolar concentration with Nextera XT DNA Library Preparation Kit and Nextera XT Index Kit (Illumina) following the manufacturer's protocol. High-quality, full-length reads of the V3 and V4 region were generated with Illumina MiSeq using paired-end (2×300-bp) and MiSeq v3 600-cycle kit (Illumina). Sequencing data were processed by MiSeq Reporter software (MSR) or BaseSpace to generate FASTQ Workflow and classify the organisms based on Greengenes database (<http://greengenes.lbl.gov/>). Demultiplexing was performed using the perfect index recognition (mismatch = 0) and by removing PhiX reads. A total 17,390,362 pass-filter reads were analyzed for the 16S rRNA composition (entries classified with no genus or species were filtered as well). The obtained data were studied to determine the differences in relative abundance at phylum, genus, and species level (number of species, Shannon index, Simpson index, and Sorensen coefficient) levels among different treatment groups.

The Shannon Index (H) was calculated by the following equation:

$$H = - \sum_{i=1}^s p_i \ln p_i$$

While the Simpson Index (D) was calculated by the following equation:

$$D = \frac{1}{\sum_{i=1}^s p_i^2}$$

Where, p is the proportion (n/N) of individuals of one species found (n) divided by the total number of individuals found (N), ln is the natural log, Σ is the sum of the calculations, and s is the number of species.

The Sorensen's Coefficient (CC) was calculated by the following equation:

$$CC = \frac{2c}{S_1 + S_2}$$

Where C is the number of species the two communities have in common, S1 is the total number of species found in community 1, and S2 is the total number of species found in community 2.

*Statistical analysis.* Student's t-test in MS Excel was used to determine the statistically significant difference ( $p < 0.05$ ) between the growth of *Lactobacillus* spp. and EDL933 in controls and treatments. The obtained data were compared among O-rf, PBS control, and MRS broth control. We used ANOVA to determine significance of the pH among different time points.

## Results

*Effect of probiotics, synbiotics, or postbiotics containing CFCSs on the growth and survival of EDL933.* In this study, experimentally added probiotics (LC<sub>wt</sub> and LC<sub>CLA</sub>) and the synbiotic combination of LC<sub>wt</sub> and peanut flour (LC<sub>wt+PF</sub>) could not reduce the growth of an experimentally added EDL933 (or pathogenic *E. coli* serotypes,  $p > 0.05$ ) in the RF significantly but numerically.

However, comparing the numerical changes the effect of synbiotic combination (LC<sub>wt+PF</sub>) was found to be more efficient as we observed a numerical growth repression of pathogenic *E. coli* (3.73 logs) at 72 h of incubation than only probiotic either LC<sub>wt</sub> or LC<sub>CLA</sub> alone (Fig.1 e). On the other hand, none of these treatments impacted on the growth of probiotics including experimentally added LC<sub>wt</sub> and LC<sub>CLA</sub> as well as *Lactobacillus* spp. naturally present in the RF as compared to the control (PBS) at the time points we assessed (Fig. 1 A-E). Further, treatment with PBS (control) and with the live probiotics (either LC<sub>wt</sub> or LC<sub>CLA</sub> as well as LC<sub>wt</sub> along with the prebiotic (LC<sub>wt+PF</sub>)) did not alter the initial pH significantly as compared to the pH of RF at respective time points.

Surprisingly, postbiotics containing CFCSs collected from the growth of LC<sub>wt</sub> in the presence of peanut flour (CFCS<sup>wt+PF</sup>) exhibited growth suppression of EDL933 significantly ( $p < 0.05$ ) initiated within 12 h (pH 5.01) of incubation and during the subsequent time points (24, 48, and 72 h) retained its antipathogen potential by exhibiting numerical growth suppression ( $p > 0.05$ ) as compared to MRS broth control. After 72 h of incubation, CFCS<sup>wt</sup> and CFCS<sup>CLA</sup> inhibited the growth of EDL933 significantly ( $p < 0.05$ ) whereas a complete growth reduction was observed from CFCS<sup>CLA</sup> (pH 8.07); while CFCS<sup>wt</sup> (pH 8.5) reduced pathogen count to 0.48 logs (Fig. 1 e). Experimentally added postbiotics present in the CFCSs (i.e., CFCS<sup>wt</sup>, CFCS<sup>wt+PF</sup>, and CFCS<sup>CLA</sup>) did not show any detrimental impact on the existing *Lactobacillus* spp. in RF, rather it helped to retain the number of existing *Lactobacillus* spp. of RF as compared to the MRS broth (control) (Fig. 1 A-E). Statistically significant ( $p < 0.05$ ) growth stimulation of *Lactobacillus* spp. was observed from CFCS<sup>wt+PF</sup> and CFCS<sup>CLA</sup> as compared to control (MRS broth) at later hours of incubation (48 and 72 h) (Fig. 1C, 1D). The highest growth of *Lactobacillus* spp. was documented as ~8 logs at 48 h (pH 8.04) in presence of CFCS<sup>wt+PF</sup> whereas the presence of CFCS<sup>CLA</sup> was responsible to increase the growth to ~7.95 logs (pH 8.1) as compared to the MRS broth control (~7.2 logs, pH 8.2) (Fig. 1C). However, unlike the probiotic and synbiotic group, addition of CFCSs caused a significant shift in the pH toward the acidic range was noted as compared O-rf (pH 7.2) when MRS broth (pH 5.61), and the postbiotics collected in MRS broth (i.e., CFCS<sup>wt</sup> (pH 5.31), CFCS<sup>wt+PF</sup> (pH 5.2), and CFCS<sup>CLA</sup> (pH 5.33)) were added to the RF. The pH of the added substances is one of the likely factors to have had an impact on our observed outcome. Overall, postbiotic treatments always showed remarkable effect on inhibition of EDL933 in RFs as compared to the respective probiotic only or combination of prebiotic and probiotic (Fig. 1 a-e).

*Notable effect of probiotics, synbiotics, or postbiotics containing CFCS on commonly present phyla in RFs.* The metagenomic analysis targeting 16S rRNA indicated that Bacteroides and Firmicutes are the two major phyla comprising approximately 70-75% of the rumen bacterial community in general. In the beginning of the experiment (0 h, pH 7.2), Bacteroides and Firmicutes comprised about 50.93% and 21.51%, respectively of the O-rf bacterial community (Fig. 2A). The notable presence of other substantial phyla such as, Tenericutes (4.79%) and Proteobacteria (3.46%) were also recorded at 0 h in O-rf. Addition of different substances to the RF for this study i.e., PBS and MRS broth (regarded as control), and the inoculation of probiotics (LC<sub>wt</sub> and LC<sub>CLA</sub>), and incorporation of synbiotics (LC<sub>wt+PF</sub>), and postbiotics (CFCS<sup>wt</sup>, CFCS<sup>wt+PF</sup>, and CFCS<sup>CLA</sup>) to the RFs did not unsettle the relative abundance of aforesaid major bacterial phyla significantly ( $p > 0.05$ ) at 0 h (Fig. 2A). However, due to the treatments with probiotic, synbiotic, or postbiotics and incubation conditions, we observed remarkable shifts in

the relative abundance of major bacterial phyla at distinct time points of the ongoing incubation (24 h and 48 h) as compared to 0 h and the respective time points as well (Fig. 2A-C). With the progression of incubation time abundance of Proteobacteria heightened in O-rf comprising 35.4% and 39.63% of the bacterial community at 24 h (pH 7.31) and 48 h (pH 7.79), respectively which was significant ( $p < 0.05$ ) as compared to the abundance at 0 h. Bacteroides ( $p < 0.05$ ) and Firmicutes ( $p > 0.05$ ) in O-rf declined correspondingly allying with incubation hours (Fig. 2A-C). The observed difference in the relative abundance of major bacterial phyla at 24 h and 48 h as compared to the O-rf at the respective time point of incubation were resultant from the nature of added substances (i.e., probiotics, synbiotics, or postbiotics). After 24 h of incubation, Bacteroidetes maintained its predominant abundance under all the cultural conditions as compared to O-rf though no statistically significant difference was observed. However, the proportion of Bacteroidetes started to decline significantly ( $p < 0.05$ ) after 24 h of incubation as compared to O-rf except LC<sub>wt</sub> (Fig. 2B-C). Interestingly, as compared to the MRS broth control at 24 h, postbiotics present in CFCS<sup>wt</sup> (decreased the abundance to 29.44%) and CFCS<sup>wt+PF</sup> (increased the abundance to 39.90%) exhibited statistically significant ( $p < 0.05$ ) opposing effect on the abundance of Bacteroidetes. A suppressive pattern ( $p > 0.05$ ) on the abundance of Firmicutes in presence of probiotics and synbiotics were observed at both 24 h and 48 h of incubation. On the other hand, a significantly ( $p < 0.05$ ) stimulatory effect was observed from postbiotics on the abundance of Firmicutes as compared to O-rf and MRS broth control as well. More specifically, at 48 h the proportion of Firmicutes was 58.15%, 72.11%, and 77.30% in presence of CFCS<sup>wt</sup>, CFCS<sup>wt+PF</sup>, and CFCS<sup>CLA</sup>, respectively. Among them effects of CFCS<sup>wt+PF</sup> and CFCS<sup>CLA</sup> was statistically significant ( $p < 0.05$ ) while CFCS<sup>wt</sup> was not, as compared to MRS broth control.

A critical observation was made on the proportion of Proteobacteria by comparing the difference of their abundance which was correlated to the variation of treatments. The addition of probiotic (either LC<sub>wt</sub> or LC<sub>CLA</sub>) could not restrain nor encourage the abundance of Proteobacteria significantly ( $p > 0.05$ ) as compared to O-rf at both time points. However, the presence of synbiotic (LC<sub>wt+PF</sub>) increased the abundance of Proteobacteria to 40.44% (pH 7.5) and 52.96% (pH 7.6) at 24 h and 48 h, respectively (Fig. 2B-C). On the contrary, postbiotics (i.e., CFCS<sup>wt</sup>, CFCS<sup>wt+PF</sup>, and CFCS<sup>CLA</sup>) as well as the MRS broth significantly ( $p < 0.05$ ) underpinned the proportion of Proteobacteria (Fig. 2B-C) throughout the study which was comparable to the abundance of Proteobacteria at 0 h time point. Among other major phyla, the abundance of Tenericutes was increased ( $p < 0.05$ ) at 24 h of incubation in the presence of postbiotics whereas it was declined ( $p > 0.05$ ) at 48 h as compared to the MRS broth treated control. In the other growth conditions (i.e., presence of probiotics or synbiotic) the proportions of this phylum were lower as compared to O-rf at 24 h ( $p > 0.05$ ) and 48 h ( $p < 0.05$ ).

*Multidirectional effects of various treatments on the abundance of Lactobacillus spp., Escherichia spp. and other major genera.* Although two of the bacteria genera, *Lactobacillus* spp. and Shiga-toxin producing pathogenic *E. coli*, were focused on this study; ultimately the applied treatments impacted the abundance of commensal *Escherichia/Shigella* spp. which was included in the representative data due to the understandable limitation of the sequencing technique we used. In this study, the proportion of *Lactobacillus* spp. were observed in the O-rf was 0.36%, 0.2% and 0.03% at 0, 24, and 48 h, respectively; while the abundance of *Escherichia/Shigella* spp. in O-rf was found to be 0.0005%, 0.04%, and 0.02% at the same

respective time points (Fig. 4 A and B). Experimental addition of probiotic strains (either LC<sub>wt</sub> or LC<sub>CLA</sub>), as well as the synbiotic (combination LC<sub>wt</sub> with peanut flour) did not show any significant ( $p > 0.05$ ) impact on the abundance of *Lactobacillus* spp. throughout the study as compared to the control (PBS). The similar abundance tendency was observed for *Escherichia* spp.; however, their abundances were reduced significantly ( $p < 0.05$ ) in the presence of synbiotic (LC<sub>wt+PF</sub>) at 24 h (0.19% reduction as compared to PBS control (Fig. 4 B).

At the same incubation condition, the presence of postbiotics created more favorable environment for the growth of *Lactobacillus* spp. which was demonstrated by their statistically significant ( $p < 0.05$ ) higher abundance at 48 h as compared to MRS broth control. Among the postbiotics, CFCS<sup>wt+PF</sup> had the greatest influence on *Lactobacillus* spp. (abundance 1.26%) while their abundance in presence of CFCS<sup>CLA</sup> and CFCS<sup>wt</sup> were documented as 0.833% and 0.44%, respectively. In terms of the inhibitory property on *Escherichia* spp. the postbiotics were proved to be superior to the probiotics and synbiotics. A continuous statistically significant ( $p < 0.05$ ) inhibition on the abundance of *Escherichia* spp. was found from all postbiotics (Fig. 4) which also correlates with our culture-based observation.

Besides *Lactobacillus* spp. and *Escherichia* spp. a clear distinguishing pattern in the predominant bacterial genera were also observed in presence of probiotic, synbiotic, and their postbiotics (Fig. 3 A-C) at different time points. In the beginning of the treatment (0 h), *Prevotella* was the most predominant genus comprising about one fourth of the rumen bacterial composition (Fig. 3 A). At 24 h other genera were found to be predominant depending on the treatment composition. In presence of the LC<sub>wt</sub>, LC<sub>wt+PF</sub>, and LC<sub>CLA</sub> the predominant genus was found to be *Prevotella*, *Acinetobacter*, *Acrobacter*, and *Comamonas* whereas at 48 h another genus named *Acholeoplasma* became predominant. We observed a relatively different bacterial composition at 24 h in presence of MRS broth and postbiotics collected in MRS broth as compared to the probiotics and synbiotics, which was dominated by *Prevotella*, *Rummeliibacillus*, and *Clostridium*. At 48 h the proportion of *Prevotella* went down whereas *Rummeliibacillus*, *Lysinibacillus* were found to be engrossed (Fig. 3 B-C).

*Comparison of the microbial diversity at species level during various treatments.* To understand the effect of treatments at species level, we analyzed the diversity of bacterial community through calculating the total number of species, alpha and beta indices which was compared with the primary control (i.e., O-rf). Over the incubation period in O-rf, the calculated total number of species were 778, 1203.5, and 1173 at 0, 24, and 48 h, respectively. A numerical difference was documented in presence of the experimentally added substances but none of them were statistically significant. The highest total number of species was documented at 24 h when the RF was supplemented with MRS broth (2135, pH 7.5) (considered as control for the postbiotics) followed by presence of CFCS<sup>wt</sup> (1422.5, pH 5.5); however, they were not statistically significant due to the variation among replicates (Fig. 5 A-C).

Diversity within a single community was analyzed via calculating alpha indices i.e., Shannon (H) and Simpson (D) index. The H and D indices are mathematical measures of species diversity which consider the number of total species present in a community as well as the number of individuals per species. More specifically, the H is an information statistic index whereas D favors the dominant species. The diversity of the RF was statistically ( $p > 0.05$ ) unaffected under the experimental circumstances. However, under specific condition, such as in

the presence of CFCS<sup>wt+PF</sup> or CFCS<sup>CLA</sup>, promoted the species diversity (calculated through Shannon index) of RF significantly at 24 h (Fig. 5 D-F). Whereas at 48 h time point, the presence of synbiotic (LC<sub>wt+PF</sub>) and its postbiotic reduced the Shannon index as compared to the O-rf significantly ( $p < 0.05$ ) (Fig. 5F). A supportive effect toward species richness was also observed via Simpson index where any suppressive effect was not documented. Moreover, a statistically significant stimulation toward diversity was observed in presence of LC<sub>wt+PF</sub> at both 24 h and 48 h of incubation (Fig. 5 G-I). We also calculated Sorensen coefficient to understand the diversity shared between O-rf and other treatments (Fig. 5 J-L). A value closer to 1 indicates the more similarity while a value closer to 0 represents less similarity. A significant ( $p < 0.05$ ) similarity in the bacterial diversity at specie level was observed due to the presence of all treatments throughout the incubation period.

## Discussion

EHEC is one of the notorious members of diarrheagenic *E. coli* (i.e., pathogenic *E. coli*) mainly residing in the intestinal tract of healthy cattle (Forano et al., 2013; Nataro and Kaper, 1998). Besides, other pathogenic *E. coli* strains are also a part of ‘normal flora’ which coexist with the harmless (i.e., commensal) *E. coli*. The commensal *E. coli* are also a part of normal gut flora of warm-blooded animals including humans. The pathogenic *E. coli* including EHEC cause sickness when transmitted to susceptible host body via contaminated foods and drinks (Doyle, 1991). Several serotype of EHEC, i.e., O157:H7 which causes bloody diarrhea, hemorrhagic colitis, and HUS is also frequently reported to be associated with foodborne outbreaks worldwide (Vanaja et al., 2013). Data from both *in vitro* and *in vivo* studies have demonstrated the antagonistic effect of probiotic bacteria, prebiotics, and postbiotic components on various foodborne bacterial pathogens by suppressing or altering their growth and virulence properties via distinct mechanisms (Aditya et al., 2020a; Alvarado-Martinez et al., 2020; Tabashsum et al., 2020). Previously published studies from our laboratory also reported that probiotic strains, specifically *L. casei* can inhibit the growth and alter pathogenic properties of EDL933 and the effects of *L. casei* can be amplified by supplementing prebiotic like components such as peanut and cocoa flour (Aditya et al., 2020a; Peng et al., 2015b). Since majority of these findings were conducted under highly controlled laboratory environment, we aimed to reduce the EDL933 load in a heterogenous ecosystem using natural components. RF collected from dairy cattle, inoculating with probiotics as well as supplementing postbiotics. Therefore, the survival of EDL933 was assessed by objectively exposing them to different probiotics, synbiotic, and postbiotics in collected RFs which was considered as a representative of cattle rumen.

In agreement with the previous *in vitro* findings (Aditya et al., 2020a; Peng et al., 2015c, 2018), these simulated rumen-based cultural approach indicated that addition of postbiotics or metabolites present in CFCSs (i.e., CFCS<sup>wt</sup>, CFCS<sup>wt+PF</sup>, and CFCS<sup>CLA</sup>) as well as MRS broth (a commercially available microbiological medium designed to support the growth of *Lactobacillus* spp.) could inhibit growth and survival of EDL933 better than the probiotics itself (LC<sub>wt</sub> and LC<sub>CLA</sub>) and synbiotic combination of probiotic and peanut flour (LC<sub>wt+PF</sub>) which favors the growth of *L. casei* (Fig. 1 A-E and a-e). Usually, the natural environment/ecosystems in which all microorganisms, including the pathogens, grow, multiply and communicate with each other via different types of interactions such as mutualism, synergism, commensalism, parasitism, amensalism, competition, etc. (Tshikantwa et al., 2018). This complex web of interaction creates

numerous microenvironments with nuances in temperature, oxygen availability, pH, redox potential, availability of critical nutrients and presence of microbial inhibitory components which allows the dynamic microbiotic shift reflecting inhibition, survival, and stimulation of microbial communities (Chen et al., 2020).

In this study, a rapid statistically significant ( $p < 0.05$ ) growth suppressive effect of CFCS<sup>wt+PF</sup> (as compared to MRS broth) against EDL933 was documented after 12 h of treatment. Our findings also demonstrated that the postbiotics in CFCS<sup>wt</sup> and CFCS<sup>CLA</sup> effectively controlled the growth of EDL933 where CFCS<sup>CLA</sup> was able to inhibit EDL933 completely ( $p < 0.05$ ) at 72 h of incubation. In contrast, the direct application of probiotics along with prebiotic component failed to demonstrate the significant inhibitory effect on EDL933. Since pH is one of the primary determinants of the microbial existence and multiplication (Jin and Kirk, 2018), therefore pH at each time points of treatments was also measured. We found that the pH of RF due to the addition of the postbiotics shifted from moderately acidic to slightly basic range with the progression of the incubation which does not seem to be the only reason of the observed superior antagonism against EDL933 (Aditya et al., 2022)

Supplementing ruminant feed with plant-based bioactive components and/or probiotics has many established benefits toward animal health and productivity including improved immune response, better fiber degradation, fermentation, maximum nutrient utilization, better growth, and milk production, etc. (Arowolo and He, 2018). In addition, it helps to maintain a balanced microbiome in rumen which limits the proliferation of many pathogenic microbes in rumen (Pachepsky et al., 2006; Arowolo and He, 2018). To measure the effects of the used treatments on the rumen microbiome, we also performed metagenomic analysis, since culture-based technique can not truly represent the slow-growing or viable but non culturable (VBNC) bacteria. In agreement with previously published studies, we also found that most predominant phyla of bovine rumen were Bacteroidetes, Firmicutes, and Proteobacteria (Bi et al., 2018). A balanced proportion Bacteroidetes and Firmicutes is important for gut homeostasis (Stojanov et al., 2020). In general, Bacteroidetes helps to maintain a healthy gut by digesting polymeric organic matter (Kim and Milner, 2007), whereas the Firmicutes harbor many probiotic bacterial genera (Thomas et al., 2011). On the contrary, Proteobacteria contains majority of gram-negative foodborne bacterial pathogens (Méndez-Salazar et al., 2018). An imbalance in the intestinal microbiota is usually caused by increased abundance of Proteobacteria. In the current study, an increase in the abundance of Proteobacteria was documented when the probiotics (LC<sub>wt</sub> and LC<sub>CLA</sub>) and/or prebiotic (LC<sub>wt+PF</sub>) were added whereas the postbiotics suppressed its abundance very efficiently. The most efficient suppression was observed from CFCS<sup>wt+PF</sup> and CFCS<sup>CLA</sup> at 48 h which is relatable to the presence of modified metabolic components of wild type *L. casei* and the presence of conjugated linoleic acid (CLA).

At genus level, presence of probiotics, synbiotics, and postbiotics had different impact in the abundance of different genera. For example, in presence of CLA-rich metabolites *Prevotella*, *Anaeroplasm*, *Lactobacillus*, *Treponema*, and *Alistipes* were the predominant phyla. However, when probiotic bacteria (LC<sub>wt</sub>, LC<sub>wt+PF</sub>, and LC<sub>CLA</sub>) were directly applied, probably due to the difficulties in colonization a different set of genera bloomed such as *Prevotella*, *Fibrobacter*, *Acinetobacter*, and *Solibacillus*. The abundance of other genus was not affected by the compositional variation of the treatments, such as *Tangfeifania*. The metagenomic analysis indicated an increase in the genus *Lactobacillus* and a suppression in *Escherichia/Shigella* spp.

Modulation of microbiome would be possible in any complex environment (e.g., soil, GI tract, rumen, etc.) if the designed or targeted microbes could overcome the physical (e.g.,

temperature, air composition), chemical (e.g., pH, nutrient composition), and biological (i.e., competition with other microbial flora) constraints using appropriate nutrients (Pandey et al., 2015; Weimer, 2015). On the other hand, when postbiotics were applied; the direct competition was avoided which was reflected in preferable and achievable outcome. This observation of our study indicates that promoting the probiotic (i.e., beneficial) microbial community of a complex environment can effectively control the pathogenic flora of the ecosystem. We also investigated the variety of the bacterial community by calculating the total number of species, alpha and beta indices, and comparing them to the primary control to understand the effect of treatments at the species level (i.e., O-rf). Under most experimental conditions, the diversity of the rumen fluid was statistically unaffected ( $p > 0.05$ ). However, under some conditions, such as the addition of CFCS<sup>wt+PF</sup> and CFCS<sup>CLA</sup>, the species diversity of RFs at 24 hours was greatly increased (as measured by the Shannon index). The Shannon index of O-rf was dramatically lowered when the synbiotic (LC<sub>wt+PF</sub>) and its collected metabolites were present for 48 hours. In the absence of any suppressive effect, a supporting effect toward species richness was also confirmed using the Simpson index and Sorenson coefficient.

Our study indicates that postbiotics which contain numerous probiotic-derived metabolites such as organic acids (e.g., lactic, acetic, formic acids, linoleic acid), peptides or proteins (e.g., nisin and numerous bacteriocin derivatives), unsaturated fatty acids (including CLA), flavonoids, catechol, hydrogen peroxide, etc. (Aditya et al., 2020a; Peng et al., 2015a); can retain their concerted differential effects on stimulating growth of beneficial microbes as well as limiting certain or targeted pathogenic bacteria in the complex ecosystem. Application of these postbiotics as feed supplement can play a potential role reducing antibiotic usage in farm animal production and improving safety of animal products. Unnecessary and improper administration of antibiotics to cattle e.g., doxycycline, tetracyclines, macrolides, etc. for growth supplements or treating bacterial infection may lead to events such as anorexia, diarrhea, belching, regurgitation, and even death (Chen et al., 2020). Alternative strategies for the similar outcome can be exploited to suppress the overall prevalence of pathogenic flora specifically EHEC in the reservoir. Although our study demonstrates promising strength of postbiotics to be used to control EDL933 in rumen ecosystem; there are few limitations which should be considered. Here, we did not use any technique to support the growth of strict anaerobic bacteria of RFs. Besides, the influence of dietary variation on the abundance of rumen microbiome was passed over. To make a concrete conclusion, *in vivo* studies are needed.

## Conclusion

The composition of microbial community can be manipulated *in situ* by directly supplementing the diet with postbiotics derived from *L. casei* under different growth conditions (i.e., CFCS<sup>wt</sup>, CFCS<sup>wt+PF</sup>, and CFCS<sup>CLA</sup>) which is restrictive in allowing its utilization; hence it supports a specific subset of microbial growth (i.e., Bacteroidetes and Firmicutes) while suppressing others (Proteobacteria in general). The mixture of different postbiotics when applied in optimum concentration can be a potential alternative of antibiotic growth promoters and minimize the pathogen load at preharvest/farm level.

List of figures, captions, and legends

Figure 1.

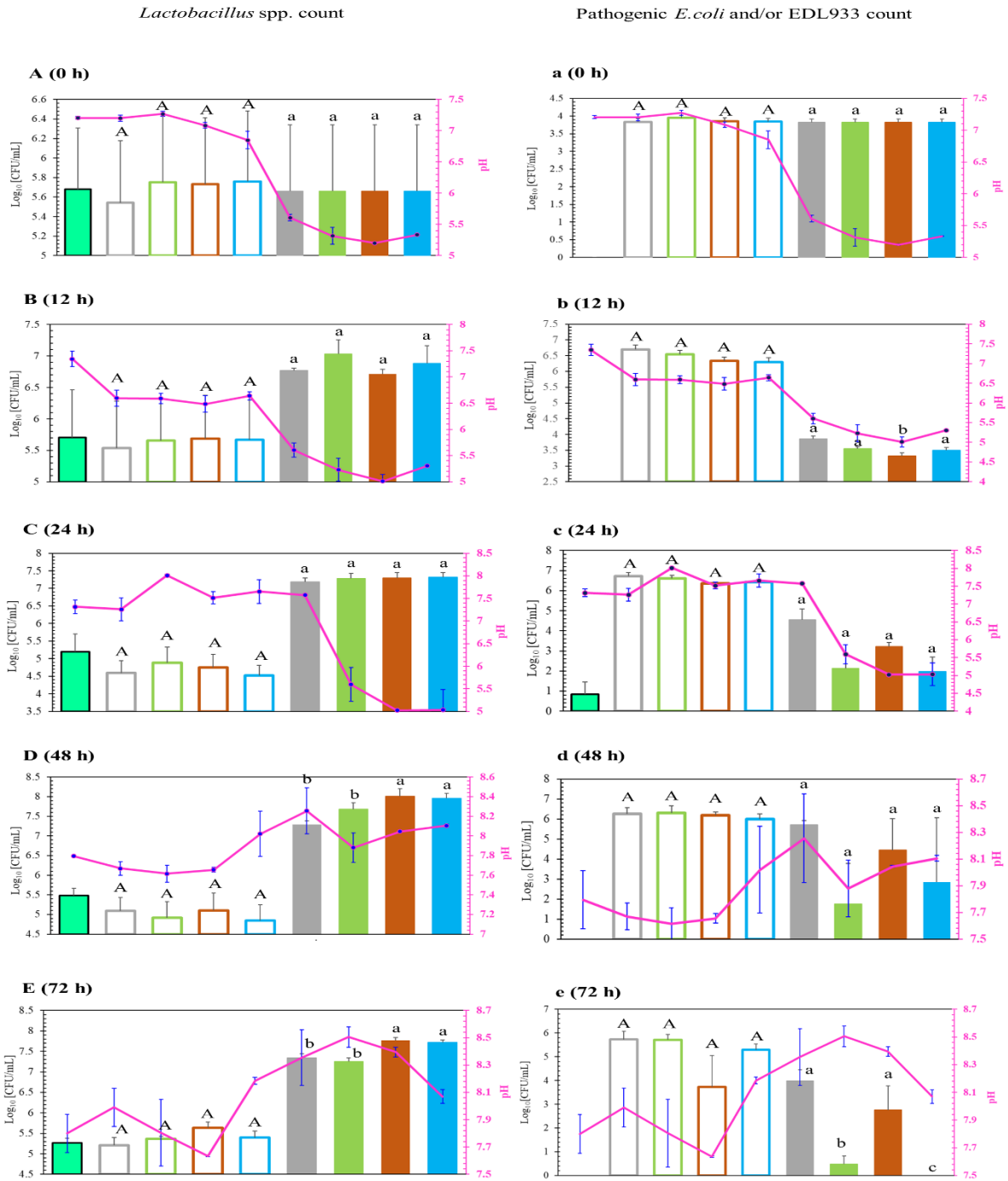


Figure 1. Effect of the presence of probiotics alone (either LC<sub>wt</sub> or LC<sub>CLA</sub>), synbiotic combination of LC<sub>wt</sub> and peanut flour (0.5%) (LC<sub>wt+PF</sub>), and their total metabolites containing postbiotics (CFCS<sup>wt</sup>, CFCS<sup>wt+PF</sup>, or CFCS<sup>CLA</sup>) in RFs to promote *Lactobacillus* spp. while exerting antagonism against pathogenic *E. coli* (i.e., EDL933) over 72 h. The left panel of the figure (A, B, C, D, and E) is showing the total count of *Lactobacillus* spp. at 0, 12, 24, 48, and 72 h, respectively. The right panel (a, b, c, d, and e) is showing survived pathogenic *E. coli* numbers at 0, 12, 24, 48, and 72 h, respectively. The bars represent the average  $\pm$  standard error among replicates and different letters (uppercase or lowercase) indicates statistical significance ( $p < 0.05$ ) among the treatments.

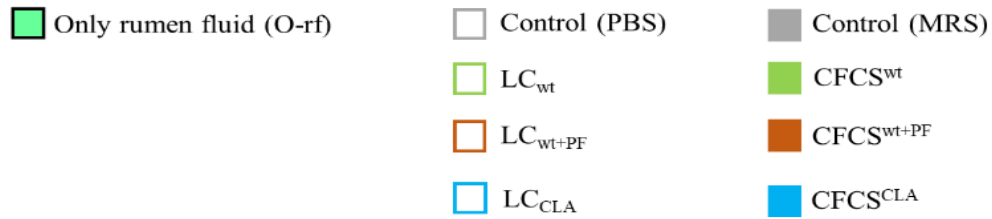


Figure 2.

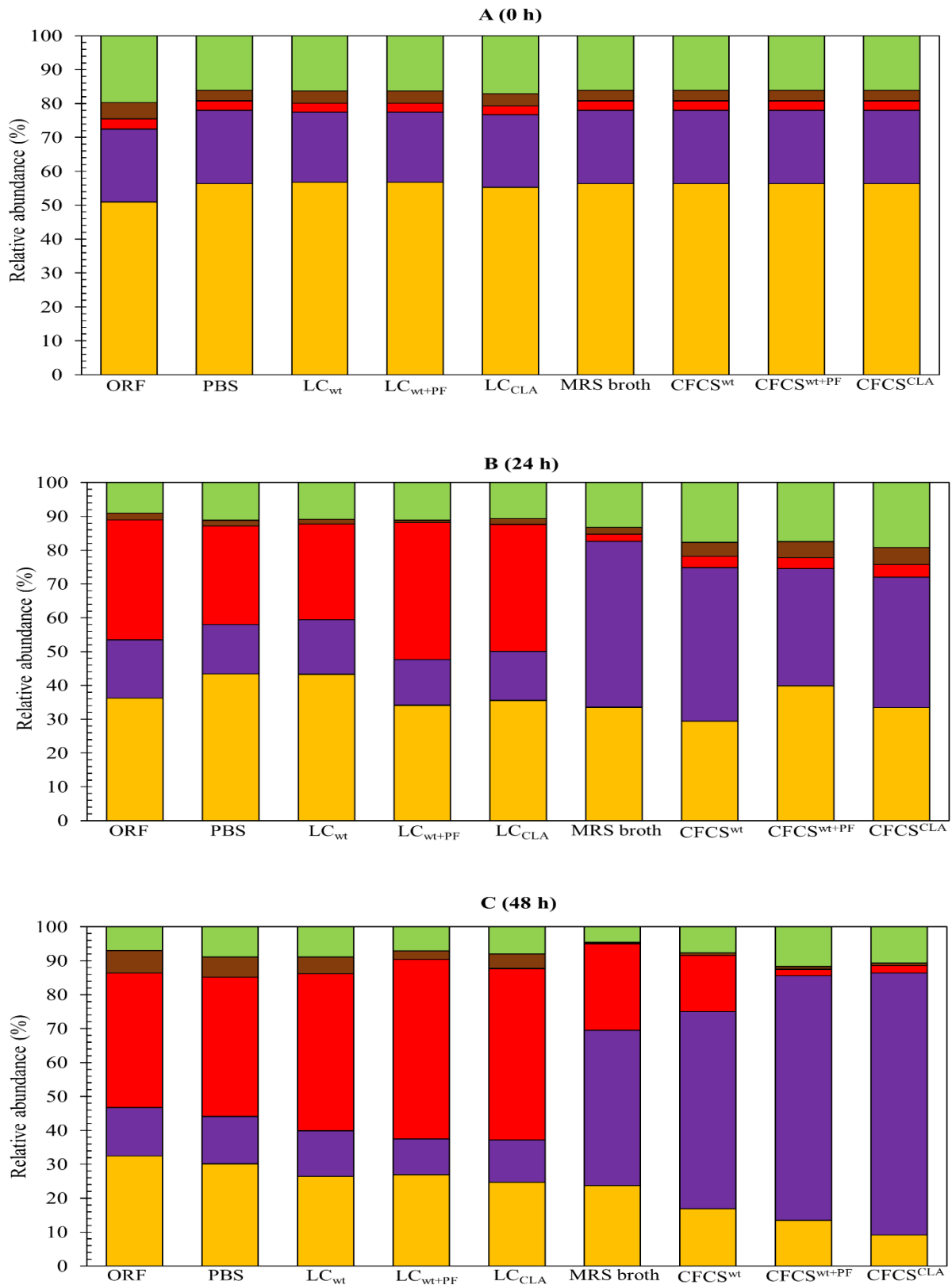


Figure 2. Relative abundance of sequences representing the rumen microbiota at phylum level at 0 h (A), 24 h (B), and 48 h (C).

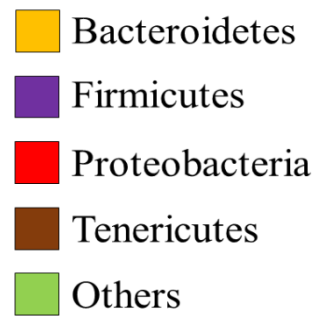


Figure 3.

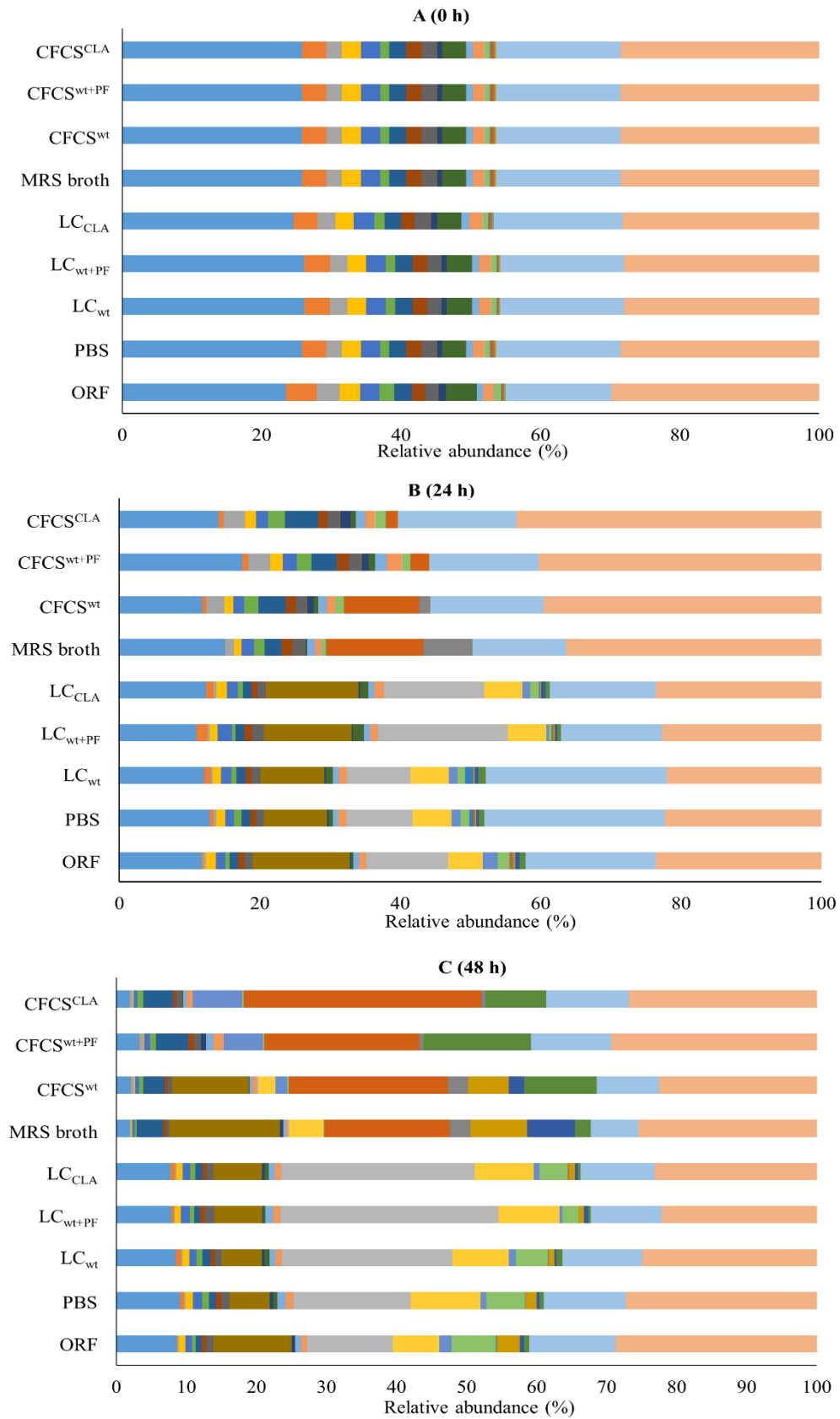


Figure 3. Comparison of bacterial composition of rumen fluids at genus level collected at 0 h (A) 24 h (B), and 48 h (C) in presence of various treatments.

- |                               |                           |                         |
|-------------------------------|---------------------------|-------------------------|
| ■ <i>Prevotella</i>           | ■ <i>Fibrobacter</i>      | ■ <i>Anaeroplasma</i>   |
| ■ <i>Paraprevotella</i>       | ■ <i>Dysgonomonas</i>     | ■ <i>Treponema</i>      |
| ■ <i>Clostridium</i>          | ■ <i>Alistipes</i>        | ■ <i>Tangfeifania</i>   |
| ■ <i>Acinetobacter</i>        | ■ <i>Erysipelothrix</i>   | ■ <i>Fibrobacter</i>    |
| ■ <i>Falsiporphyromonas</i>   | ■ <i>Succiniclasicum</i>  | ■ <i>Arcobacter</i>     |
| ■ <i>Comamonas</i>            | ■ <i>Solibacillus</i>     | ■ <i>Acholeplasma</i>   |
| ■ <i>Emticicia</i>            | ■ <i>Rummeliibacillus</i> | ■ <i>Kurthia</i>        |
| ■ <i>Sphingobacterium</i>     | ■ <i>Chryseobacterium</i> | ■ <i>Lysinibacillus</i> |
| ■ Unclassified at genus level | ■ Others                  |                         |

Figure 4.

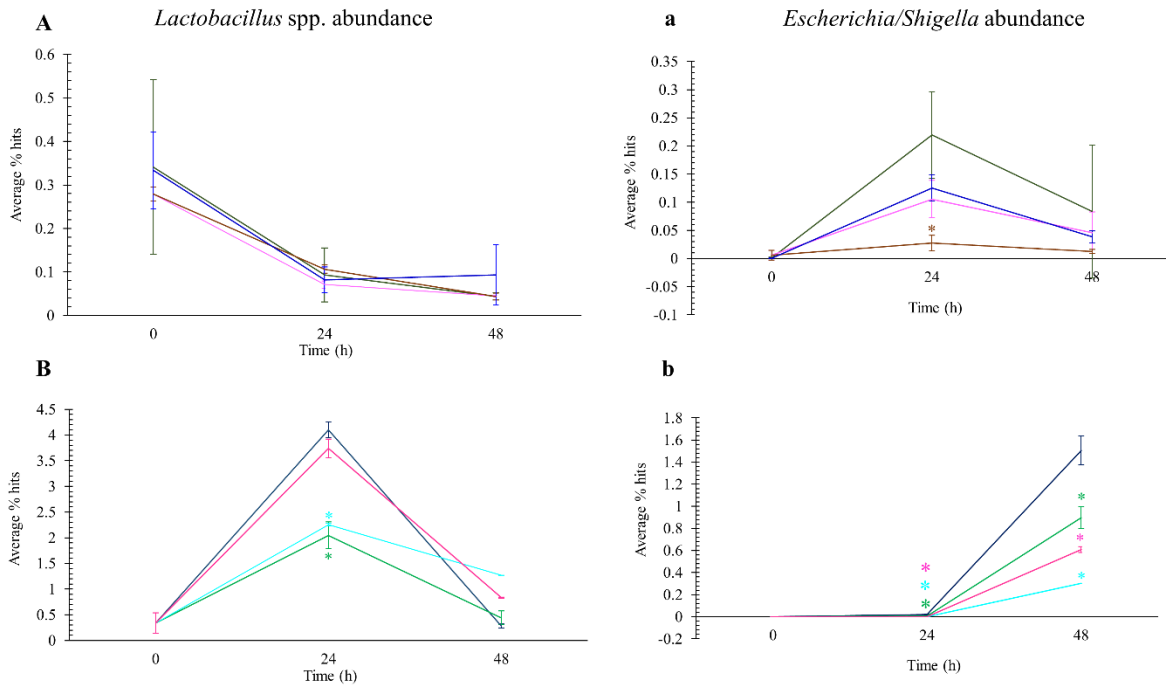


Figure 4. Comparison of *Escherichia* spp. and *Lactobacillus* spp. abundance in presence of different treatments throughout 48 h time. Asterisks (\*) indicate statistically significant ( $p < 0.05$ ) difference between the control and treatment.

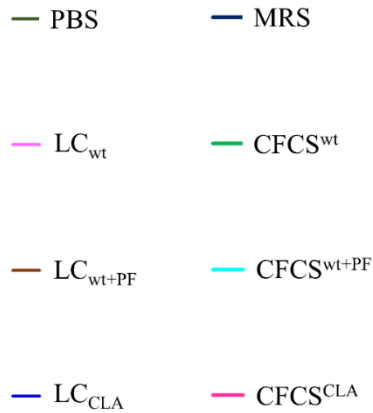


Figure 5.

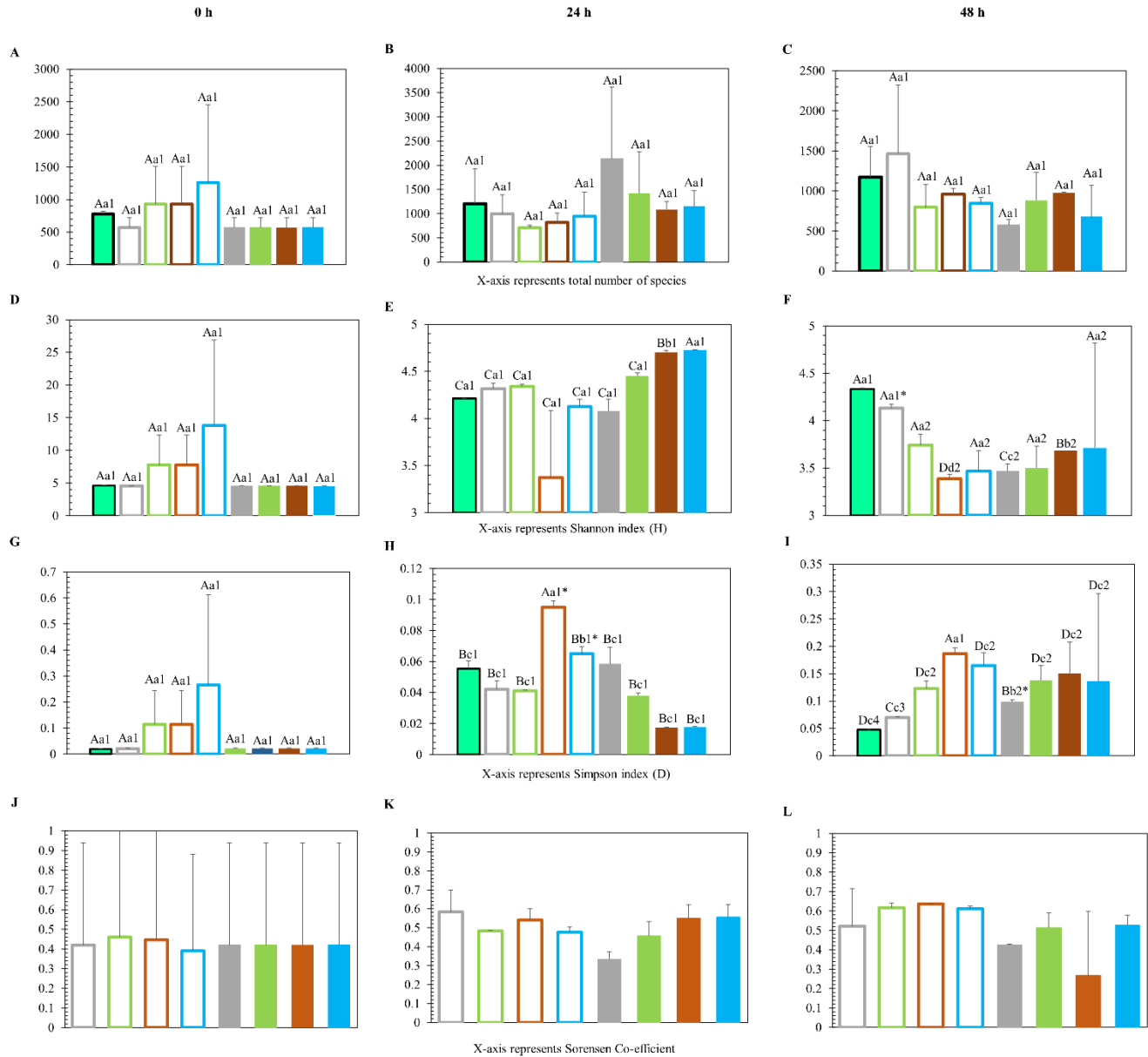
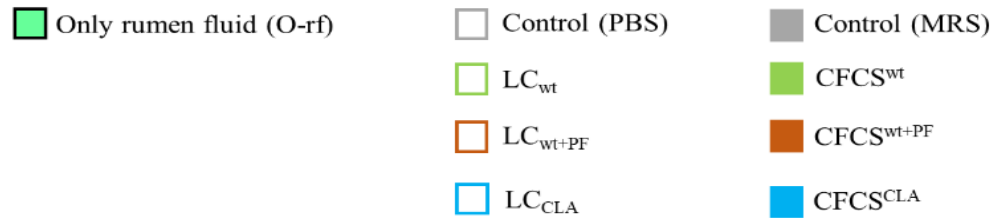


Figure 5. Comparison of the rumen microbiome in presence of different treatments at species level. Bacterial diversity at species level were compared in total number of species (A, B, C) Shannon index (D, E, F), Simpson index (G, H, I) and Sorensen coefficient (J, K, L). The bars

represent the average  $\pm$  standard deviation among replicates; different letters (uppercase or lowercase) indicate statistical significance ( $p < 0.05$ ) among the treatments.



## Chapter 4: Identify effective concentration of specific bioactive isomers of linoleic acid in metabolites produced by *L. casei*

### Introduction

The application of microorganisms to food and beverage products is traced back to 2000 BC when humans grasped the use of microbial powers to improve the flavor and shelf life of foods, even without fully understanding their mechanism of actions (Ozen and Dinleyici, 2015). With the development of advanced microbiological and molecular techniques, researchers have demonstrated the beneficial or detrimental roles of various microorganisms, and now people have become familiar with the use of microbes in food production (Kechagia et al., 2013). Due to the established health benefits of probiotics, the consumption of beneficial microbes through fermented foods and dietary supplements contributes to improving public health and host immunity (Aditya et al., 2020b).

In general, probiotics are recognized to produce various bioactive metabolites with different functionalities which play critical role in improving host health (Aditya et al., 2020b; Peng et al., 2018). Those extracellular bioactive molecules and metabolites are generated with the normal growth and physiological processes of probiotics, while their quality as well as bio-functions are influenced by a variety of factors, such as the type of substrate (prebiotics or prebiotic-like food components), probiotic species, population density, their growth phase, and kinetics (Delgado et al., 2007; Jones et al., 2008). Peanuts are one of the most nutrient dense prebiotic-like food which contain fibers, vitamins, minerals, electrolytes, bioactive compounds such as resveratrol, polyphenols, omega-6 fatty acids (such as linoleic acid, LA), etc. Nutritional richness of peanuts make it a sustainable source in achieving health benefits (Arya et al., 2016; Brenna et al., 2015). Majority of the probiotic-originated functional metabolites are polypeptides and fatty acids in nature. Among the fatty acids, LA (C18:2) and its positional and geometric isomers known as conjugated linoleic acids (CLAs) play a crucial role on host by exerting anti-inflammatory, and anti-carcinogenic potential by itself or in concert with other metabolic by-products. Besides the animal sources, these LA and CLA can also be obtained from microbes which are also normal flora of mammalian intestine. Examples of such microorganisms include *Lactobacillus*, *Bifidobacterium*, *Lactococcus*, etc. Among them, different species of *Lactobacillus* specifically *L. acidophilus*, *L. plantarum*, and *L. rhamnosus* are recognized as CLA producers where *L. rhamnosus* has the highest CLA conversion ratio among all *Lactobacillus* species (O'Shea et al., 2012; Serini et al., 2009). Moreover, *Lactobacillus* spp. mediated fermentation process often produce D-lactic acid, acetoin, formic acid, succinic acid, malic acid, ethanol, diacetyl, etc. (Díaz-Muñiz et al., 2006). They can also utilize prebiotic food components and convert to bioactive metabolites such as flavonoids, glycosyl compounds, indole, CLAs, peptides such as bacteriocins, etc. (Broberg et al., 2007; Cleveland et al., 2001; Peng et al., 2015b; Schnürer and Magnusson, 2005). Altogether these metabolites offer an extended host health benefit.

Further, an optimum proportion of probiotics in the gut microbiota and their bioactive by-products suppress the colonization of endogenous and exogenous pathogens which alleviate antibiotic associated diarrhea and travellers' diarrhea, respectively (Parvez et al., 2006). It also helps to improve chronic digestive disorders such as, irritable bowel disease (IBD), celiac

disease, Crohn's disease, ulcerative colitis (UC), etc. (Aditya et al., 2020b). Besides, these probiotic-derived metabolites have also been shown to possess inhibitory activity towards the multiplication of many common enteropathogens, though the specific mechanism is still not fully elucidated (Charernjiratragul et al., 2010; Jones et al., 2008; Mezaini et al., 2009; Ren et al., 2018). Previous studies of our lab have demonstrated that *L. casei* has relatively robust antimicrobial effect on gut pathogens including enterohemorrhagic *Escherichia coli* (EHEC) serotype O157: H7 than *L. rhamnosus*. To obtain higher conversion of LA to CLAs and a robust anti-microbial effect at the same time, our lab generated a *L. casei* mutant which contains myosin-cross-reactive antigen (*mcra*) gene from *L. rhamnosus* GG that encodes linoleate isomerase. This genetic modification enabled *L. casei* to overexpress CLAs (121-fold more than the wild type) while retaining their intense antimicrobial potential (Peng et al., 2018). We are broadly interested in exploring the anti-microbial activity of CLAs and its mechanism of actions against common foodborne pathogens specifically EHEC. This specific serotype is noteworthy for its ability to produce shiga toxin which may eventually lead to the development of hemolytic uremic syndrome (HUS) in significant portion of infected people. HUS is a major cause of acute renal failure in young children in Europe and Americas. Evidence-based strategies can prevent a significant percentage of EHEC infection, which is very practical because once an infection has been established, there are no therapies available to minimize the risk of developing HUS (Pennington, 2010).

In this study, we aimed to identify different linoleic acid isomers or CLAs in the cell-free cultural supernatant (CFCS) collected from *Lactobacillus* strains under different cultural conditions and their crucial role in stimulating the antimicrobial potential of CFCS against foodborne EHEC.

### Materials and methods

**Bacterial strains and cultural conditions.** Two probiotic strains, including a wild-type *Lactobacillus casei* (LC<sub>wt</sub>) (ATCC334) and a genetically engineered linoleate isomerase overexpressing *L. casei* (LC<sub>CLA</sub>) previously developed and reported by our research group (Peng et al., 2018, 2020b), were grown on de Man-Rogosa-Sharpe (MRS) agar or broth (Merck KGaA, Darmstadt, Germany) at 37°C under aerobic condition in a CO<sub>2</sub> incubator (Thermo Fisher Scientific Inc., Waltham, MA, USA). Shiga toxin-producing enterohemorrhagic *Escherichia coli* O157:H7 EDL933 (EDL933) (ATCC700927) was used as a representative of foodborne pathogens. EDL933 was grown at 37°C overnight on Luria-Bertani (LB) agar or broth (Becton, Dickinson and Co., Sparks, MD, USA) under aerobic conditions (Thermo Fisher Scientific Inc., Waltham, MA, USA).

**Chemicals.** The chemicals used in this study include cis-9, trans-11 CLA (Sigma-Aldrich; product no. 16413), linoleic acid methyl ester cis/trans isomers, and heptadecanoic acid was purchased from Sigma (Sigma-Aldrich; product no. L8404 and H3500, respectively). Other chemicals used as solvent such as dimethyl sulfoxide (DMSO) (BDH, Cat. No. BDH1115-4LP), glacial acetic acid (Fisher Chemical; CAS 64197), chloroform (BDH; Cat. No. BDH1109-4LG), methanol (Fisher Scientific; CAS 67561), hexane (Sigma-Aldrich; product no. 34859-1L) were purchased commercially.

*In vitro* assessment of probiotic growth and analysis of metabolites at different growth conditions. In this study, we prepared whole peanut white kernel flour (PF) as previously described by our research group (Aditya et al., 2020a) and evaluated their effect on probiotic growth by supplementing 0.5% (w/v) PF as prebiotic-like dietary component. Approximately 6 log CFU/mL of LC<sub>wt</sub> (in absence and presence of PF; labeled as LC<sub>wt</sub> and LC<sub>wt+PF</sub>, respectively) and LC<sub>CLA</sub> (I8) were inoculated in MRS broth to evaluate their growth at 24, 48, and 72 h. Based on their growth phase, total cell number, and cultural conditions; the CFCSs were collected separately after 24 h and 48 h of incubation following the method previously described by Peng *et al.* (Peng et al., 2015a, 2015c, 2015b). *L. casei* reaches stationary phase after 24 h of incubation and continue till 48 h of incubation in our laboratory culture conditions. Therefore, we considered to collect the metabolites containing CFCSs before the culture reached to late stationary phase or death phase to avoid the were accumulated cellular waste products. We did not collect CFCS from 72 h (Andrianto et al., 2018). The CFCSs collected from LC<sub>wt</sub> in only MRS broth, LC<sub>wt</sub> in MRS broth supplemented with 0.5% (w/v) PF and LC<sub>CLA</sub> in only MRS broth were referred to as CFCS<sup>wt</sup>, CFCS<sup>wt+PF</sup>, and CFCS<sup>CLA</sup>, respectively. The pH of the collected CFCSs at 24 h and 48 h time points were measured (Mettler Toledo, Columbus, OH, USA) and preserved at 4°C for further use.

Comparative study of the antimicrobial effect of CFCSs collected at different time points. We assessed the antimicrobial potential of *L. casei*-originated total metabolites present in the collected CFCSs (mentioned above) against EDL933 *in vitro* primarily considering their growth inhibition potential. We cultured EDL933 in LB broth with an inoculum of ~3.5 log CFU/mL (the infectious dose of EHEC is 10-100 colony forming units (CFU) (Rahal et al., 2012)) in presence of 25% (v/v) MRS broth (considered as control) and CFCSs collected at different time points and growth conditions (*i.e.*, 24-h and 48-h CFCS<sup>wt</sup>, CFCS<sup>wt+PF</sup>, and CFCS<sup>CLA</sup>). A portion of the untreated and treated samples were retained at 0, 4, 8, 12, 24, and 48 h of incubation which were plated on LB agar after serial dilution (original to 10<sup>-5</sup> dilution) following the methods described previously (Aditya et al., 2019; Peng et al., 2015c; Salaheen et al., 2014). The viable pathogens survived during various treatments were enumerated. Besides counting viable pathogens, the change in pH of the control and treatments at all above mentioned time points was also monitored.

Inhibition of EDL933 growth with commercially available linoleic acid isomers (CLA). To understand the effect purified CLA against EDL933 growth, we added commercially purchased CLA mixture (a mixture of t9 t12, c9 t12, t9 c12, and c9 c12 isomers), a 20 mM stock solution of CLAs was prepared by dissolving the isomers in dimethyl sulfoxide, DMSO) and adjusted to the targeted concentration with CFCS<sup>wt</sup> (24-h and 48-h) at two different concentrations, 128 µM and 64 µM following the methods described by Withey et al (2015) (Withey et al., 2015). Same volume of DMSO (32 µL and 16 µL respectively) was mixed with CFCS<sup>wt</sup> to evaluate the effects of DMSO in this part of experiment. We followed the same procedure as described in the previous section to see the growth inhibition of EDL933 with an initial concentration of ~3.5 log CFU/mL of the pathogen. Every sample was compared with the effect of either 24-h or 48-h CFCS<sup>wt</sup>.

*RT-qPCR for virulence gene expressions of EDL933.* The antimicrobial effect of the collected metabolites (24-h and 48-h) against EDL933 were also evaluated through observing the effects on the expression of their major virulence genes. RNA was extracted after treating EDL933 with different CFCSs separately for 4 h. Complementary DNA (cDNA) was synthesized followed by the qPCR analysis for particular virulence genes including *eaeA*, *tir*, *espA*, *espB*, and *espD* of EDL933, as has been described previously (Aditya et al., 2019; Peng et al., 2015c, 2018). Comparative log fold-change of EDL933 key virulence genes was calculated. The cycle threshold ( $C_t$ ) value of the target virulence genes in the presence of CFCSs was normalized to the reference gene (Aditya et al., 2019) (listed in supplementary table 1) with treatment. The relative expression of the genes was compared between respective treated and untreated conditions (Livak and Schmittgen, 2001).

*Gas chromatography-mass spectrometry (GC-MS).* The conjugated linoleic acids (CLAs) in CFCSs collected at 48 h, since a more efficient growth inhibition of EDL933 from 48 h culture of *L. casei* was observed, were detected and quantified by GC-MS according to the methodology described by Politz *et al.* (2013) (Politz et al., 2013).

Briefly, the lipids (i.e., extracellular fatty acids) were extracted from 5 mL of each CFCSs. As a control, fatty acids extracted from same volume of MRS broth were used. 12.5  $\mu$ L of 10 mg/mL heptadecanoic acid (10 mg of heptadecanoic acid was dissolved in 1 mL of absolute ethanol to prepare the solution) was introduced to the CFCSs as an internal control. Under a fume hood, 100  $\mu$ L glacial acetic acid (CAS 64197) was added to the solution, which was then vortexed to mix it. Then, 5 mL of a 3:1 (v/v) solution of chloroform (Cat. No. BDH1109-4LG) and methanol (CAS 67561) was added and vortexed vigorously, followed by 10 min centrifugation at 1000 $\times$ g. A vacuum aspirator (Welch, Monroe, LA, USA) was used to remove the aqueous layer and the interface holding the debris. The chloroform extract was then dried in a SpeedVac Vacuum Concentrator (Thermo Fisher Scientific Inc., Waltham, MA, USA), yielding a dry residue in the tubes. In a tight cap tube, 1 mL of anhydrous 1.25 M HCl in methanol (to prepare the working solution 26 mL of HCl was dissolved in 224 mL of methanol) was added and heat-treated at 50°C overnight in a tight cap tube.

After the heat incubation, the tubes were cooled to room temperature, and 1 mL of gas chromatography grade hexane (product no. 34859-1L) was added rapidly to the tubes inside a fume hood. To quench the acid-catalyzed reaction, 5 mL of 100 mg/mL NaHCO<sub>3</sub> aqueous solution was added and mixed by vortexing. To generate a stable interface, the mixture was centrifuged at 1000 $\times$ g for 10 min. About 400  $\mu$ L of the hexane layer (containing the extracted fatty acids) was collected in a gas chromatographic vial which was analyzed by GC-MS. The gas chromatography measurements were performed on an Agilent 6890N system coupled with a JEOL high-resolution magnetic sector mass spectrometer (JMS-700 MStation) with the EI ion source (70 eV). The mass spectrometer was operated in the mode of high scan speed and low resolution (1000) with a mass range from 50 to 400 Daltons. A silica capillary column (Agilent HP-5MS, 30 m length, 250  $\mu$ m I.D.) was used with helium (at 1 mL/min) as the carrier gas. Analysis was performed as follows: injection volume was 1  $\mu$ L, the split ratio was 1:10, the inlet temperature was 250°C, the column temperature was programmed from 150°C at 3 min, increased to

200°C at the rate of 25°C/min, then increased to 280°C at the rate of 5°C/min, and held at 280°C for another 4 min.

*Statistical Analysis.* SAS 9.2 software (Cary, NC, USA) was used to determine the statistical significance. One-way analysis of variance (ANOVA) followed by the Dunnett test was applied to determine significant differences of bacterial growth, pH and EDL933 virulence gene expression levels between control and treatment based on a significance level of 0.05.

## Results

*Peanut flour (PF) enhanced the growth of probiotic.* A continuous, statistically significant growth-promoting effect of 0.5% PF on LC<sub>wt</sub> up to 72 h was observed (Fig. 1). After 24 h of incubation, the growth of genetically engineered LC<sub>CLA</sub> was higher than LC<sub>wt</sub> grown in both conditions; MRS broth supplemented with PF (0.48 log CFU/mL higher) and only MRS broth (0.79 log CFU/mL higher). However, in course of incubation at 48 h we documented the highest probiotic growth which was observed in LC<sub>wt</sub> grown in the presence of PF (approx. 10.69 logs). At 48 h and 72 h, we did not find any statistically significant difference in LC<sub>wt+PF</sub> and LC<sub>CLA</sub> growth (Fig. 1). Moreover, increasing the peanut concentration up to 5% did not have any significant stimulatory effect on *L. casei* strain used in this study (data not shown).

*Concerted effect of metabolites presents in CFCSs suppress EDL933 growth.* A substantial qualitative difference was observed between the total extracellular metabolites collected after incubation for 24 h and 48 h. *L. casei* originated metabolites collected after 24 h of incubation present in CFCS<sup>wt</sup> (pH 4.65), CFCS<sup>wt+PF</sup> (pH 4.46), CFCS<sup>CLA</sup> (pH 4.81); as well as 48 h of incubation only from CFCS<sup>wt</sup> (pH 3.77), were found to repress the growth of EDL933 (Fig. 2A and Fig. 2B). However, the metabolites present in 48-h CFCS<sup>wt</sup> showed superior inhibition against EDL933 as compared to 24-h CFCS<sup>wt</sup>, CFCS<sup>wt+PF</sup>, and CFCS<sup>CLA</sup>. In the same experiment metabolites collected from 48 h of incubation from CFCS<sup>wt+PF</sup> (pH 3.48), and CFCS<sup>CLA</sup> (pH 3.75) could eradicate the pathogen EDL933 by 24 h and 48 h of treatments, respectively. The pH value in real time of each treatment (with 24-h and 48-h CFCSs) was found to be at different points of acidic pH range (5.83- 4.17) (Fig. 2C and Fig. 2D). No significant differences in pH within a single treatment was recorded throughout the treatment period.

*Isomers of CLA synergistically with other metabolites present in CFCS<sup>wt</sup> exert augmented inhibition.* We added commercially available CLA isomers to CFCS<sup>wt</sup>, collected both 24-h and 48-h and observed an augmented inhibitory activity against EDL933. A complete inhibition of EDL933 growth was observed within 24 h of treatment from the combination of CFCS<sup>wt</sup>, collected after 48 h incubation and 64 µM CLA isomers. At the same time, a higher concentration of CLA isomers (128 µM) in combination of CFCS<sup>wt</sup>, collected after 48 h of incubation, inhibited EDL933 growth but took longer period of treatment (48 h) (Fig. 2B). However, supplementing CLA isomers to the CFCS<sup>wt</sup>, collected after 24 h of incubation, was not able to reduce the EDL933 growth (Fig. 2A).

*Downregulation of EDL933 virulence genes.* We observed that the collected metabolites (both at 24-h and 48-h) downregulated the expression of several key virulence genes of EDL933 which are involved in initiating infection to the host; specifically, those related to motility (*fliC*) and effector proteins related to attaching/effacing (A/E) functions (*eaeA*, *tir*, *espA*, *espB*, and *espD*) (Fig. 4). However, metabolites of probiotics with distinct growth phases and conditions exhibited a different effect on the flagellin synthesis gene (*fliC*). To be specific, 24-h metabolites of CFCS<sup>wt+PF</sup> and CFCS<sup>CLA</sup> induced downregulation of the *fliC* gene, whereas in presence of 24-h CFCS<sup>wt</sup> or 48-h CFCS<sup>wt</sup>, CFCS<sup>wt+PF</sup>, and CFCS<sup>CLA</sup>, *fliC* was upregulated (Fig. 4A and Fig. 4B). We observed downregulation of *eaeA* in the presence of all CFCSs except for 24-h CFCS<sup>wt</sup>; however, the downregulation was more intense by 48-h collected metabolites regardless of the growth condition. Differential effects of CFCSs were observed on other critical genes essential for A/E lesion formation including *espA*, *espB*, and *espD*. All the 48-h CFCSs downregulated *espB*, *espD*, and *tir* but upregulated *espA* (Fig. 4B). Moreover, 48-h CFCS<sup>wt+PF</sup> and 48-h CFCS<sup>wt</sup> induced more intense repressive effects on these genes, in comparison with 48-h CFCS<sup>CLA</sup>. However, 24-h CFCS<sup>CLA</sup> more intensively downregulated these genes (*espA*, *espB*, *espD*, and *tir*) than 24-h CFCS<sup>wt+PF</sup> and 24-h CFCS<sup>wt</sup>.

*Detection of CLA in the collected CFCSs.* In this study, we used commercially available CLAs as standard including t9 c12, t9 t12, c9 t12, and c9 t11, with their RT recorded at 12.29, 12.36, 12.44, and 12.86 min, respectively (Table 1). We were able to identify one isomer (c9 t11) present in CFCS<sup>CLA</sup> based on the RT of c9 t11. We could not identify the other isomers due to the unavailability of standards. However, based on the RT of the specific molecular weight of CLA FAME, we recorded several CLA isomers in CFCS<sup>wt</sup> (12.87, 12.97, and 13.39 min), CFCS<sup>wt+PF</sup> (12.84, 12.96, and 13.37 min), and CFCS<sup>CLA</sup> (12.86, 12.94, and 13.36 min). We also recorded a 115-unit increase of the total amount of CLAs in 48-h CFCS<sup>CLA</sup>, compared to that in 48-h CFCS<sup>wt</sup> (Fig. 5).

## Discussion

Boosting a balanced intestinal microbiota with a healthy diet (particularly prebiotic-like foods such as cocoa, peanut, berry fruits, etc.) that contains bioactive components is becoming one of the popular alternative approaches toward avoiding antibiotics and preventing foodborne illnesses (USDA Agricultural Research Service and Allen, 2017). The normal gut flora including probiotics utilize indigestible components of prebiotic-like foods such as fructooligosaccharides, oligofructose, pectic oligosaccharides, inulin, and many others which improve the host health by providing important proteins/peptides (Aditya et al., 2019; Peng et al., 2015a; Pranckutė et al., 2016; Rurangwa et al., 2009; Salaheen et al., 2014; Tabashsum et al., 2018). In agreement with these statements, we found a statistically significant growth promoting effect of 0.5% whole peanut white kernel flour (PF) toward LC<sub>wt</sub> until 72 h (Aditya et al., 2020a; Peng et al., 2015a). Such upregulated growth of probiotic bacteria depends on various factors including pH of the medium, incubation temperature and duration, initial inoculum size, type of substrate/stimuli and its concentration. All of these factors specifically the presence of prebiotic-like components, such as PF that contains polyphenols, proanthocyanidins and

fibers, were accountable for the production of altered type and/or amount of metabolites which impacted the overall growth inhibitory property on the pathogen (Lamuel-Raventos and Onge, 2017; Ye et al., 2013). In this study, the antagonistic effects of total extracellular metabolites produced by LC<sup>wt</sup> (without peanut), LC<sup>wt+PF</sup> (with peanut), and LC<sup>CLA</sup> (engineered *L. casei* capable of overexpressing linoleate isomerase) in the cell-free cultural supernatant (CFCS) at 24-h and 48-h time points showed effective inhibitory effect against EDL933. We observed a bright-line between the CFCSs collected at 24-h and 48-h of incubation which may grossly linked to the degree of the acidity of the collected metabolites at different growth conditions. The total metabolites collected at 24-h generally exerted bacteriostatic property while 48-h ones were found to be bactericidal. Usually, pH of an environment is important for any microbial growth since it can alter their surface charge and influence the ionization state of organic compounds ultimately regulating the nutrient absorbing pathways of microbes (Ye et al., 2013). Multiple research groups have also reported that the supernatant of probiotics works best in the acidic range of pH (2-6); they also found that with the increase of alkalinity the antagonistic potential of supernatants attenuates and eventually lost at different level of alkalinity (Mirnejad et al., 2013; Peng et al., 2015c; Ren et al., 2018). Interestingly, in our study we did not find any statistically significant difference in the degree of acidity among 24-h CFCSs or 48-h CFCSs. When we compared the pH values between 24-h and 48-h respective CFCSs, we found significantly different pH value only for CFCS<sup>wt</sup>. Our findings align with the fact that acidic pH is one of the responsible factors of the observed antagonistic activity of collected supernatant in this study. We enumerated the number of viable EDL933 cells in presence of collected CFCSs and recorded the real-time pH values of each time points (Fig. 2A, 2B, 2C, and 2D). We did not detect a significant change from the initial pH throughout the treatment time in presence of CFCSs irrespective of their sources. Nevertheless, we did observe complete growth inhibition from 48-h CFCS<sup>wt+PF</sup> and 48-h CFCS<sup>CLA</sup> within 24 and 48 h, respectively. Whereas 24-h CFCS<sup>wt+PF</sup> and 24-h CFCS<sup>CLA</sup> just retarded the pathogen growth. Whereas 48-h CFCS<sup>wt</sup> and 24-h CFCS<sup>wt</sup> had significantly different pH values but neither of the treatment could exert a complete growth inhibition. Interestingly, the pH of the control (contains 25% MRS broth instead of CFCSs) dropped to a range similar to 24-h CFCSs after 12 h (Fig. 2), but any suppressive effect on the EDL933 growth was not documented. Thus, it was unreasonable to harmonize the observed growth inhibitory feature of the collected supernatant exclusively with their acidic pH. Furthermore, Jordan *et al.* (1999) reported that *E. coli* O157: H7 could survive at pH 3 even after 81 h of incubation (Jordan et al., 1999). These findings bolstered our hypothesis that only low pH was not enough to reduce *E. coli* O157: H7. In consistency with available information about the acid tolerance properties of *E. coli* O157: H7 lead us to explore the effect of compositional variation of CFCSs as well as their concentration toward the observed antimicrobial activity.

We hypothesized that isomers of linoleic acid *i.e.*, conjugated linoleic acid (CLA) could be one of the critical metabolites responsible for the observed antimicrobial property of CFCSs against EDL933. We added commercially available CLA (a mixture of t9t12, c9t12, t9c12, and c9c12) to 24-h and 48-h CFCS<sup>wt</sup> and found interesting outcomes. We observed that CLA when present at a lower concentration (e.g., 64  $\mu$ M) with 48-h CFCS<sup>wt</sup> eliminated the EDL933 within 24 h (Fig. 3B). However, a higher concentration (128  $\mu$ M) was less efficient and was able to eliminate EDL933 within 48 h

of treatment. At the same time, addition of CLA at 64  $\mu\text{M}$  and 128  $\mu\text{M}$  concentration to 24-h CFCS<sup>wt</sup> was not found to exert any better suppressive effect on EDL933 growth; rather we found a numerical increase of EDL933 growth as compared to that of 24-h CFCS<sup>wt</sup> (no CLA added) ( $p > 0.05$ ). In a nutshell, we documented an improved inhibition when CLA was present in the 48-h CFCS<sup>wt</sup>, however, when we performed the same experiment with only CLA (not in combination with 24-h or 48-h CFCS<sup>wt</sup>) we did not find any effect on the pathogen growth (data not shown), which also agrees with the findings of other studies (Withey et al., 2015). So, our study implies that the CLA isomers itself are unable to bring any inhibitory effect on EDL933 at the concentrations we tested, but at lower concentrations (such as 64  $\mu\text{M}$ ) synergistically with other compounds they play a crucial role against EDL933. The precise mechanism of the observed antipathogenic activity of linoleic acid isomers is yet to be fully elucidated. Some studies have reported that the long chain fatty acid interfere with bacterial fatty acid synthesis specifically by inhibiting enoyl-acyl carrier protein reductase (FabI) (Zheng et al., 2005).

We noticed different degrees of downregulation of key virulence genes of EDL933 in the presence of CFCSs (both 24-h and 48-h) which might be linked to the variation of the composition of CFCSs. The observed difference in flagellar gene expression and associated motility might be an adaptation response to the metabolites (also their concentration) e.g. CLAs and other fatty acids present in CFCSs (Lackraj et al., 2016). Similarly, most of the genes causing histological lesion in intestinal lining (A/E lesion) were found to be downregulated in the presence of various CFCSs, for instance, *eevA* (encodes intimin which is responsible for intimate attachment to epithelial cells that leads to microvillus effacement) (Donnenberg et al., 1993; Franzin and Sircili, 2015). This observation broadly indicates that metabolic diversity and their concentration are critically responsible for the antagonistic effect on EDL933 growth and virulence gene expression.

Since the 48-h *Lactobacillus* metabolites were more effective against EDL933 than the 24-h ones, we analyzed the presence of CLAs in these CFCS samples by GC-MS, by comparing their retention time (RT) with commercially available standard CLAs (Table 1). However, the commercial unavailability of all 28 purified CLAs to be used as reference was a major limiting factor for this part of the study. Meanwhile, in accordance with Kankaanpää *et al.* (Kankaanpää et al., 2004), we detected the presence of several CLAs in the growth medium of *Lactobacillus* (MRS broth), which was another constraint for the study. It should be noted that the data we obtained cannot confidently demonstrate if the CLA was produced from *Lactobacillus* during growth or simply accumulated from the cultural broth. However, the RT of the CLAs present in the growth media was constant, and we detected several CLA peaks in the CFCSs with different RTs. Moreover, we also found that CLA at a lower concentration (64  $\mu\text{M}$ ) experimentally added augments the inhibitory property of 48-h CFCS<sup>wt</sup>. This led us to agree with the conception of possible CLA bioconversion by linoleate isomerase originated from *Lactobacillus* and their associated antimicrobial property (Banni, 2002; Ogawa et al., 2001). Another limitation of this study was using one reference gene (16 rRNA, a housekeeping gene) to normalize and interpret the level of virulence gene expression. However, the gene expression study was reported from five biological replicates. In this study, expression of

*stx* gene (encodes for shiga toxin) was not evaluated because the expression of critical virulence genes required for A/E lesion formation was considered only.

The findings of this study possess the merit to delve into the mechanistic approaches of CLAs at appropriate concentration and other probiotic-originated metabolites against foodborne pathogens, specifically EHEC. Further, the outcomes of this study might be helpful to prevent EHEC-related foodborne illnesses more efficiently using bioactive probiotics or a combining approach of probiotic and prebiotic-like dietary components.

## List of Tables

Table 1. Study of different linoleic acid isomers based on their retention time by GC-MS. The same lowercase letters are indicating the same retention time of CLA present in different CFCS samples

Retention time of CLA (min)					
CLA standards	MRS broth	MRS broth + PF	CFCS <sup>wt</sup>	CFCS <sup>wt+PF</sup>	CFCS <sup>CLA</sup>
12.29 (t9 c12)	12.84 <sup>a</sup>	12.86 (c9 t11) <sup>c</sup>	12.87	12.84 <sup>a</sup>	12.86 (c9 t11) <sup>c</sup>
12.36 (t9 t12)	12.92	12.94 <sup>d</sup>	12.97	12.96	12.94 <sup>d</sup>
12.44 (c9 t12)	13.16	13.19	13.39	13.37 <sup>e</sup>	13.36 <sup>b</sup>
12.86 (c9 t11) <sup>c</sup>	13.36 <sup>b</sup>	13.37 <sup>e</sup>			

List of figures, legends, and captions

Figure 1.

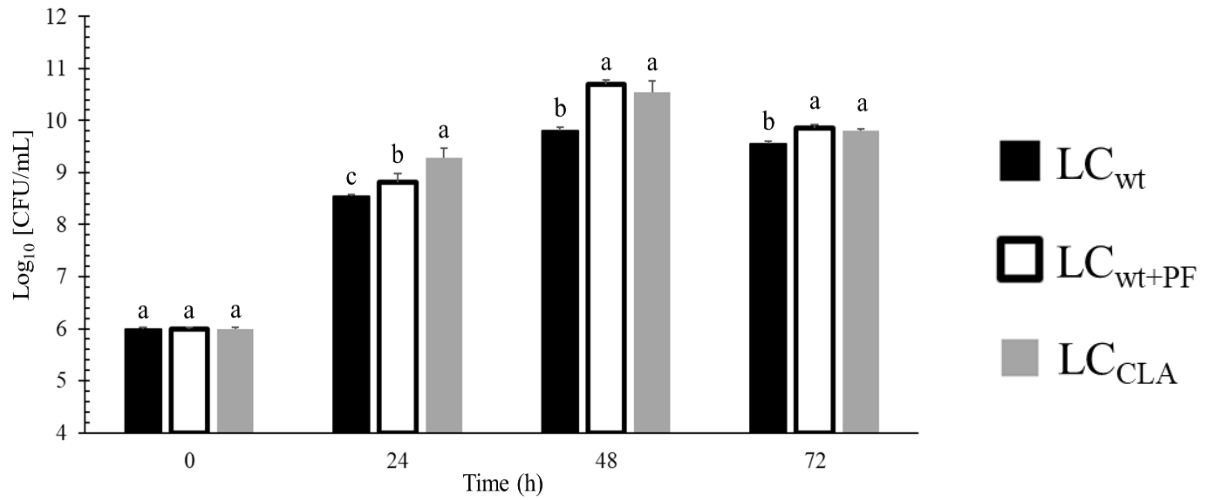


Figure 1. Growth stimulation of wild type *L. casei* ( $LC_{wt}$ ) in presence of 0.5% peanut flour (PF) and genetical modification. The bars represent the average  $\pm$  standard deviation among replicates and different lowercase letters within a time point indicate significant difference between control and treatment ( $p < 0.05$ ).

Figure 2.

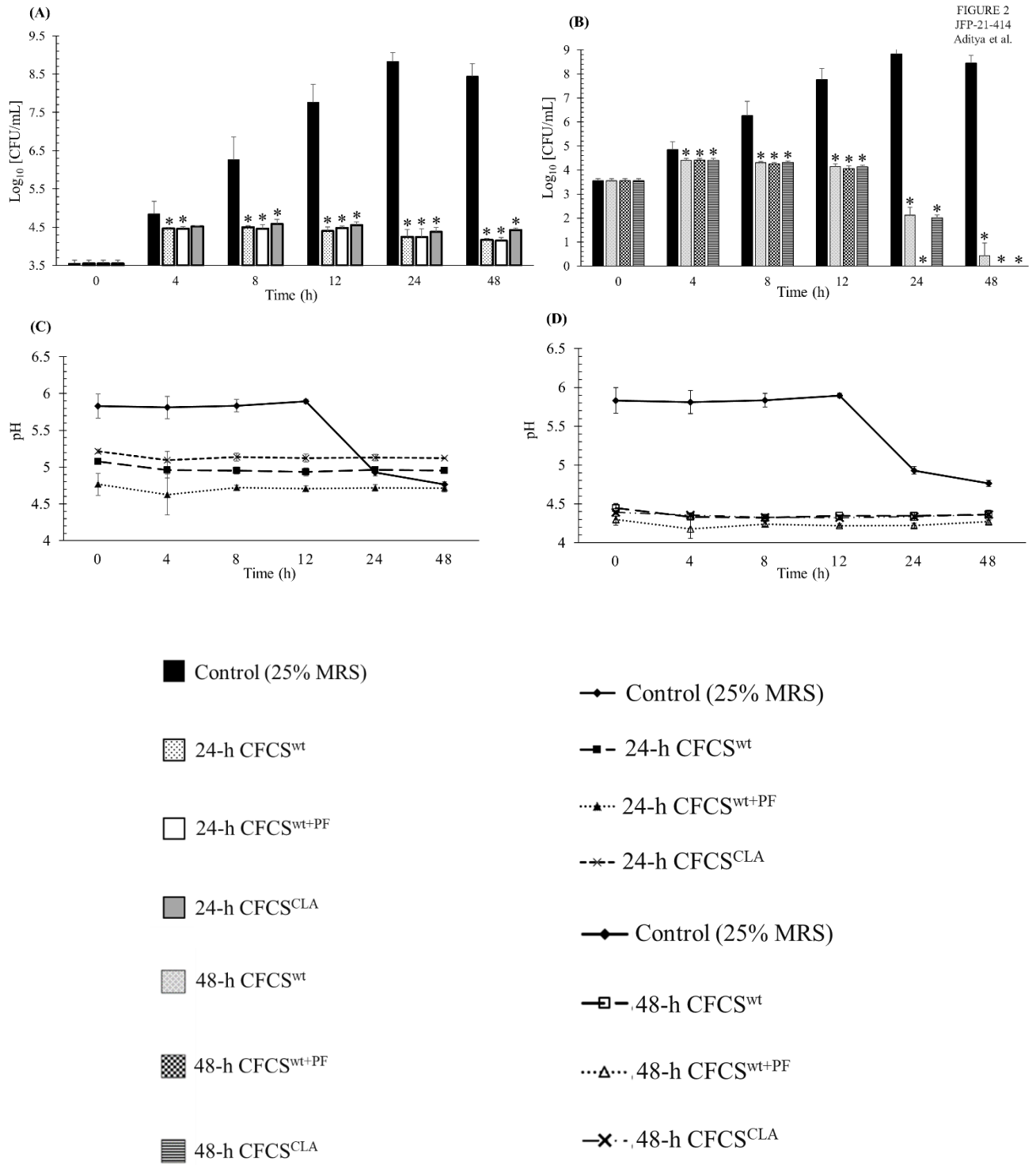


Figure 2. Suppressive effect of total metabolites (CFCS<sup>wt</sup>, CFCS<sup>wt+PF</sup>, and CFCS<sup>CLA</sup>) collected at 24-h (A) and 48-h (B) against the growth of EHEC EDL933 with real-time pH in presence of 24-h CFCSs (C) and 48-h CFCSs (D). The bars represent the average± standard deviation among replicates and the asterisks (\*) indicate a significant difference between control and treatment ( $p < 0.05$ ).

Figure 3.

FIGURE 3  
JFP-21-414  
Aditya et al.

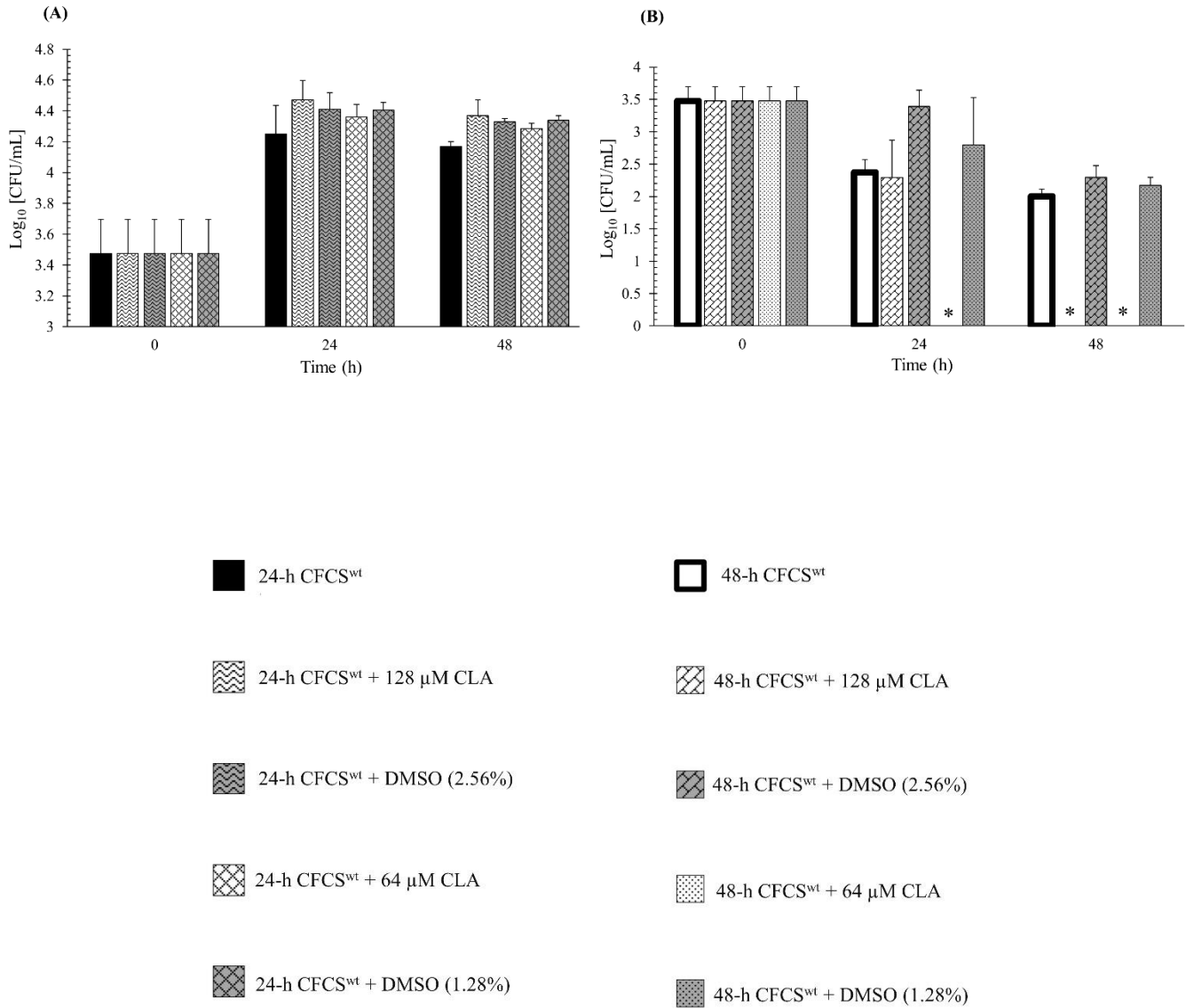


Figure 3. Inhibitory effect of CFCS<sup>wt</sup> collected at 24-h (A) and 48-h (B) in combination of different concentration of CLA dissolved in DMSO against EDL 933. The bars represent the average  $\pm$  standard deviation among replicates and the asterisks (\*) indicate a significant difference between control and treatment (p < 0.05).

Figure 4.

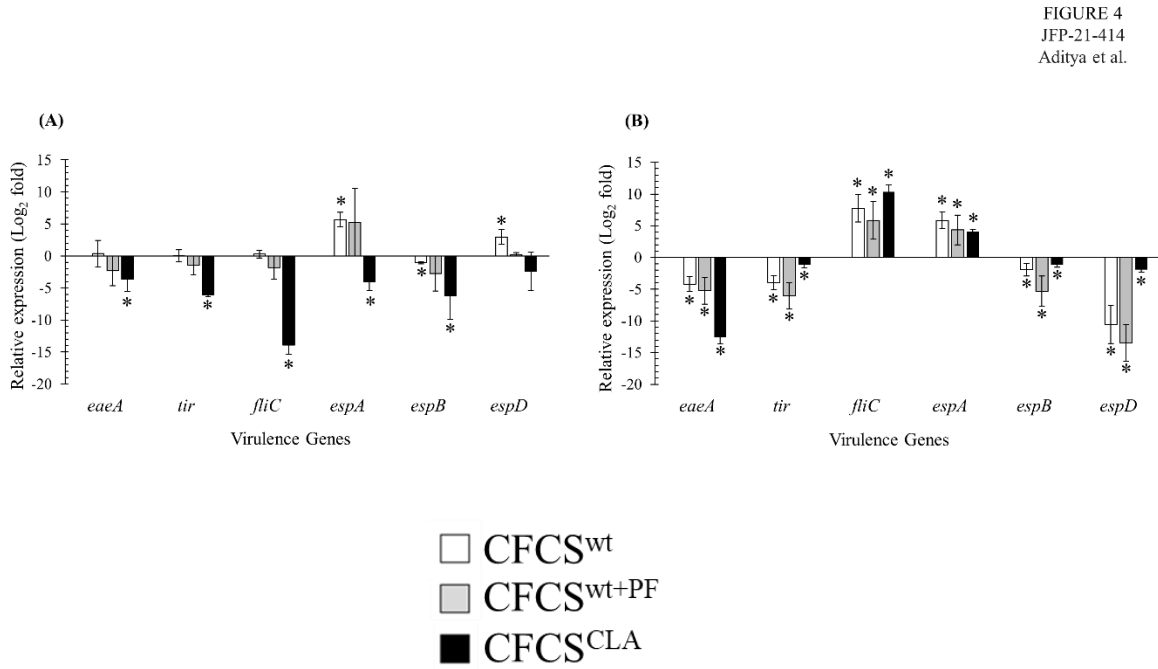


Figure 4. Relative expression of virulence genes of EDL933. Effect of CFCSs collected at 24-h (A) and 48-h (B). Bars represent relative fold-change between control and individual treatment. Error bars are showing the  $\pm$  standard deviation among replicates. Asterisks (\*) indicate a significant difference between control and treatment ( $p < 0.05$ ).

Figure 5.

FIGURE 5  
JFP-21-414  
Aditya et al.

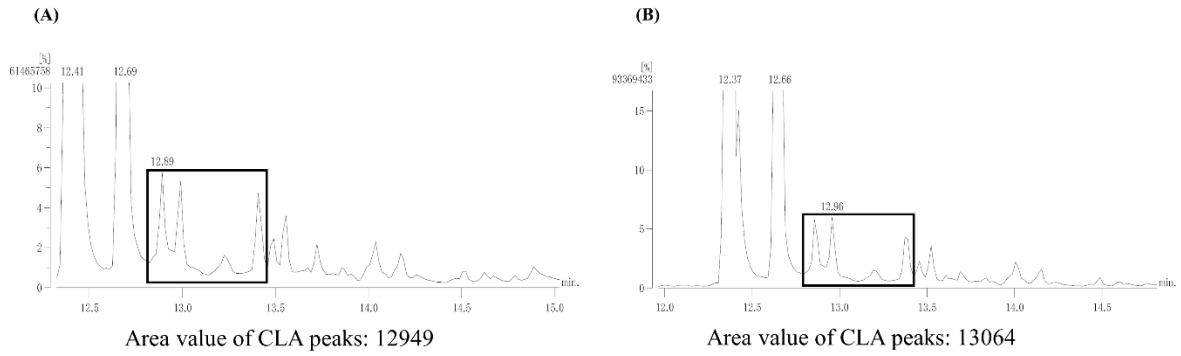


Figure 5. The chromatogram of CFCS<sup>wt</sup> (A) and CFCS<sup>CLA</sup> (B) obtained by GC-MS.

## Chapter 5: Mechanism of metabolites produced by *L. casei* on lysis of EHEC

### Introduction

Most of the human and animal bacterial infections are treated following the anti-pathogen strategies targeting to either directly eliminate or control growth and proliferation of the pathogen in the host using conventional antibiotics and antimicrobial therapeutics (Callaway et al., 2013). However, the widespread application and/or misuse of antibiotics is raising the concern of antibiotic-resistant bacteria, which is limiting the effectiveness of the prophylactic application of the existing antibiotics (Llor and Bjerrum, 2014; Ventola, 2015). Besides, some bacterial infections, including enteric infections with enterohemorrhagic *Escherichia coli* O157:H7 (EHEC), cannot be treated with traditional synthetic antibiotics because of severe consequences of cytotoxic or adverse effects of the pathogen. For example, antibiotic therapy in EHEC infection can induce the rapid release of Shiga-like toxin (stx) in the gastrointestinal (GI) tract by the death of a vast number of pathogens, which may lead to hemolytic uremic syndrome (HUS) or kidney failure (Aditya et al., 2019; Cordonnier et al., 2017). Further, it also negatively modulates the normal inter-colonic microbial balance which increases the systemic absorption of the toxin (Peng et al., 2015a). Many other therapeutics, such as anti-motility agents, narcotic analgesics, and anti-inflammatory non-steroidal drugs which may alleviate diarrhea, pain, or fever, is also suggested to be avoided in treating EHEC infections because of their observed association with HUS development and lowering the renal blood flow (Bell et al., 1997; Cimolai et al., 1992; Murray and Brater, 1993).

Recent research focusing on the pro-commensal strategy to control foodborne bacterial infections is gaining attention (Callaway et al., 2013). The pro-commensal approach aims to promote the growth of probiotics (e.g. *Lactobacillus* spp., *Bifidobacterium* spp., *Streptococcus* spp., etc.) and suppress the pathogen number or growth in the gut microbial ecosystem (Fuller, 1989b; Ouwehand et al., 2002). Adding a specific nutritional component for beneficial microbes, i.e. prebiotic or prebiotic-like dietary products (colloquially termed as ‘health-foods’ originating from the plant or animal sources e.g. berry fruits, cocoa, peanut, green tea, etc.), confers selective growth benefits to probiotics and result in the production of a diverse array of their metabolic byproducts (Charernjiratragul et al., 2010; Fuller, 1989b; Reid and McCormick, 2002; Schrezenmeir and de Vrese, 2001). In the gut environment, probiotics exert their beneficial potential by competitively excluding the pathogens/opportunistic pathogens, preventing pathogenic adhesion to mucosal surfaces and colonization. The most promising feature of probiotics already established from *in vitro* and *in vivo* studies is their antimicrobial activity which is conferred through their metabolic byproducts (Kechagia et al., 2013; Mercenier et al., 2008; Ming et al., 2018; Peng et al., 2018; Saarela et al., 2000). Probiotics alone or in combination with prebiotics (known as a synbiotic strategy) produce many cell-associated and extracellular molecules as well as bioactive metabolites through their normal physiological processes. The formation of these compounds depends on the substrate, population density, particular species and strains of probiotics, and their kinetics (Delgado et al., 2007; Jones et al., 2008). The antibacterial potential of probiotics has been proven to be a concerted effect of these compounds against pathogen growth. Detrimental effects against the growth of both Gram-positive and Gram-negative foodborne pathogens, such as *Listeria monocytogenes*, *Campylobacter jejuni*, *Enterococcus faecalis*, *Salmonella enterica*, EHEC, *Vibrio*

*parahaemolyticus*, and *V. cholerae* are observed to be potentially controlled by pro-commensal strategy (Chareunjiratragul et al., 2010; Jones et al., 2008; Mezaini et al., 2009; Nigatu\* et al., 2015; Ren et al., 2018).

Probiotics have been reported to produce various metabolites, such as flavonoids, glycosyl compounds, steroids, indole, indazole, benzoic acid, gluco-phospholipid, catechol, hydrocinnamic acid, salicylic acid, ferulic acid, caffeic acid, lactic acid, acetic acid, formic acid, linoleic acid, phenyllactic acid, vanillic acid, azelaic acid, hydrocoumaric acid, hydroferulic acid, hydrocaffeic acid, 2,3-butadione, reuterin, acetaldehyde, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), hydroxyl radical, peptides or proteins, such as the bacteriocins, and countless derivatives of these compounds (Broberg et al., 2007; Cleveland et al., 2001; Peng et al., 2015a; Schnürer and Magnusson, 2005). Besides foodborne illnesses, many of the lifestyle-based human health complications e.g. cardiovascular disease, diabetes, obesity, etc. can be prevented and more efficiently treated with a combination of existing drug therapy and healthy dietary choice (Drexler and Medicine (US), 2010; Ramalingum and Mahomoodally, 2014). Consumption of probiotic bacteria in fermented dairy products or as a supplement has become very popular due to their reported valuable association with lactose intolerance, improved gut health which is linked to the decreased risk of various other complications of the GI tract like irritable bowel disease (IBD), celiac disease, etc. (Kechagia et al., 2013; Ramalingum and Mahomoodally, 2014; Salminen et al., 2005). Similarly, many of the probiotics originated bioactive metabolites, e.g. linoleic acid isomers or conjugated linoleic acid (CLA), have been reported to possess potential anti-carcinogenic, anti-inflammatory, and anti-microbial activities (Banni, 2002; Belury, 2002; Peng and Biswas, 2017). Apart from dietary sources, this essential omega-6 fatty acid is obtained from microbial biosynthesis of many probiotic species e.g. *Bifidobacterium* spp., *Lactobacillus acidophilus*, *L. brevis*, *L. casei*, *Corynebacterium* spp., etc. (Kishino et al., 2002; Ogawa et al., 2001).

Furthermore, the quantity and quality of metabolites generated by *L. casei* could be improved by peanut flour, and they exhibit more intensive antimicrobial effects towards the growth of common foodborne pathogens (Peng et al., 2015a). However, the mechanism by which these metabolites work against pathogens and their effective concentrations are yet to be elucidated. In this study, we aimed to investigate the underlying mechanism of extracellular metabolites, including CLA, collected from the culture of *L. casei* strains in various conditions against EHEC growth, their cell membrane integrity as well as genomic DNA.

## Materials and methods

*Bacterial Strains and Their Growth Conditions.* In this study, Shiga toxin-producing enterohemorrhagic *E. coli* O157: H7 EDL933 (EHEC EDL933) (ATCC700927) was used as a representative of a foodborne pathogen. This bacterium was grown at 37°C overnight on Luria-Bertani (LB) agar or in LB broth (Becton, Dickinson and Co., Sparks, MD, USA) as required under aerobic conditions (Thermo Fisher Scientific Inc., Waltham, MA, USA). Two probiotic strains, including wild type *Lactobacillus casei* (LC<sub>wt</sub>) (ATCC334) and an engineered linoleate isomerase over-producing *L. casei* (LC<sub>CLA</sub>), previously generated in our laboratory (Peng et al., 2018) were grown at 37°C in aerobic conditions on de Man-Rogosa-Sharpe (MRS) agar or in MRS broth (Merck KGaA, Darmstadt, Germany) in a CO<sub>2</sub> incubator (Thermo Fisher Scientific Inc., Waltham, MA, USA) as required.

*Peanut Flour Preparation and Modulating Metabolites from Probiotics.* Jumbo Virginia in-shell peanuts (*Arachis hypogaea*) packed and labeled by Royal Oak Peanuts (Drewryville, Virginia, USA) was purchased from a local vendor, unshelled, and the red skin was removed by hand to collect the raw white kernel parts. Later, they were manually grounded into a fine powder and a 10% (w/v) peanut suspension in sterile deionized water was prepared by overnight stirring. Following aseptic techniques, the suspension was sieved by a kitchen fine mesh sieve strainer (LiveFresh, Darwen, UK) to separate larger peanut fractions from the peanut suspension. The pH of the sieved peanut suspension was measured and sterilized under UV irradiation for 3 h and cultured it on LB agar to ensure sterility. Later, this suspension was added in MRS broth to achieve a final concentration of 0.5% (v/v) to naturally modulate the metabolites of LC<sub>wt</sub>.

*Culture Condition of Lactobacillus Strains and Collection of Cell-free Culture Supernatants (CFCSs).* CFCSs were collected from the individual cultures of *Lactobacillus* strains in MRS broth as described previously by Peng et al. (Peng et al., 2015c) with slight modification. Briefly, 0.5% (v/v) whole peanut flour suspension was added as supplementation in MRS broth to stimulate the growth of LC<sub>wt</sub> and enhance its metabolite production. Approximately 10<sup>6</sup> colony-forming units (CFU)/mL of LC<sub>wt</sub> were inoculated to the fresh MRS broth with or without peanut flour and the metabolites in cell-free culture supernatants were collected at 48 h of incubation by centrifuging at 4000×g for 20 min, followed by filtration of the supernatant with a sterile syringe filter (0.2 µm pore size) (VWR International, Radnor, PA, USA). LC<sub>CLA</sub> was grown without peanut flour and CFCS was collected following the same procedure. In all conditions, we cultured the *L. casei* strains (LC<sub>wt</sub>, LC<sub>wt+PF</sub>, and LC<sub>CLA</sub>) under aerobic condition in 5% CO<sub>2</sub> incubator. The collected CFCSs from LC<sub>wt</sub>, LC<sub>wt</sub> in presence of 0.5% peanut, and LC<sub>CLA</sub> were labeled as CFCS<sup>wt</sup>, CFCS<sup>wt+PF</sup>, and CFCS<sup>CLA</sup>, respectively, and preserved at 4°C until further use.

*Assay of EHEC EDL933 Inhibition with CFCSs Collected from LC<sub>wt</sub> or LC<sub>CLA</sub>.* The effect of the collected metabolites against EHEC EDL933 growth was determined quantitatively *in vitro* following the method previously described in triplicate (Aditya et al., 2019; Peng et al., 2015a; Salaheen et al., 2016). Briefly, individual culture tubes containing LB broth and one of the CFCSs (CFCS<sup>wt</sup>, CFCS<sup>wt+PF</sup>, or CFCS<sup>CLA</sup>) in a ratio of 3:1 (v/v) was used as a treatment, where the same ratio of LB and MRS broth was considered as control. For the initial pathogen load, the optical density of EHEC EDL933 was fixed at 0.1 at 600 nm (OD<sub>600</sub>) by spectrophotometer (PerkinElmer, Waltham, MA, USA) which was further diluted by 100-fold to achieve an inoculum size of ~10<sup>4</sup> CFU/mL. 50 µL of the diluted EHEC EDL933 suspension was inoculated to control and treatment tubes, each having a final volume of 5 mL. The number of viable cells in the presence of each type of CFCS was estimated at 4, 8, 12, 24, and 48 h time points by serial dilution in phosphate-buffered saline (PBS; pH 7.4), followed by plating on LB agar.

*Quantitative Assay for Gene Expression of EHEC EDL933 Treated with CFCSs.* For comparing EHEC EDL933 gene expressions in the presence or absence of CFCSs, RNA extraction, and

cDNA synthesis were executed in triplicate as previously described (Peng et al., 2018; Salaheen et al., 2016). Using the cDNA (40 ng) as a template, the q-PCR reaction mixture was prepared according to PerfeCTa<sup>®</sup> SYBR<sup>®</sup> Green FastMix<sup>®</sup> protocol (Quanta Biosciences, Beverly, MA, USA) and amplified in an Eco Real-Time PCR system (Illumina, San Diego, CA, USA) with 30 s denaturation at 95°C, followed by 40 cycles of 95°C for 5 s, 55°C for 15 s, and 72°C for 10 s. The relative expression level of the target genes in treatment was calculated by the comparative log fold change. The C<sub>t</sub> (cycle threshold) value of target genes in treatment was normalized to the reference gene (Aditya et al., 2019) (all genes used in this study are listed in Table 1) in treatment then the relative expression of the genes was compared between respective treated and untreated conditions (Livak and Schmittgen, 2001).

*Fluorescence Microscopy of EHEC EDL933 Treated with CFCSs.* The live/dead cells of EHEC EDL933 treated with CFCSs was determined using the BacLight<sup>™</sup> bacterial viability kit (L7012) (Molecular Probes, Inc., Eugene, OR, USA) following the standard protocol provided by the vendor (Farkas et al., 2017). Briefly, the optical density of the bacterium was fixed at (OD<sub>600</sub>) 0.2 in LB broth which was treated with 25% (v/v) of CFCS<sup>wt</sup>, CFCS<sup>wt+PF</sup>, and CFCS<sup>CLA</sup> separately. For control, bacterial cells were grown in LB broth mixed with 25% (v/v) MRS broth. The control and treatments were cultured at 37°C under aerobic conditions. At 4, 24, and 48 h time points, 1 mL of bacterial suspension from each culture tube was collected, washed three times, and resuspended in PBS. Then the cells were stained with an equal volume of SYTO 9 and propidium iodide (PI) dye. After incubating in the dark for 15 min, 5 µL of the stained bacterial suspension was taken on a microscope slide and fixed with 0.5% (w/v) agarose. A coverslip was swiftly placed on the specimen and visualized under Zeiss AxioObserver fluorescence microscope (Zeiss, White Plains, NY, USA) with 100× oil immersion objective lens. GFP and DsRed filter sets were used to capture images of the specimens.

The remaining stained bacterial suspension was analyzed for the fluorescence intensity by a Cytation 5 spectrophotometer (BioTek Instruments, Inc. Winooski, VT, USA) at 530 and 630 nm for SYTO 9 and PI, respectively. For this step, EHEC EDL933 treated with isopropanol was used as a positive control to confirm cell death.

*Scanning Electron Microscopy (SEM) of EHEC EDL933 Cells Treated with CFCSs.* The optical density of EHEC EDL933 culture was measured and adjusted at 0.2 (OD<sub>600</sub>) in LB broth and incubated overnight at 37°C under aerobic conditions in the presence of either 25% (v/v) of MRS broth (served as a control), CFCS<sup>wt</sup>, CFCS<sup>wt+PF</sup>, or CFCS<sup>CLA</sup>. Both control and CFCSs treated EHEC EDL933 cells were harvested and washed with PBS three times. After that, cells were fixed with 2.5% (v/v) glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for 1 h. Five microliters of the fixed bacterial suspension were taken and spread on a polycarbonate membrane filter (pore size 0.2 µm) (Millipore Sigma, Burlington, MA, USA). Then the membranes were washed thrice with PBS followed by dehydration with a series of increasing aqueous ethanol concentrations, each for 15 min (30%, 50%, 70%, 80%, and 100% (v/v)) (Farkas et al., 2017). The membranes were stored overnight under anhydrous calcium sulfate (Peng et al., 2018). The EHEC EDL933 cells on the membrane were gold coated and observed under Hitachi SU-70 FEG scanning electron microscope (Hitachi Ltd., Japan) at an accelerating voltage of 10 kV.

*DNA Degradation Assay.* From an overnight culture of EHEC EDL933 (inoculum size  $\sim 10^4$  CFU/mL) in LB broth, 1 mL of the bacterial suspension was harvested and quickly spun to collect the pellet. Genomic DNA was extracted from this pellet with Trizol<sup>TM</sup> reagent (Invitrogen, Waltham, MA, USA) according to the Trizol Reagent (DNA isolation) user guide (Chomczynski and Sacchi, 1987). The extracted genomic DNA was treated with CFCSs to investigate their DNA damaging potential (Brudzynski et al., 2012). DNA (90  $\mu$ g/mL) was mixed with CFCSs in a ratio of 3:1 and incubated at 37°C for 4 h. DNA treated with LB/MRS broth in the same ratio considered for the control. After the treatment, 1  $\mu$ L of the 5 $\times$  loading dye was added, and the mixture was loaded onto a 1% agarose gel. Gel electrophoresis was performed according to a protocol described previously (Brudzynski et al., 2012).

*Statistical Analysis.* SAS 9.2 software (SAS Institution Inc., Cary, NC, USA) was used to determine the statistical significance. The one-way analysis of variance (ANOVA) followed by Dunnet test was applied to determine significant differences of EHEC EDL933 growth and gene expression levels between control and treatment based on a significance level of 0.05.

## Results

*Growth Inhibitory Effect of CFCSs on EHEC EDL933.* Quantitative assessment of the antagonistic effect of the metabolites collected in CFCS<sup>wt</sup>, CFCS<sup>wt+PF</sup>, and CFCS<sup>CLA</sup> exhibited statistically significant bactericidal property against EHEC EDL933 as compared to the control (Figure 1). Metabolites of LC<sub>wt</sub> which was enhanced by peanut flour supplement (CFCS<sup>wt+PF</sup>) exhibited the strongest inhibitory potential by eliminating EHEC EDL933 growth within 24 h, whereas metabolites collected from LC<sub>wt</sub> without the supplement (CFCS<sup>wt</sup>) could not eradicate the pathogen even after 48 h of the treatment. However, a continuous bacteriostatic effect was observed on CFCS<sup>wt</sup>, which reduced the growth of pathogen by 6.7 logs ( $p < 0.05$ ) as compared to the control at 48 h. The CFCS collected from LC<sub>CLA</sub> (CFCS<sup>CLA</sup>) which contained more CLA than the other two CFCSs (CFCS<sup>wt</sup>, CFCS<sup>wt+PF</sup>) exhibited a stronger bactericidal effect than CFCS<sup>wt</sup> against EHEC EDL933 by eradicating its growth within 48 h (Figure 1).

The antagonistic effect of the CFCSs on the growth of EHEC EDL933 was also observed at both lower and higher concentrations. A lower concentration of all the collected CFCSs (e.g. 10% v/v) exerted a bacteriostatic effect on the pathogen which was attenuated after 4-8 h depending on the *Lactobacillus* strains and the growth condition specifically the presence or absence of peanut flour as a growth supplement. Whereas a higher concentration (e.g.  $\geq 50\%$  v/v) of CFCSs regardless of the cultural conditions eradicated EHEC EDL933 within 12 h as compared to the negative control (without CFCS, data not shown).

*Alteration of EHEC EDL933 Gene Expression Indicates Adverse Growth Environment.* The relative expression of several critical genes of EHEC EDL933 cells, specifically the genes involved in cellular structure and cell-division, were examined in the presence of CFCS<sup>wt</sup>, CFCS<sup>wt+PF</sup>, and CFCS<sup>CLA</sup> (Figure 2). The most crucial gene for bacterial cell division, *ftsZ* which binds with the Z-ring to facilitate cell division was significantly downregulated (Figure 2A) in

the presence of all of three CFCSs but the suppressive effect by CFCS<sup>CLA</sup> was more intense than the other two CFCSs (CFCS<sup>wt</sup>, CFCS<sup>wt+PF</sup>), indicating the presence of metabolites produced by LC<sub>CLA</sub> is important but their concentration, possibly CLA, is critical in intensive inhibition of growth and cell division of EHEC EDL933.

On the other hand, genes associated to maintain membrane structure (*htpX*, *cpxP*, *pspA*, *pspB*, *pspC*, and *pspE*) of EHEC EDL933 were upregulated due to the treatment with CFCSs collected from LC cultures (Figure 2B). We found that expression of these genes was upregulated at various folds ranging from 2 to 8.2 which could be linked to the notion of membrane targeting activity of the metabolites. We also evaluated the expression of some genes known as molecular chaperones (*ibpA*, *ibpB*, *clpB*, *grpE*, *dnaJ*, and *dnaK*) which are expressed in stress as well as in non-stressed conditions. In this study, CFCS<sup>wt+PF</sup> and CFCS<sup>CLA</sup> upregulated the expression of all chaperon genes that were tested, however in the presence of CFCS<sup>wt</sup>, *clpB* and *dnaK* genes were found to be downregulated, indicating that the effect is attributed to the variation in CFCS composition in terms of concentration (Figure 2C). The upregulation of the DNA replication and SOS response-related genes (*recA*, *lexA*, *tus*, and *yebG*) indicated that the CFCSs regardless of the source might also have a DNA damaging activity (Figure 2D) arising from the metabolites.

*Membrane Integrity of EHEC EDL933 is Compromised by CFCSs.* In membrane integrity assay, we observed that the extracellular metabolic byproducts of *L. casei* strain present in CFCS<sup>wt</sup>, CFCS<sup>wt+PF</sup>, and CFCS<sup>CLA</sup> influenced the normal cell membrane permeability of EHEC EDL933 cells by directly disrupting the membrane integrity (Figure 3). To compare the membrane damage, the CFCS treated and non-treated EHEC EDL933 cells were visualized after staining with fluorescent nucleic acid dyes SYTO 9 and propidium iodide (PI). In general, green fluorescence is observed from a population when stained with SYTO 9, since the dye stains the DNA of both bacteria with the intact and damaged membrane. On the other hand, PI can penetrate bacterial cells only when the normal cell membrane integrity is compromised. When co-stained the red fluorescence overrides the green one because of their stronger affinity to DNA than SYTO 9. We observed a significant progression in cell membrane disruption by all treatments over the 48 h period (Figures 3A and 3B), which was reflected by the progressive abundance of red fluorescence as compared to the control. Fluorescent micrographs (Figure 3A) of the CFCSs-treated cells showed an increasing number of red fluorescent cells with the progression of treatment time (up to 48 h). A discernable variation in the number of red fluorescent cells was observed among the treatments. A similar proportion of damaged cells were observed from CFCS<sup>wt+PF</sup> and CFCS<sup>CLA</sup> at 48 h while CFCS<sup>wt</sup> had comparatively less damaged cells at the same time point. However, a significant number of green fluorescent EHEC EDL933 cells were detected under the microscope while the growth inhibition assay showed complete elimination of the pathogen. These findings can be related to the diverse mechanism of growth-inhibitory action of CFCSs besides bacterial cell membrane disruption that arises due to the diversity and concentration of metabolites present in CFCSs.

The spectrophotometric observation (Figure 3B) of the CFCS treated EHEC EDL933 cells showed a growing proportion of membrane damaged cells which were reflected by the increasing red fluorescence (at 630 nm wavelength). For this assay, EHEC EDL933 cells treated with 70% isopropanol was used as a positive control for bacterial membrane damage as isopropanol lyse bacterial cells by dissolving the membrane. The fluorescence from the isopropanol treated EHEC EDL933 cells remained static throughout the experiment because of

their rapid mode of action. On the other hand, red fluorescence of EHEC EDL933 cells cultured without CFCSs was significantly lower than other treatments that exactly coincided with the fluorescent microscopy and growth inhibition experiment.

*Morphological Deviation of EHEC EDL933 Membrane Treated with CFCSs.* The effects of the CFCSs collected from different cultural conditions i.e. CFCS<sup>wt</sup>, CFCS<sup>wt+PF</sup>, and CFCS<sup>CLA</sup> on EHEC EDL933 membrane showed significant divergence (Figure 4). The morphology of CFCS-treated EHEC EDL933 cells was visualized to compare the effect of metabolites and their concentration using SEM. The electron micrographs of overnight (18 h) CFCSs-treated cells showed distinct morphological attributes of the membrane as compared to the control. Untreated EHEC EDL933 cells' morphology was undamaged (Figures 4 A1, A2, A3), while all the CFCS-treated EHEC EDL933 cells exhibited a diverse array of membrane disruption and deviation from the normal structure. The metabolites present in CFCS<sup>wt</sup> disrupted both outer and inner membranes from random directions which made a hollow opening on the pathogen (Figures 4 B1, B2). In addition to the membrane disruption, a bleb-like structure was observed uniquely when the pathogen was treated with CFCS<sup>wt+PF</sup> (Figure 4 C1). Whereas, some EHEC EDL933 cells exhibited a crinkly appearance with a smooth surface in the presence of CFCS<sup>CLA</sup> (Figures 4 D1, D2). This particular appearance was also uniquely seen in CFCS<sup>CLA</sup> treated cells which might be the concerted effect of CLA along with other metabolites. Dislocation of the plasma membrane from the bacterial cell wall was observed in all three types of CFCS treated EHEC EDL933 cells (Figures 4 B3, C3, and D3). Although no obvious disruption of the outer membrane was apparent in these cells, the cytoplasmic content was seemed to be released possibly through a porous opening on the displaced membranes.

*DNA Degradation by CFCSs.* The effect of CFCSs on EHEC EDL933 genomic DNA was investigated by agarose gel electrophoresis. All the collected CFCSs (CFCS<sup>wt</sup>, CFCS<sup>wt+PF</sup>, and CFCS<sup>CLA</sup>) were found to be destructive to the DNA of EHEC EDL933 within the 4 h of treatment (Figure 5). However, an interesting fact was observed from CFCS<sup>wt+PF</sup> since there was no visible band in the lane (Figure 5, lane 6) while CFCS<sup>wt</sup> and CFCS<sup>CLA</sup> lanes showed bands at around 100 bp. This might be connected to the presence of metabolites that are coming from the peanut flour.

This study was also performed with pH adjusted CFCSs. The original pH of the CFCS<sup>wt</sup>, CFCS<sup>wt+PF</sup>, and CFCS<sup>CLA</sup> were 3.71, 3.48 and 3.75, respectively. After adjusting the pH to 5.43 [the pH of 3:1 (v/v) mixture of LB and MRS broth], the same effect was recorded as of pH non-adjusted CFCSs (data not shown). Therefore, this observation indicated that the destruction of DNA was not the effect of acidic pH rather the concentration of metabolites present in the CFCSs.

## Discussion

The growth of probiotics and their production ability of extracellular metabolites can be enhanced by supplementing prebiotics which is indigestible by the hosts, such as inulin, fructooligosaccharides, pectic oligosaccharides, oligofructose, and polyphenols in both culture condition and feed/food (Pranckutė et al., 2016; Rurangwa et al., 2009). A similar effect has been observed by supplementing prebiotic-like foods known as functional foods, such as peanut,

cocoa, or berry fruits (Peng et al., 2015a; Salaheen et al., 2016; Tabashsum et al., 2018). The nourishing effects of functional foods on human health are associated with the content and concentration of fatty acids, plant-based protein, vitamins, minerals, fiber, antioxidants, sterols, flavonoids, and phenolics (Arya et al., 2016; Grosso et al., 2015; Lamuel-Raventos and Onge, 2017). However, sometimes some of these components may be linked to undesirable conditions e.g.  $\alpha$ -arachin protein present in peanut white kernel is thought to be the cause of peanut allergy (Barnett et al., 1983; Johns and Jones, 1916). Besides, fungal infestation e.g. *Aspergillus flavus* on grainy functional foods is linked to aflatoxin mediated carcinogenesis (Achar et al., 2009). Considering these adverse effects, cost, availability, and persistent applicability all prebiotics cannot always stimulate the growth and metabolite production of probiotics for hosts. Hence, engineered probiotics capable of producing critical metabolites without the presence of prebiotic components are an alternative option (Peng et al., 2018). Further, such type of modified probiotic strains could be more effective in the fermentation of dairy products and vegetables. In this study, we qualitatively compared the specific effects arising from the metabolites of a probiotic bacterium when it grows alone, in presence of a prebiotic-like supplement, and aimed to achieve a similar beneficial effect by genetically engineering a probiotic strain through overproducing one of the bioactive metabolites, CLA.

In agreement with Peng et al. (2018) reported previously, we also observed a gradual decline in the growth of EHEC exerted by all collected CFCSs from *L. casei* strains in a time-dependent manner (Peng et al., 2015a, 2018). However, CFCS<sup>wt+PF</sup>, collected from the *L. casei* in the presence of peanut flour, exhibited a stronger inhibitory effect than CFCS<sup>wt</sup> as well as CFCS<sup>CLA</sup>, as expected. By genetic modification of *L. casei*, we could increase the conversion of CLA only 21 folds (Peng et al., 2018) while in the presence of peanut flour (0.5%) they might have produced more CLA and other metabolites as well. Hence, CFCS<sup>CLA</sup> which contained a higher amount of CLA inhibited the growth of EHEC more efficiently than CFCS<sup>wt</sup> which was collected from wild-type *L. casei* without the peanut flour supplement. Lamuel-Raventos et al. (2017) reported that nutritionally indigestible components of peanuts like polymerized polysaccharides and polyphenols are enzymatically converted by gut microbes into a diverse array of metabolic byproducts encompassing organic acids, fatty acids, and H<sub>2</sub>O<sub>2</sub>, etc. (Lamuel-Raventos and Onge, 2017) that might have conferred the relatively rapid antagonistic effect of CFCS<sup>wt+PF</sup> against EHEC growth in our study.

Previously, we also reported that CFCS<sup>CLA</sup> impaired physicochemical properties of bacterial pathogens such as auto-aggregation, surface hydrophobicity, and biofilm formation of several enteric bacterial pathogens more efficiently than CFCS<sup>wt</sup> (Peng et al., 2018; Tabashsum et al., 2018; Vu et al., 2009). As the physicochemical properties play a major role in pathogenic bacterial adherence to host cells, secretion of effector proteins, and initiate colonization (Aditya et al., 2019; Cordonnier et al., 2017; Sorroche et al., 2012), we also compared the expression of genes which are involved in such physicochemical properties and secretion of proteins in the presence of CFCSs collected from LC<sub>wt</sub> and LC<sub>CLA</sub>. Major virulence genes involved in EHEC Type-III secretion system (T3SS) i.e. *eaeA*, *espA*, *espB*, *espD*, *tir*, and *ler* are downregulated presumably by the polyunsaturated fatty acid components of CFCSs (Cardenal-Muñoz and Ramos-Morales, 2011; Peng and Biswas, 2017; Peng et al., 2018). Altered physicochemical properties and virulence gene expression of EHEC are concurred with its reduced adherence to host intestinal epithelial cells. A paucity of receptor-like molecules on the host cell surface is one

of the reasons for reduced adherence since the receptors are already settled with the metabolites present in CFCSs (Bernet et al., 1994; Matsuo et al., 2012; Peng et al., 2015c).

It is already explicitly established that the components of CFCSs create an adverse condition that induces the pathogen to adaptively regulate its genes in response to such stressful conditions for their survival (Seshasayee et al., 2009; Wall et al., 2004). We observed a significant downregulation of *ftsZ*, the key regulator of EHEC cell division, which coincides with our observed bacteriostatic effect of CFCSs regardless of the source in the first five hours of treatment. In an unfavorable growth environment, such as nutrients insufficiency, adverse pH, and presence of growth inhibitory compounds bacteria usually delay their proliferation due to the inhibition of septal ring (Z-ring) proteins specifically FtsZ or DnaA or disruption of other factors involved in Z-ring formation (Arends and Weiss, 2004; Jonas, 2014). When the Z-ring is unable to form septa, the cells continue to grow like long aseptate filaments which may not form colonies upon plating (Addinall et al., 1997; Vicente et al., 2006). In this study, a stronger suppression on *ftsZ* gene was observed when the EHEC EDL933 cells were treated with CFCS<sup>CLA</sup> which might be correlated to the higher concentration of metabolites including CLA.

In a stressful environment, bacterial cells produce defective and misfolded membrane proteins much higher than they would produce in a favorable growth condition. To retain membrane integrity, the bacterial cells need to correct or destroy the misfolded proteins (Sakoh et al., 2005). In our study, we observed an overexpression of *htpX* and *cpxP* genes which are involved in the quality control of membrane proteins of EHEC in the presence of CFCSs. We also observed an upregulation of genes that are involved in phage shock protein operon (*pspA*, *pspB*, *pspC*, and *pspE*). Yoshitani *et al.* (2019) found that *htpX* along with *ftsH* lyse the abnormal inner membrane (IM) proteins of EHEC (Yoshitani et al., 2019). Besides, Shimohata et al. (2002) reported that the accumulation of misfolded proteins in the IM may occur due to the inefficiency or loss of *htpX* or *ftsH* which further activates Cpx stress response proteins located in periplasmic space (Shimohata et al., 2002). The Psp proteins are thought to be a part of the interrelated stress response system to manage the membrane. An increased permeability of IM caused by the mislocalization of secretin proteins activates the psp-system. Secretins are components of the export system essential for virulence, biofilm formation, and antibiotic resistance of EHEC (Joly et al., 2010; Srivastava et al., 2017). Under non-stress conditions, Psp proteins (except PspA) are almost undetectable where PspA along with HtpX and DnaK represses the psp-system (Elderkin et al., 2002). When a stress signal is received by IM-bound PspB and PspC and transferred to PspA thereby downregulating it and increasing other Psp proteins (Jovanovic et al., 2006; Nonaka et al., 2006; Weiner et al., 1991). In this study, we observed an upregulation in all *psp* (*pspA*, *pspB*, and *pspC*) genes, while PspA was expected to be downregulated in CFCSs-induced stress conditions. However, researchers also found that when any gene is only expressed under adaptive control (in this study *pspB*, *pspC*), there might be a delay to produce enough protein to adjust to the situation and during such time could lead to death or abnormal cellular morphology (Brissette et al., 1990; Price et al., 2013).

To evaluate the effect of CFCSs on the EHEC EDL933 cell membrane, we studied the EHEC membrane intactness and found evidence of membrane disruption caused by metabolites present in CFCSs. Our fluorescent microscopic and spectrophotometric data indicated that during the earlier hours of treatment, membrane disruption was less which was apparent by the lower signal intensity of red-fluorophore, propidium iodide (PI). The PI is a widely used stain to detect dead cells; this dye can only penetrate cells with a disrupted membrane, intercalates to DNA

irrespective of base-composition, or degradation with one dye molecule binding per 4-5 base pairs (Stocks, 2004; Waring, 1965). In our study, the proportion of membrane damaged cells increased with longer CFCS treatment duration, albeit there was a considerable proportion of green-fluorescent signal from SYTO 9 dye indicating the presence of bacteria with intact membrane. This variance of our fluorescent microscopic observation from the time-dependent growth reduction of EHEC was understood after the EHEC membrane morphology analysis by SEM. The electron micrographs revealed several membrane altering effects of CFCSs on EHEC. The control cells displayed a normal intact surface whereas the treatments exhibited a multitude of the wrinkled-irregular membrane, bleb-like surface, deep craters on both sides of the cell, shrinkage of the inner membrane, blisters, etc. The ‘bleby’ surface morphology was observed uniquely in CFCS<sup>wt+PF</sup> treatment. This type of structure is formed at places on the outer membrane due to the deficiency of lysine, which can result in the detachment of the outer membrane from the underlying layer of peptidoglycan (Chatterjee and Chaudhuri, 2012). Lysine is an essential amino acid for humans which is present in peanuts and is also required for bacterial membrane protein synthesis (Gillner et al., 2013). Peanut originated components later metabolized by *L. casei* in our study have presumptively interfered with the EHEC lysine biosynthetic pathway hence resulted in the bleb-like appearance. In all treatment, no obvious disruption was apparent in some cells other than a detachment of the inner membrane from the outer membrane, which explains the dissimilarity of our fluorescent microscopic and EHEC EDL933 growth reduction observation.

Heat shock proteins induced under stress usually act as protease (e.g. HtpX, Lon, DegP), chaperons (e.g. ClpB, DnaJ, DnaK), or chaperon helper (e.g. IbpA, IbpB) (Lewis, 2019). We observed the expression of some SOS pathway and chaperon related genes of EHEC EDL933 in the presence of collected CFCSs. LexA and RecA are two major proteins of SOS response in EHEC EDL933 where the cell’s response to DNA damage is mediated by expression of about 40 genes of SOS regulon by LexA repressor and RecA (Campoy et al., 2005). In normal growth condition, LexA represses/limit the expression of SOS genes (Friedberg et al., 2005). Upon encountering DNA damage stimuli, *RecA* becomes activated which facilitates the autolysis of LexA thereby initiating the expression of SOS genes that are involved in DNA damage tolerance, DNA repair, and delay of the cell cycle (Kreuzer, 2013). In our study, we observed a parallel overexpression of both *lexA* and *recA*. Upregulation of another two genes *tus* and *yebG* which are related to stopping DNA replication by blocking the movement of the replication fork and assisting in SOS response, respectively, indicated a DNA damaging stimuli presented by the CFCSs components (Lomba et al., 1997; Roecklein et al., 1991). Following the gene expression results of this study, we observed the DNA damaging effect of CFCS on EHEC EDL933 genomic DNA on the agarose gel. Such an effect could be attributed to the acidic pH of the CFCSs, but the same outcome was observed with pH adjusted CFCSs. So, the DNA damaging effect is linked to the specific compounds of CFCS possibly H<sub>2</sub>O<sub>2</sub> (Brudzynski et al., 2012). The residual product size of the cleaved DNA on agarose gel poses an interesting observation that requires further investigation to reveal their sequences.

Molecular chaperones are proteins that assist a wide range of other proteins and enzymes to ensure their correct tertiary and quaternary conformation and functionality *in vivo* (Bukau et al., 2000; Saibil, 2013). All living cells constitutively maintain an intense regulation of chaperon gene expression to maintain consistency in protein folding to cope up with any growth

perturbation (Morimoto, 1998). Therefore, chaperones or heat shock proteins are always expressed but expressed differently during stress conditions. In this study, an upregulation of the chaperon gene expression (*ibpA*, *ibpB*, *clpB*, *grpE*, *dnaJ*, and *dnaK*) in the presence of CFCSs was observed. Our findings in chaperon gene expression indicated stressed conditions created by CFCSs. However, as chaperones are involved in multiple complex cross-talks that are active in various conditions, further extensive analysis is needed to evaluate whether the upregulation is directed by solely CFCS-induced stress conditions.

Though evaluating the mechanism of total CFCS rather than finding out the individual metabolite responsible for exerting the antibacterial effect on EHEC is a limitation, but to our knowledge, this is the first study to address the mechanism of action of metabolites produced by probiotics against foodborne *E. coli* O157: H7. Further in-depth and extensive research is needed to find out the responsible metabolites by investigating specific protein-ligand interaction through combining different computational approaches. The orchestrated effect of metabolites present in the CFCSs of probiotics on the membrane and genomic DNA of EHEC could be potentially targeted to develop a therapeutic for EHEC infections including HUS.

List of tables

Table:1 Primers used for RT-qPCR analysis

Function	Gene	Primer sequence (5'-3')	Reference
Housekeeping gene	16 S rRNA	F: CGTTACCCGCAGAAGAAGC R: GTGGACTACCAGGGTATCTAATCC	(Aditya et al., 2019)
Cell division protein	<i>FtsZ</i>	F: TTGGGTATCCTGACCGTTGC R: AGCAGTTTGTTCGTTTCGGGAT	Used in this study
Membrane heat shock proteins	<i>HtpX</i>	F: GGCAACCCGCTGATCTACTT R: AGCGCGGCAATCATTTTCTC	"
	<i>cpxP</i>	F: TTTCTGCGGTGACAAGACGA R: TCAGGCGATAACTGGCATCC	"
	<i>pspA</i>	F: ATCCACAGAAACTGGTGCGT R: GTTTCTTTTCTGCCAGCGCA	"
	<i>pspB</i>	F: ACCGATCTGGTTATGGCTGC R: TTCCAGCGCCTGAATACGTT	"
	<i>pspC</i>	F: GTGCGTATCCTGGTGGTGTT R: CACCAAAGGCCATGTTGTCC	"
	<i>pspE</i>	F: AGTGAAAGAGCGCATTGCCA R: CGTGGGTATATCCCATCTCGC	"
SOS response and DNA replication related genes	<i>recA</i>	F: CAGGCAGTTGCATTTCGCTTT R: TCTACGGCGAACTGGTTGAC	"
	<i>lexA</i>	F: GGTCGTTGTTCGCACGTATTG R: CTGCTGACGAAGGTCAACGA	"
	<i>tus</i>	F: TCCTGGCACAGCTGGAAAAA R: ACTTCGCGTTCTGTGGTAGG	"
	<i>yebG</i>	F: CATAGCGAAAGGGCTTCACG R: AGCAAAAAGGAAGCCGATGC	"
Chaperon proteins	<i>ibpA</i>	F: CGACGAACAAAAAGAGCGCA R: ACCAGGTTAGCACCACGAAC	"
	<i>ibpB</i>	F: AGCGACGATAACCACTACCG R: GCCCTTGATGCAGCCATTTT	"
	<i>clpB</i>	F: GGCCGAGGAACAGGAATGAA R: ATTGGTCAGAACGAAGCGGT	"
	<i>grpE</i>	F: GCACAACATCCAGCATCGAC R: TGCCGGTGATTGATAGCCTG	"
	<i>dnaJ</i>	F: TCATGGTTCTGGTCAGGTGC R: TGCCGGGATTTTAACGGACA	"
	<i>dnaK</i>	F: GAAAGTTGCACTGCAGGACG R: GGTTAACGTCTTTACGCGGC	"

List of figures, figure legends, and captions

Figure 1.

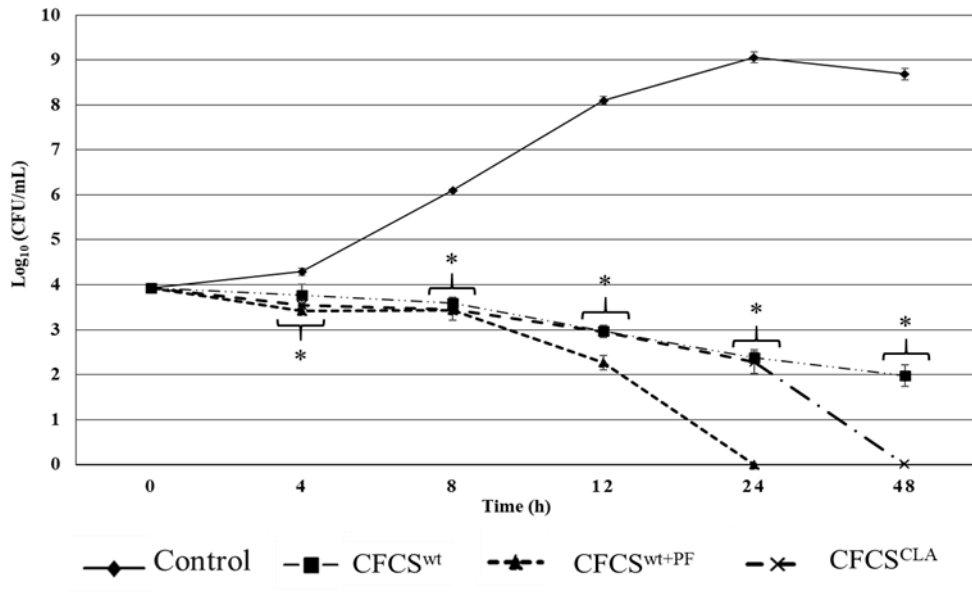


Figure 1: Antagonistic effect of crude metabolites (CFCS<sup>wt</sup>, CFCS<sup>wt+PF</sup>, and CFCS<sup>CLA</sup>) against the growth of EHEC EDL933 over 48 h time. The bars represent the average  $\pm$  standard deviation among replicates and the asterisks (\*) indicate a significant difference between control and treatment ( $p < 0.05$ ).

Figure 2.

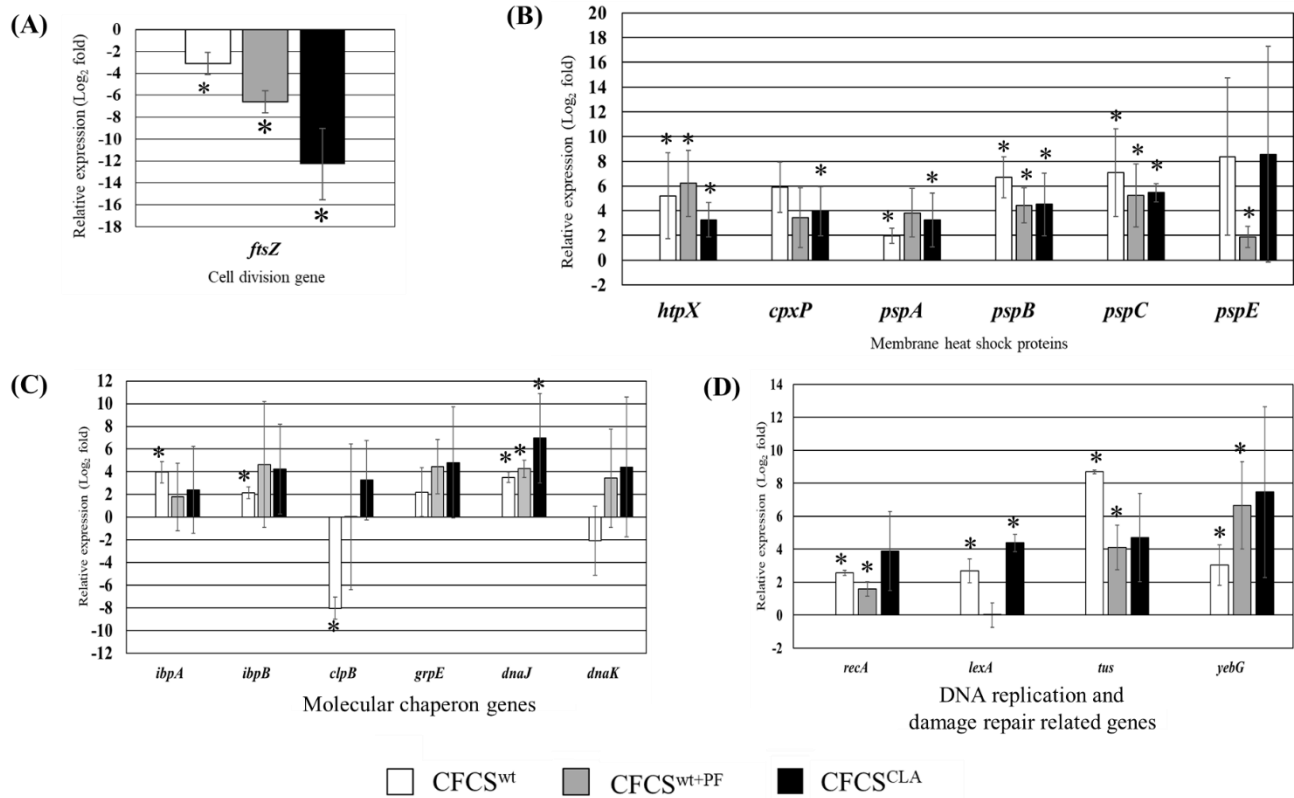
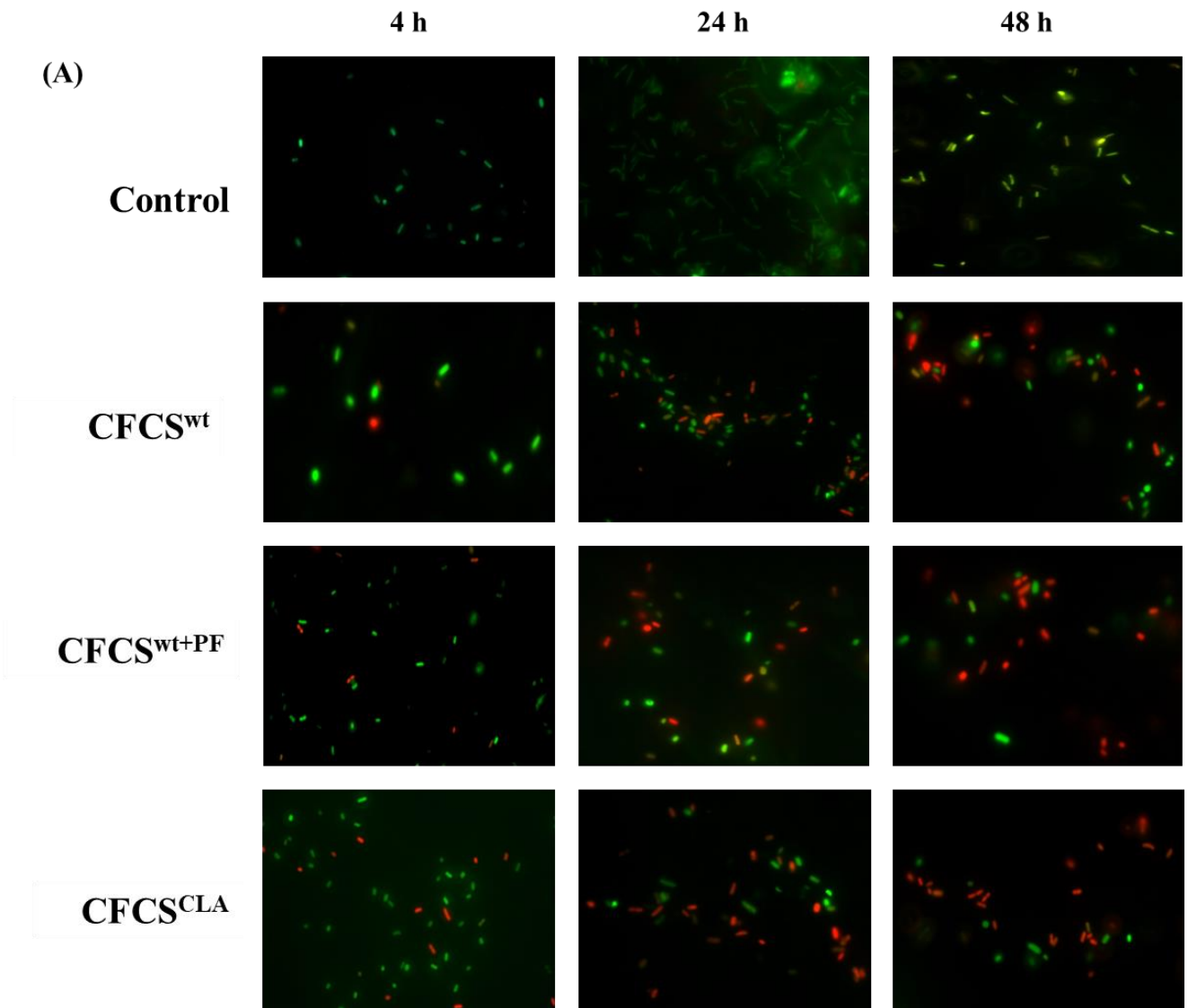


Figure 2: Relative expression of genes related to EHEC EDL933 cell division (A), membrane heat shock proteins (B), various molecular chaperon genes (C), and DNA replication and damage repair-related genes (D). The bars represent relative fold-change between control and individual treatment. The error bars are showing the  $\pm$  standard deviation among replicates, and the asterisks (\*) indicate a significant difference between control and treatment ( $p < 0.05$ ).

Figure 3.



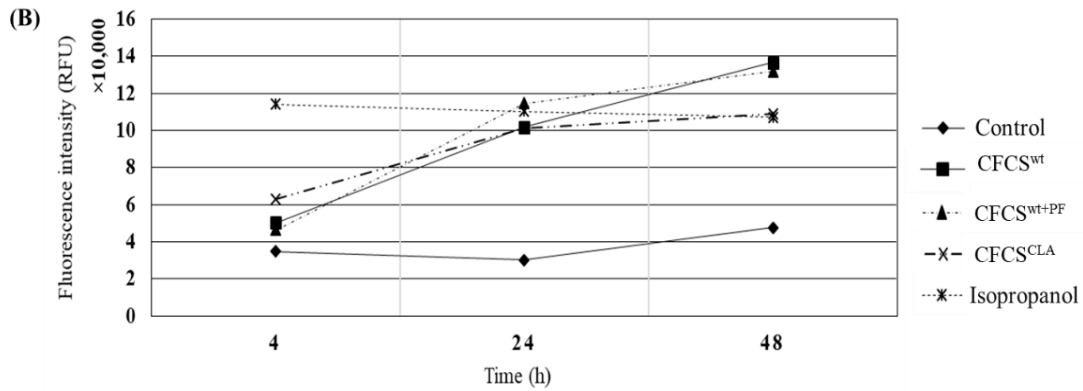


Figure 3. Fluorescent micrographs showing altered EHEC EDL933 cell membrane permeability (A) in the presence of *Lactobacillus* metabolites. An increase in the extent of membrane disintegration was visible because of the presence of CFCS<sup>wt</sup>, CFCS<sup>wt+PF</sup>, and CFCS<sup>CLA</sup> throughout 4, 24, and 48 h. The green fluorescence represents bacterial (EHEC EDL933) cells with an intact membrane whereas red fluorescence indicates a disrupted membrane. Increasing the intensity of red fluorescence (relative fluorescence units, RFU) over the incubation period for treatments indicated an increasing proportion of dead cells in the cultural conditions (B).

Figure 4.

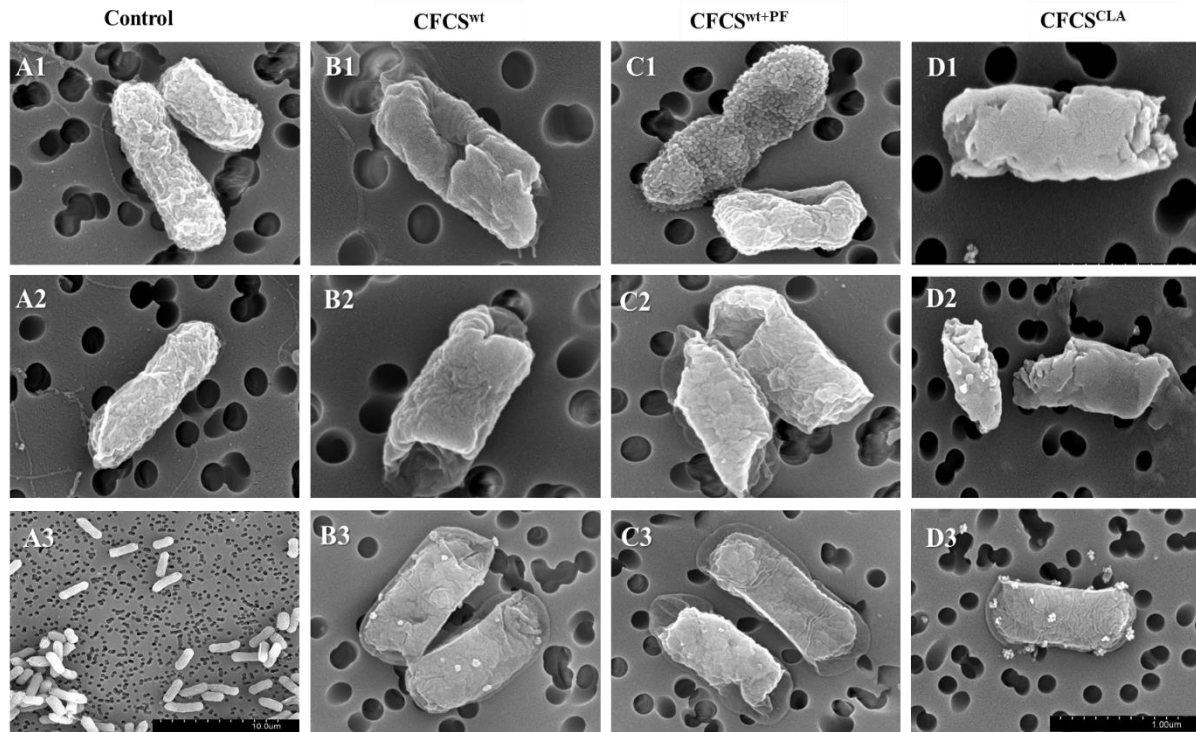


Figure 4. Scanning electron micrographs showing the effect of metabolites in CFCSs on the EHEC EDL933 cell surface after overnight (18 h) incubation. No membrane disruption was visible on the control group (A1, A2, A3), whereas dents were apparent at the ends of the cells (B1, B2, and C2). Apart from the dents, bleb-like structures were also visible (C1), while a crumpled and blistery surface was also observed (D1, D2, and D3).

Figure 5.

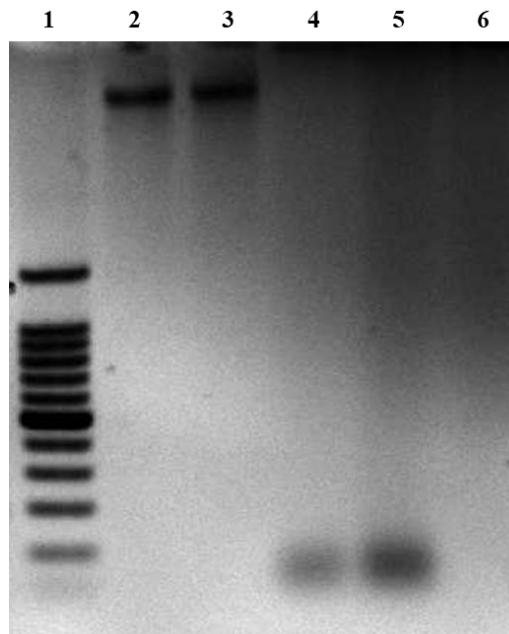


Figure 5. DNA degradation of EHEC EDL933 treated in the presence of CFCSs for 4 h. For the agarose gel: lane 1 represents a 100 bp DNA ladder. Lane 2 represents EHEC EDL933 genomic DNA without any treatment, Lane 3,4,5, and 6 represent EHEC EDL933 genomic DNA separately treated with 25% (v/v) of LB/MRS broth mixture (control), CFCS<sup>wt</sup>, CFCS<sup>CLA</sup>, and CFCS<sup>wt+PF</sup>, respectively.

## Chapter 6: Investigate metabolites produced by *L. casei* in production and neutralization of Shiga toxin of EHEC using Gb-3 receptor containing mammalian cells

### Introduction

Shiga toxin producing *Escherichia coli* (STEC) is one of the more infamous bacteria out of 31 most common attributable foodborne pathogens in the United States (US) and the world as well (Oporto et al., 2019; Singha et al., 2022). According to Centers for Disease Control and Prevention's (CDC) National Enteric Disease Surveillance program, STEC is responsible for about 265 thousand cases of sicknesses, with 3.6 thousand hospitalizations and 30 deaths annually in the US (Scallan et al., 2011; CDC, 2012). Like other foodborne pathogens, STEC generally causes abdominal cramps and diarrhea, which can be self-limiting in some patients, but can also progress into bloody diarrhea (WHO, 2018). This pathogen remains a significant public health concern due to its ability to produce Shiga toxin (Stx) (KAPER and O'BRIEN, 2014; Kim et al., 2020). Stx is a major virulence factor in STEC, as it confers a unique pattern of pathogenicity that especially creates complications at the time of treatment when compared to other virotypes of pathogenic *E. coli* (Kaper et al., 2004). Depending on various factors, such as host immune status, age of the patient and serotype of the pathogen, STEC can cause hemorrhagic colitis (HC, commonly referred to as "bloody diarrhea") and a type of "kidney disease" known as hemolytic uremic syndrome (HUS), which is mediated by Stx. HUS is characterized by thrombocytopenia, hemolytic anemia, acute renal failure, chronic renal sequelae, neurological complications such as seizure, stroke, coma, and even death (Bai et al. 2022, WHO, 2018).

Ruminants specifically cattle are natural reservoir of STEC and are usually harmless to the ruminant host, but pathogenic to humans upon transmission via fecal-oral route (Amézquita-López et al., 2018). The subtype of STEC that has been linked to HC and HUS in humans are referred to as enterohemorrhagic *E. coli* (EHEC) (Levine, 1987). Interestingly, there are also some STEC that have been reported to cause dysentery in young calves (Lee et al., 2008). The baffling nuances in the structure of STEC contributes to its major antigenic variability and host specificity (Wang and Reeves, 1998). Like other virotypes of *E. coli*, STEC can be further grouped into serotypes which are classified based on the presence and pattern of specific surface antigen structure known as O- and H-antigens. The O-antigen is a part of outer membrane lipopolysaccharide (LPS) and consists of unique repeats of multiple oligosaccharide units. To date, based on O-antigen more than 470 serotypes of STEC have been identified (Zhang et al., 2022). In addition to the O-antigen, identification of H-antigen (a flagellar protein) adds more specificity to the STEC serotype identification which is an important information for further characterizing and investigating foodborne outbreaks (CDC 2012). Traditionally, *E. coli* are serotyped against 186 O-antigen and 53 H-antigens for outbreak inspection (Fratamico et al., 2016; Wang et al., 2003).

Although Stx is the main virulence factor of STEC, the presence of Stx gene does not ensure effective pathogenesis. Their complex pathogenesis is regulated by the overexpression of an array of genes whose protein products are not always directly associated with virulence (e.g., metabolic genes) and also depends on the presence appropriate receptors found on the host cell (Panel et al., 2020). Receptor specificity is linked to the O-antigen of STEC outer membrane. For

instance, *E. coli* O157: H7 is a deadly pathogen to humans because the Stx produced by this pathogen binds to the globotriaosylceramide (Gb<sub>3</sub>) receptor which is found on renal endothelial cells (Melton-Celsa, 2014). However, if the same pathogen were to be transmitted to adult cattle, even at a very high number, i.e., 10<sup>10</sup> colony forming units (CFU), it would not cause any sickness. Unfortunately, the cattle still shed the pathogen in feces along with all other commensal *E. coli* as a natural process (Solomon et al., 2002; Stein and Katz, 2017). Some STEC serotypes, such as O5, O26 and O111, have been reported to be cause diarrhea in both neonatal calves and humans (Janke et al., 1990; Lee et al., 2008). Besides, the Stx produced by different STEC serotypes also has different nature which is not thoroughly understood. For instance, *E. coli* O26 also produce the similar Stx as that of *E. coli* O157, which causes similar illness, but it does not lead to HUS (CDC, 2014).

Certain serotypes of STEC, such as O157:H7, O103, O121, O26, O103, O104:H4, O145, continue to be discussed in the context of foodborne outbreaks all through the year (CDC 2019). Among these, O157 serotype gets the highest media attention because it is responsible for more than 75% of all *E. coli* outbreak-mediated illnesses (IFSAC 2021). Moreover, the hospitalization rate related to this serotype is 46.2% and the total illness related cost is about 254.8 million dollars annually in the US (Carstens et al., 2019; Hoffmann et al., 2012). Since cattle are the major reservoir of O157 serotype, it is easily transmitted via beef, dairy products, and ready to eat fresh produce which are contaminated by O157 possibly coming from compost and irrigation water (Agga et al., 2022). Moreover, traditional farm practice, such as mixed-crop livestock farms where different animal and plant crops are grown together, may also contribute to the pathogen transmission (Salaheen et al., 2015).

Unlike other virotypes of *E. coli*, such as enterotoxigenic *E. coli* (ETEC, causative agent of traveler's diarrhea), antibiotics are not prescribed to treat *E. coli* O157:H7 infections due to its controversial association with Stx production which is known to lead to HUS (Kakoullis et al., 2019). The illness is usually self-limiting with immune supportive therapies such as replenishing fluid. However, for HC and HUS cases hospitalization and advanced care such as transfusion, dialysis may be required (Goldwater and Bettelheim, 2012). Few other strategies, such as development of monoclonal antibodies and vaccines against Stx, are being explored with limited success in clinical trial so far (Mühlen and Dersch, 2020). The major challenge of human specific STEC serotype research is the lack of appropriate animal models. Although rabbits, mice, and pigs are frequently used, they do not exhibit the same spectrum of disease progression as humans (Ritchie, 2014). Among all these inexorable limitations, one of the successful management strategies of *E. coli* O157:H7 mediated illness is the use of probiotic bacteria which outcompetes the *E. coli* O157:H7 within the environment of the gastrointestinal (GI) tract by various mechanisms. Probiotics have been shown to confer favorable effects to its hosts by producing critical metabolites with selective antibacterial potential, by blocking the specific cellular receptors and making them inaccessible to pathogens, or by simply nutritionally outcompeting the pathogen (Aditya et al., 2020a; Kim et al., 2020; Peng et al., 2018). Probiotics alone or in combination with prebiotic-like dietary components produces countless derivatives of organic acids, flavonoids, hydrogen peroxide, peptides, different fatty acids, etc. (Aditya et al., 2020a). Previous studies from our research group found that total metabolites collected from *Lactobacillus casei* which also included conjugated linoleic acid (CLA), synergistically exhibit bactericidal effect on *E. coli* O157:H7 EDL 933 (Aditya et al., 2022). In addition to down-regulation of virulence genes, we also found that the metabolites exert multidirectional effect on

EDL933 such as disruption of cell membrane, restricting cell division, and damaging DNA (Aditya et al., 2020a).

In this *in vitro* study, we explored the association of *L. casei* originated metabolites specifically CLA on the Stx production ability of *E. coli* O157: H7 EDL933. This study also aimed to assess the neutralization potential of these metabolites towards Stx, which was assessed by evaluating changes in cytotoxicity within a Gb<sub>3</sub> receptor containing mammalian cell line.

## Materials and methods

*Bacterial strains and their cultural conditions.* Stx-producing enterohemorrhagic *E. coli* O157: H7 EDL933 (EDL933) (ATCC700927) was used as a representative of a Stx-producing foodborne pathogen. This bacterium was revived on Luria-Bertani (LB) agar (Becton, Dickinson and Co., Sparks, MD, USA) plate from a glycerol stock by subculturing and incubating at 37°C overnight under aerobic conditions (Thermo Fisher Scientific Inc., Waltham, MA, USA). Two strains of *Lactobacillus* spp. also sub-cultured from glycerol stock on de Man-RogosaSharpe (MRS) agar (Merck KGaA, Darmstadt, Germany) at 37°C in aerobic conditions with 5% CO<sub>2</sub>. They are wild type *Lactobacillus casei* (LC<sub>wt</sub>) (ATCC334) and a genetically engineered linoleate isomerase over-converting *L. casei* (LC<sub>CLA</sub>), formerly generated by our research group (Aditya et al., 2020a; Peng et al., 2018).

*Mammalian cells and their growth condition:* Gb<sub>3</sub> receptor containing mammalian cell, HCT 116, was cultured in Dulbecco's Modified Eagle Medium (DMEM) (Corning Cellgro, Manassas, VA, USA) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS) (Atlanta Biologicals®, Lawrenceville, GA, USA) (DMEM + 10% FBS). To prevent bacterial contamination 50 µg/mL of gentamycin (IBI Scientific, Peosta, IA, USA) was also added to the cell culture media. The cells were cultured and incubated at standard condition (37°C, 5% CO<sub>2</sub>, and 95% humidity) (Thermo Fisher Scientific Inc., Waltham, MA, USA) until it reached a confluent cell monolayer covering 90% of the flask surface. For evaluating changes in gene expression and cell cytotoxicity assays, cells were seeded in 24- and 96-well culture plate, respectively and incubated at the same condition as stated above until they were 70-85% confluent and observed under light microscopy. When the cell culture attained desired confluency, cells were washed with DMEM three times and again incubated with the corresponding treatments (described later) for further assays (Aditya et al., 2019).

*Preparation of treatments and evaluate their effect on EDL933 growth.* Two probiotic bacterial strains, LC<sub>wt</sub> and LC<sub>CLA</sub>, were cultured in DMEM<sup>+10%FBS</sup> under the incubation condition described above for *Lactobacillus* spp. The probiotic strains were allowed to grow until 48 h in order to later extract the metabolites that were present in the respective cell free culture supernatants, L-CFCS<sup>wt</sup> and L-CFCS<sup>CLA</sup>, of each. These were collected to store and use as treatments following the protocol described by Aditya et al., 2020, 2022. Besides, 64 µM of CLA prepared in DMEM<sup>+10% FBS</sup> to understand the effect of probable effect of CLA (Aditya et al., 2022). The antimicrobial potential of the collected total metabolites (L-CFCS<sup>wt</sup> and L-CFCS<sup>CLA</sup>) at a ratio of 1:3 (v/v) and CLA (64 µM) were evaluated against EDL933 at different time points, i.e., 0, 3, 6, 12, and 24 h, as described previously by Aditya et al., 2020. The

minimum inhibitory concentration (MIC) of ciprofloxacin was determined against EDL933 and their growth pattern was also observed at a time dependent manner at a sub-lethal concentration (CLSI, 2019).

*Collection of CFCS from EDL933 under various conditions.* To understand the association between the collected total *Lactobacillus*-originated metabolites (L-CFCS<sup>wt</sup> and L-CFCS<sup>CLA</sup>), CLA, and/or ciprofloxacin and Stx production ability of EDL933, we collected their CFCS after 48 h of incubation following the procedure described in Aditya et al., 2022. Briefly, ~4 logs of EDL933 were inoculated in DMEM with 10% FBS, supplemented separately with 25% of L-CFCS<sup>wt</sup>, L-CFCS<sup>CLA</sup>, 64  $\mu$ M of CLA, and 7.8 ng/ml of ciprofloxacin. The collected CFCSs from EDL933 culture were labeled as E-CFCS<sup>wt</sup>, E-CFCS<sup>CLA</sup>, E-CFCS<sup>CLA-64 $\mu$ M</sup>, and E-CFCS<sup>cipro</sup> according to the specific inhibitory treatment present in the cultures. One set of samples were incubated only in DMEM<sup>+10%FBS</sup> was considered as control, E-CFCS<sup>EDL933</sup>. All CFCSs collected from EDL933 were stored at 4°C until further use.

*Collection of Stx produced by EDL933 in minimal media and evaluate their gene expression.* To induce Stx production by EDL933, one set of samples were cultured in M9 minimal media (M9) with an inoculum of ~4 logs without any antibiotic and another set of samples were incubated with sublethal concentration (7.8 ng/ml) of ciprofloxacin (M9+C). Both sets of samples were incubated at standard incubation condition for 48 h. The CFCS of EDL933 in M9 and M9+C was collected after centrifugation and filtration (Aditya et al., 2020a).

For comparing Stx gene expression, EDL933 cultures were collected after 5 h incubation from both M9 and M9+cipro media. RNA was extracted separately from these EDL933 cultures using TRIzol<sup>®</sup> reagent (Ambion by Life Technologies Co., Carlsbad, CA, USA) and cDNA was synthesized according to protocol previously described (Aditya et al., 2020a; Peng et al., 2018). Then, 40 ng of cDNA was used as a template for analyzing gene expression. The q-PCR reaction mixture was prepared according to PerfeCTa<sup>®</sup> SYBR<sup>®</sup> Green FastMix protocol (Quanta Biosciences, Beverly, MA, USA) and amplified in an Eco RealTime PCR system (Illumina, San Diego, CA, USA) with 30 s denaturation at 95°C, followed by 40 cycles of 95°C for 5 s, 55°C for 15 s, and 72°C for 10 s. The relative expression level of the target genes in treatment conditions were calculated by the comparative log fold change. The cycle threshold (C<sub>t</sub>) value of the target genes in the treatments were normalized to the reference gene (all genes used in this study are listed in Table 1) in control/treatment and then the relative expression of the genes were compared between respective treated and untreated conditions (Livak and Schmittgen, 2001).

*Cell cytotoxicity assay.* Viability of HCT 116 cells when cultured with CFCS collected from EDL933 separately was assessed using a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT) according to the protocol described previously (Riss et al., 2004). This is a colorimetric assay that measures the metabolically active cells which is visualized by formation of purple colored formazan crystals. Briefly, the HCT116 cells were cultured in a monolayer under standard condition in 96-well plate until 75% confluency was

achieved. After washing the monolayer three times with DMEM, it was incubated with 25% (v/v) of E-CFCSs, such as E-CFCS<sup>EDL933</sup>, E-CFCS<sup>wt</sup>, E-CFCS<sup>CLA</sup>, E-CFCS<sup>CLA-64 $\mu$ M</sup>, and E-CFCS<sup>cipro</sup>. As a positive control, commercially purchased Stx was used at concentration 2  $\mu$ g/ml. Cells that were cultured in absence of any of these treatments was considered as negative control while wells containing only DMEM<sup>+10% FBS</sup> media was used a blank. Following a 24 h and 48 h incubation period, 10  $\mu$ l MTT reagent was added to the wells and incubated again until purple intracellular precipitation was visible (within 2-4 h) under microscope. After that, 100  $\mu$ l DMSO was added to the wells and incubated in dark overnight. The absorbance of the plate was measured at 570 (OD<sub>570</sub>) nm and the equation below was used to calculate the cell viability:

$$\% \text{ viability} = [(OD_{570} \text{ of the treatment} - OD_{570} \text{ of blank}) / (OD_{570} \text{ of control} - OD_{570} \text{ of blank})] * 100.$$

The data was represented as a relative cell death as compared to the growth of cells that were grown without any treatment.

*Statistical analysis.* Student's t-test was performed in MS Excel to determine the statistically significant difference ( $p < 0.05$ ) between the growth and gene expression of EDL933 in presence or absence of treatments. Significant difference in the relative cell death was also determined by t-test as compared to the control (cells cultured without any treatment).

## Results

*Subversion of EDL933 growth under various treatment conditions.* Growth of EDL933 incubated with exposure to the various treatment conditions that were under evaluation was also measured at multiple pre-determined bouts of time to determine the range of inhibition potential for each of the treatments (Figure 1). A suppressive effect against EDL933 was documented in the early hours of incubation for bacteria treated with L-CFCS<sup>wt</sup> and L-CFCS<sup>CLA</sup> (Figure 1 A) with a difference of the duration of potency. More specifically, L-CFCS<sup>wt</sup> significantly reduced (~1.1 logs) EDL933 growth as compared to the control at 6 h, however after 12 h, their antimicrobial activity was attenuated, as shown by an uptick in the treatment group growth that has a 9 log CFU/ml similar to that of the untreated control at the same timepoint. Meanwhile, L-CFCS<sup>CLA</sup> significantly continued to exert antimicrobial pressure for 12 h before being attenuated and showing a similar growth pattern to the untreated control. When using purified isomers of LA, there was also a significant reduction of pathogen number (~1.6 logs) at 6 h (Figure 1 A) followed by their potency loss in subsequent incubation hours. As a positive control of this assay, a DNA targeting antibiotic known to induce Stx production, ciprofloxacin, was implemented to compare the experimental treatment to established antibiotics, as well as to account differences in the growth pattern of EDL933 when grown and treated in DMEM+10%FBS. The laboratory strain, i.e., EDL933, demonstrated to be sensitive to ciprofloxacin, with an MIC of 15 ng/ml, which lead to the use of a sub-lethal concentration (7.8 ng/ml) that allowed for the evaluation of the growth pattern of EDL933 under prolonged exposure to antimicrobial pressure. Results showed that conventional antibiotic, ciprofloxacin, exerted a continuous inhibitory effect on the bacteria over the various time points.

*Measurement of cell cytotoxicity in presence of Stx-containing E-CFCSs.* Changes in the cytotoxicity of E-CFCSs that were prepared through the different treatment conditions outlined in this study, were evaluated in a cell line of HCT116 by using an MTT assay, which specifically measures the metabolic activity of live cells within a given sample as an indicator of viability. After incubating HCT116 cells with pure Shiga toxin (2µg/ml) and 25% of the different types of E-CFCSs, such as E-CFCS<sup>cipro</sup>, E-CFCS<sup>wt</sup>, E-CFCS<sup>CLA</sup>, E-CFCS<sup>CLA-64µM</sup>, and E-CFCS<sup>EDL933</sup>, for 24 and 48 h separately, the relative cell cytotoxicity was measured as compared to the cells where no treatment was applied (Figure 3). Pure Stx lead to a decrease in viability of 55.21%, which can be attributed to the induction of cell death within 24 h of exposure. After 48 h under the same condition the documented cell death was 46.97%.

Exposure of host cells to E-CFCS<sup>EDL933</sup> demonstrated the highest level of cytotoxicity as seen in a decrease of cell viability rate of 66.27% and 63.60% when tested at 24 and 48 h respectively. In presence of E-CFCS<sup>cipro</sup> and E-CFCS<sup>CLA-64µM</sup> were shown to induce a similar rate of cytotoxicity after 24 h, with a decrease rate in viability of 49.85% and 51.11%, respectively. Interesting, at 48 h E-CFCS<sup>cipro</sup> was found to be more cytotoxic (70.75%), whereas cell death by E-CFCS<sup>CLA-64µM</sup> were almost negligible (0.55%).

By pre-treating the EDL933 with the total metabolic components of L-CFCSs, there was a reduction in the cytotoxicity of the subsequent E-CFCSs that was collected after treatment i.e., E-CFCS<sup>wt</sup> and E-CFCS<sup>CLA</sup>. This reduction in cytotoxicity was documented from E-CFCS<sup>CLA</sup>, in a 51.64% and 62.7% increased cell survivability as compared to the ones directly challenged with Stx and with E-CFCS<sup>EDL933</sup> at 24 h, respectively. Similar effect was also observed from E-CFCS<sup>wt</sup> at both 24 h and 48 h of incubation.

*Ciprofloxacin induces Shiga toxin genes of EDL933.* Changes in relative gene expression of Stx associated genes was measured in EDL933 grown in M9 minimal media (M9) and supplemented with the sublethal concentration of ciprofloxacin (7.8 ng/ml) (M9+C) to induce Stx production after 5 h of treatment (Figure 3). Two Stx genes (*stx2A* and *stx2B*, representing the active and binding subunit, respectively) were significantly upregulated when the bacteria were cultured in M9 media 8.81- and 9.63-fold ( $p < 0.05$ ), respectively as compared to control (EDL933 grown in LB broth). Sublethal concentration of ciprofloxacin also upregulated the Stx gene expression 11.31- and 10.57-fold ( $p < 0.05$ ), respectively.

Two DNA damage repairing gene, *recA* and *lexA* were also targeted for measuring their relative gene expression, considering their association with Stx production. In M9 media, *recA* and *lexA* was upregulated by 8.65- and 9.14-fold, respectively. Whereas presence of ciprofloxacin decreased their expression to 1.19- and 1.11-fold than only M9 media.

*Assessment of L. casei-originated metabolites in the neutralization of Stx present in E-CFCS<sup>M9</sup> and E-CFCS<sup>M9+C</sup> on pre-treated host cell line.* When evaluating the neutralizing potential of L-CFCSs, there was a significantly lower percentage of cell cytotoxicity induced by purified Stx and Stx present in E-CFCS<sup>M9</sup> and E-CFCS<sup>M9+C</sup>, when the cells were pretreated with either L-CFCS<sup>wt</sup>, L-CFCS<sup>CLA</sup>, or 64 µM of CLA (Figure 4). When host cells were not pretreated with L-

CFCSs or purified CLA, we found more than 70% cell death as compared to the control (where no pretreatment nor Stx was applied).

The specific nature of the metabolites that was used to pretreat the host cells, i.e., L-CFCS<sup>wt</sup>, L-CFCS<sup>CLA</sup>, or pure CLA, influenced extent of cell viability induced by pure Stx and Stx present in E-CFCSs. More specifically, upon pretreatment with L-CFCS<sup>wt</sup> an increase in cell viability was observed which was documented by 35.52%, 44.94%, and 34.11% cell deaths as compared to the no pretreatment group ( $p < 0.05$ ). Pretreatment with L-CFCS<sup>CLA</sup> and pure CLA demonstrated similar type of cell cytotoxicity toward Stx (Figure 4). However, L-CFCS<sup>CLA</sup> was showed better capability than CLA alone since we observed less cell cytotoxicity (29.01%) when they were exposed to Stx present in E-CFCS<sup>M9+C</sup>.

## Discussion

EHEC or *E. coli* O157:H7 is a major enteric pathogen which is specifically recognized by its Stx production ability and subsequent association to the development of HUS in patients (Bai et al.). Though the occurrence of HUS can be attributed to being mediated by *E. coli* O157:H7 outbreaks, the etiological agents for this complication can differ significantly as more virulent strain emerges in every outbreak. This indicates that there are certain bacterial factors that play a role in the developing HUS, which are still not clearly understood (Kulasekara et al., 2009). The largest *E. coli* O157:H7 outbreak in the US was in 1999 where 781 people were infected, occurred from contaminated drinking water. About 9% of the sick people were hospitalized among which 2% developed HUS and two deaths occurred in that outbreak (Rangel et al., 2005). Another outbreak occurred in 2006 due to contaminated spinach, where 102 (51%) were hospitalized and 31 (16%) developed HUS, with three deaths (CDC, 2006). However, further genomic analysis of an isolate from previous outbreaks known to have led to the development of HUS confirmed the presence of Stx-encoding genes (*stx2* and *stx2c*) (Pacheco and Sperandio, 2012). In addition to Stx production, genomic analysis of other isolates revealed the presence of *stx2* gene in other pathogenic *E. coli* strains such as enteroaggregative *E. coli* (EAEC, *E. coli* O104: H4). EAEC inherently forms biofilm in the intestine which makes it more difficult to treat. The combination of formation of biofilm and production of Stx, caused 908 HUS cases with 34 deaths in that outbreak (Pacheco and Sperandio, 2012; Sheikh et al., 2001). This indicate that like other pathogens, STEC also acquire new virulence properties which makes it even more difficult to treat (Kulasekara et al., 2009).

Generally, antibiotics are a popular therapeutic option to treat bacterial infections. But using them for the purpose of treating STEC is still controversial. Some *in vitro* studies have shown that roxithromycin, rokitamycin, clindamycin, minocycline, doxycycline, and macrolide decrease the amount of Stx, but they did not show significant bactericidal effect (Murakami et al., 2000). On the contrary, there are ample *in vitro* studies proving the association of antibiotic use and further Stx release. The sub-inhibitory and sub-lethal concentration of polymyxin B, trimethoprim, sulphamethoxazole, ciprofloxacin, cefixime, tetracycline, quinolone, azithromycin, and gentamycin have been shown to increase the Stx released as the bacterial cell wall is broken down (Grif et al., 1998; Ito et al., 1997; Karch et al., 1986; Karmali et al., 1985; Murakami et al., 2000; Yoh et al., 1999). The findings of this study were also in agreement with

this information as shown in the recording of higher cell cytotoxicity rates in the CFCS of EDL933 bacteria pre-treated with sub-lethal concentration of ciprofloxacin (7.8 ng/ml) (Figure 2 and 4), suggesting that this effect was attributable to the production of Stx because of these treatments. Different *in vivo* studies also have shown inconclusive results of antibiotic usage. Some studies concluded about the beneficial role of antibiotics even no correlation as well (Bell et al., 1997; Cimolai et al., 1992; Murray and Brater, 1993). So, it is difficult to make a concrete conclusion about the helpful role of synthetic therapeutics to treat EHEC infected patients because of the inability of large randomized, double-blind, placebo-controlled human trials (Panos et al., 2006).

Among many of the novel approaches on controlling STEC transmission and disease progression, utilization of the natural components is the most promising (Peng et al., 2018). The synergistic mechanism of action exerted by the metabolites produced by probiotic bacteria to control the growth and suppressing some virulence properties of STEC is well established (Aditya et al., 2019, 2020a; Peng et al., 2018). However, the molecular mechanism of these metabolic components on the Stx-production ability of STEC is still being thoroughly explored. In the human intestine, the direct interaction between gut microbiome including probiotic bacteria and their metabolites such as organic acids may suppress Stx production from STEC (Nawrocki et al., 2020). Other acellular factors from probiotic bacteria e.g., colicins, microcins, and other bacteriocins might suppress or promote Stx production while inhibiting the STEC growth (Carey et al., 2008; Nawrocki et al., 2020). Getting a wholistic idea about the association of any type of treatment (either synthetic or natural) is difficult because *in vivo* models do not demonstrate enough infection due to lack of Gb<sub>3</sub> receptors (Peng et al., 2020b). The understandable experimental shortcomings make it difficult to evaluate all possible factors related to Stx production and neutralization. As a result, to understand mechanistic details, we must depend on *in vitro* studies.

In our *in vitro* assay, overall, we found a relatively lower evidence of cell cytotoxicity in the MTT assay. Overall, the metabolites present in L-CFCS<sup>wt</sup>, L-CFCS<sup>CLA</sup> and purified 64 μM of CLA induced less Stx production from EDL933 which resulted in less cytotoxicity. Pretreatment of host cells with these metabolites also conferred protection against Stx by neutralization.

## Figures and Figure legends

Figure 1.

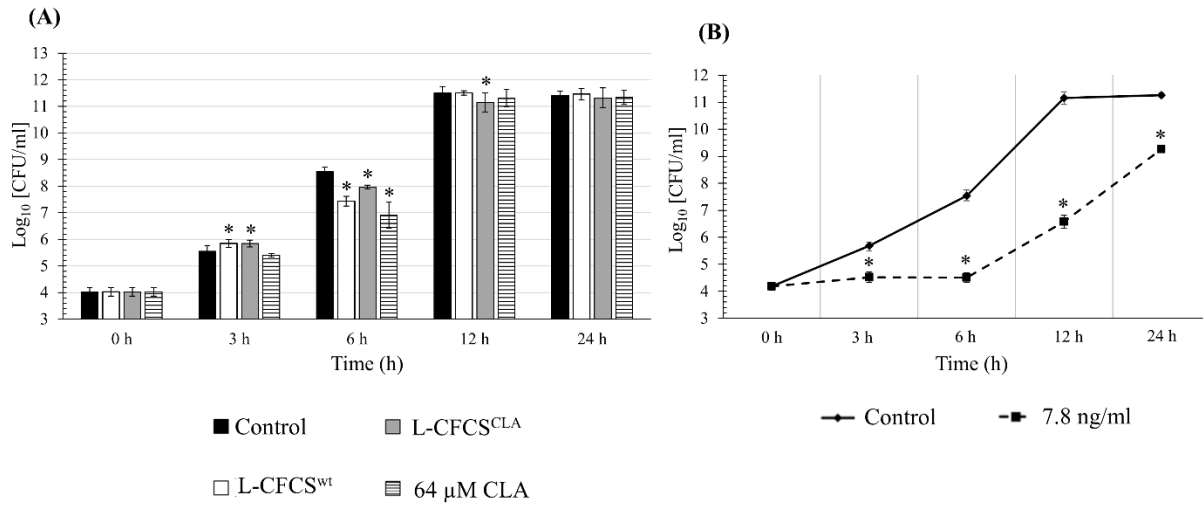


Figure 1: Growth suppressive effect of total metabolites against EDL933 collected from different strains of *L. casei* as well as CLA (A) and sub-lethal concentration of ciprofloxacin (B) at different time points of incubation. The asterisks (\*) indicate statistical significance ( $p < 0.05$ ) as compared to the control (no treatment present).

Figure 2.

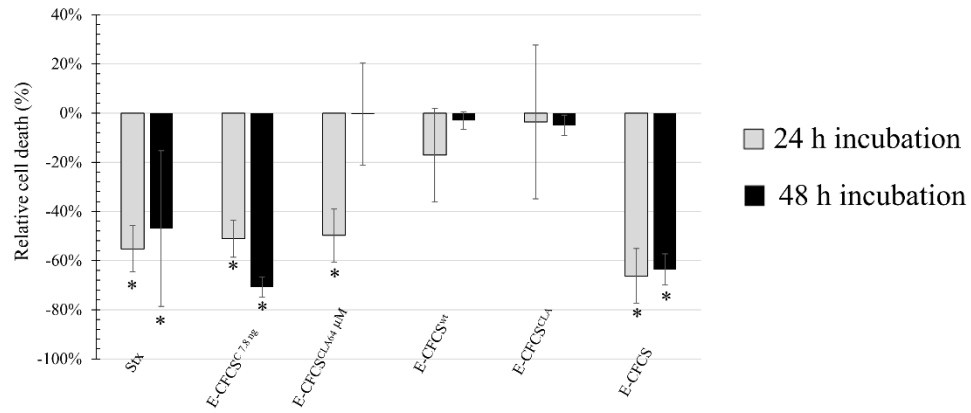


Figure 2. Evaluation of cytotoxin effect of Stx on the Gb3 containing host cells (HCT116) present in CFCSs collected under various conditions from EDL944. The asterisks (\*) indicate statistical significance ( $p < 0.05$ ) as compared to the control (only HCT116 cells).

Figure 3.

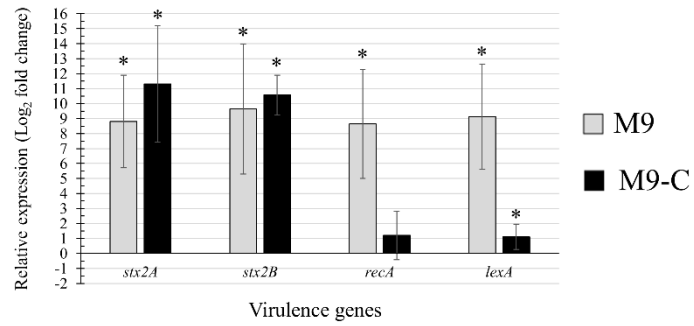


Figure 3. Upregulation of Stx gene of EDL933 in presence of sublethal concentration of ciprofloxacin as well as M9 media. The asterisks (\*) indicate statistical significance ( $p < 0.05$ ) as compared to the control.

Figure 4.

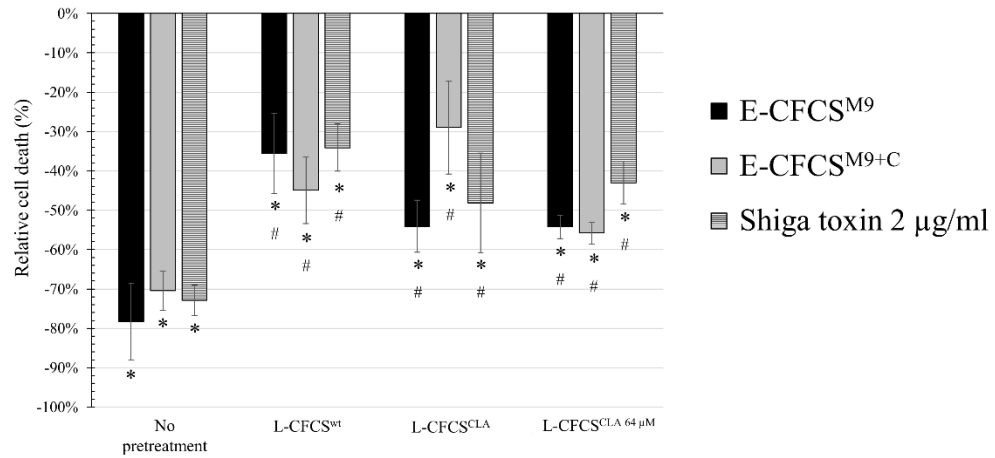


Figure 4. The relative cell death following various pretreatments. The asterisks (\*) indicate statistical significance ( $p < 0.05$ ) as compared to the control (only HCT116 cells) whereas, the hash marks (#) indicate significant difference as compared to the cells that did not receive any pretreatments.

## Summary and Future Directions

Various elements of a conventional dairy farm environment, such as water, feed, feces, soil, compost, and lagoon water harbored ~8.39% pathogenic *E. coli* among them ~21.64% was identified as STEC. Although antibiotic usage as a growth promoter is no longer practiced in the US, ~95.87% of pathogenic *E. coli* was found to be resistant to commonly used antibiotics whereas only ~4.12% was sensitive. Seasonal variation, i.e., summer and winter climatic conditions, played a role in the relative abundance of *E. coli* in the cattle grazing land soil; but carried equal risk of pathogen transmission from farm environment to susceptible hosts. This warrants proper precautions for outdoor recreational activities specifically food preparation, and agritourism.

As a preventative strategy against the pathogenic *E. coli* specifically EHEC, application of *L. casei*-originated metabolites in combination of 0.5% peanut flour was observed to be more effective than the *L. casei* alone. An engineered *L. casei* manipulated to convert higher amount of CLA was also very effective as compared to wild type *L. casei*. These combinations were also effective to control EHEC in a complex cultural condition such as collected rumen fluid (RF). Metabolites collected from the engineered *L. casei* showed stronger inhibition which was identified by their ability to eliminate the EHEC from RF within 72 h. None of the treatments showed negative impact on the relative abundance of rumen beneficial phylum, such as Bacteroidetes and Firmicutes.

*In vitro* studies documented that, purified CLA worked synergistically with other metabolites of *L. casei* and augmented their growth inhibitory property against EHEC at a lower concentration (64  $\mu$ M). Through evaluating the mechanism of total metabolites, we observed a multidirectional effect of the metabolites on the EHEC. The orchestrated effect of metabolites has been observed to cause disruption of cell membrane, interfering with cell division, and damage their genomic DNA. These metabolites of *L. casei*, as well as purified CLA, were not found to induce Shiga toxin production by EHEC since no significant host cell cytotoxicity was observed.

Further in-depth and extensive research can be performed to find out the responsible metabolites by investigating specific protein-ligand interaction through combining different computational approaches. The orchestrated effect of metabolites present in the CFCs of probiotics on the membrane and genomic DNA of EHEC could be potentially targeted. Since the metabolites of *L. casei*, specifically CLA, does not induce Shiga toxin production, it can be explored to develop a therapeutic for EHEC infections including HUS.

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