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# Lactic acid bacteria from traditionally fermented African food affect the diarrhoeagenic potential of enteroaggregative *Escherichia coli*


**by**

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# DECLARATION

I, Wisdom Selorm Kofi Agbemavor, declare that the thesis, which I hereby submit for the degree of PhD (Food Science) at the University of Pretoria, is my work and has not previously been submitted by me for a degree at this University or any other tertiary institution.

**Signature:** ……  ……

SEPTEMBER 2022

# DEDICATION

I dedicate this work to the Almighty God for His love and the gift of life; to my parents, Mr Wellington Korblah Doe Agbemavor, Madam Alice Korshiwor Sokah, and my children, Sedem, Senam and Sefakor.

# ACKNOWLEDGEMENT

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# ABSTRACT

**Lactic acid bacteria from traditionally fermented African food affect the diarrhoeagenic potential of enteroaggregative *Escherichia coli***

By

Wisdom Selorm Kofi Agbemavor

**Supervisor: Professor Elna Maria Buys**

**Degree: PhD Food Science**

Enteroaggregative *Escherichia coli* (EAEC) is one of the several aetiological agents accounting for acute and persistent (childhood) diarrhoea. Diarrhoea has several associated medical symptoms, including abdominal pains, the passage of watery stools, and the malabsorption of nutrients and water. Its acute form leads to stunting, cognitive growth impairment, hospitalisation, loss of productive hours, reductions in household and gross national income [Gross Domestic Product (GDP)] and even death in its severest form. Despite the several medical and pharmacological breakthroughs for treating persistent diarrhoea, the inadequacy of these interventions necessitated the exploration of other prophylactic and therapeutic techniques, including the use of probiotics. Probiotic bacteria confer beneficial effects on their host when consumed in adequate quantities. The competence of probiotics depends on their ability to undergo aggregation, surface hydrophobicity, and the production of antimicrobial substances against such (diarrhoeagenic) pathogens. Probiotics adhesion to the intestinal epithelium is a pre-requisite and strategy to colonise the gastrointestinal tract (GIT) to circumvent several pathogenic invasions. Some fundamental mechanisms underlying probiotics' mechanism of action include competitive exclusion, displacement, and inhibition of pathogens from adhesion to the intestinal epithelial cells (IECs) whilst playing significant roles in maintaining epithelial barrier

integrity and function. Thus, probiotics application as preventive medicine is emerging for controlling foodborne and inflammatory bowel infections.

Selected EAEC strains previously isolated from unpasteurised fresh milk samples were obtained. The unpasteurised fresh milk samples were collected from vendors in the Mpumalanga Province of the Republic of South Africa. Two lactic acid bacteria (LAB) strains, *Lactobacillus plantarum* FS2 and *Pediococcus pentosaceus* D39, with promising probiotic potentials, were obtained. These two LAB were previously isolated from traditionally fermented West-African food, *ogi*. The study was conducted in four main sub-studies (phases) with different specific objectives contributing to the primary research aim.

The aggregation and adhesion to hydrocarbons results from the first sub-study involving abilities exploration of the two bacteria indicate that the LAB (*Lactobacillus plantarum* FS2 and *Pediococcus pentosaceus* D39) and EAEC showed sufficient cell surface hydrophobicity (32.7 – 50.7 %) with diverse auto-aggregation (4.6 – 46.9 %) and co-aggregation (14.3 – 33.4 %) abilities. The auto-aggregation varied with different bacterial (LAB and EAEC) strains. The bacterial co-aggregation, cell surface hydrophobicity, and the competitive exclusion of EAEC by the selected LAB strains depended on the incubation (treatment) time and the bacteria strains involved. Contrary to other studies, no correlation was detected between auto-aggregation and adhesion to hydrocarbons for both EAEC and LAB strains. Similarly, auto-aggregation and adhesion to Caco-2 cells lacked correlation for the two combating bacteria. The LAB strains also demonstrated different levels of inhibition against the selected EAEC strains *in vitro*. These results indicate that two LAB from the traditionally fermented West African cereal, *ogi*, are potentially worth considering for further applications in fermented functional foods and food products that can be explored to enhance human health.

The second phase of the current study focused on determining the adhesion abilities of selected EAEC and LAB strains to IECs. This study continued to evaluate the competence of the LAB strains to

competitively exclude, displace and inhibit the selected EAEC strains from securing adhesion to the IECs. Results from this sub-study indicate that all the tested bacteria strains uniquely adhered to the Caco-2 cells. All LAB strains competitively excluded, displaced, and inhibited the EAEC strains from adhesion in different capacities in a strain-strain-dependent manner ($P < 0.05$). Overall, *Lactobacillus plantarum* FS2 demonstrated the highest adhesion to the IECs. The capability to competitively exclude, displace and inhibit the EAEC strains from adherence depended on both the pathogen and the LAB strains, implying several underlying mechanisms of action. Except for the EAEC strains, the low pH (2.5) gut stress factor did not affect ($P > 0.05$) LAB adhesion to the IECs. *In vitro* bile salt conditioning (pH 6.5) did not affect ($P > 0.05$) the adhesion of both LAB and EAEC strains. Thus, this sub-study shows that the tested LAB showed specific hindrance to EAEC adhesion to Caco-2 cell monolayers as demonstrated during the competitive exclusion, displacement, and inhibition. All the LAB strains, notably *Lactobacillus plantarum* FS2, showed excellent antagonistic effects against the selected EAEC, which can prevent gastroenteritis.

The third sub-study assessed potential probiotic LAB abilities to mitigate changes caused by diarrhoeagenic EAEC (D-EAEC) to the intestinal epithelium. Results from this study phase revealed that the treatment of polarised Caco-2 cell monolayers with LAB (*Lactobacillus plantarum* FS2 and *Pediococcus pentosaceus* D39) eased D-EAEC-imposed disruptions of intestinal epithelial barrier integrity and function as illustrated by the measurement of trans-epithelial electrical resistance (TEER) and the inflammatory cytokine [interleukin 8 (IL-8)] induction. Additionally, the results indicate that EAEC and LAB infection mode (as in competitive exclusion, displacement, and exclusion), as well as treatment time, significantly ($P < 0.05$) affected TEER, inflammatory cytokine (IL-8), and bacterial adhesion to the IECs. Furthermore, TEER and IL-8 induction due to infection with D-EAEC (K2) demonstrated a bacterial dose-dependent effect. Thus, the two LAB isolates conferred protective effects against disruptions caused by D-EAEC to the epithelial barrier integrity and function, indicating that both LAB have the potential to maintain and enhance their hosts' gut health.

The last phase of the study aimed at assessing the abilities of two LAB (*Lactobacillus plantarum* FS2 and *Pediococcus pentosaceus* D39) to protect, prevent, and alleviate disruptions caused by D-EAEC to the intestinal epithelial barrier function using Caco-2 cells. EAEC challenged IECs demonstrated very low TEER scores ($2.50 \pm 0.05\ \Omega.cm^2$) with significantly higher ($P < 0.01$) phenol red flux points contrary to their respective experimental controls and LAB ($10^9$ CFU/mL) treated cells. However, EAEC-influenced hyperpermeability was re-established significantly following competitive exclusion, displacement, and inhibition of EAEC by the (potential) probiotic LAB strains. Meanwhile, enterocyte treatment with EAEC cells leads to the translocation of significantly high numbers of EAEC cells (0.8 – 3.8 $\log_{10}$ CFU/ml) across it. In conclusion, the two potential LAB from *ogi* retained and reinstated the intestinal barrier structure and function by boosting important tight junction proteins expression and distribution, which can be used for food supplements, additives, and functional foods to treat various (gastrointestinal) diseases.

Thus, the four research sections (chapters) point to one thing. The two potential probiotic lactic acid bacteria [isolated from the traditionally fermented African food (cereal)] reduced the diarrhoeagenic potential of the enteroaggregative *Escherichia coli* from the unpasteurised milk samples. These findings implied that the two potential probiotic lactic acid bacteria have tremendous potential to prevent and treat gastrointestinal-related infections in different capacities. However, there is the need to further back these findings with clinical studies for potential clinical adaptations.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| $A_0$ | Initial Absorbance (Optical Density) Reading |
| $A_f$ | Final Absorbance Reading |
| A-AA | Auto-aggregation Ability |
| AAF | Aggregative Adhesive Fimbriae |
| A-AS | Auto-aggregation Score |
| $A_{CCIM}$ | Area of Cell Culture Insert Membrane |
| AIDS | Acquired Immune Deficiency Syndrome |
| ANOVA | Analysis of Variance |
| APEC | Avian Pathogenic *E. coli* |
| AS | Adhesion Score |
| $A_t$ | Absorbance Reading (Optical Density) at a given time |
| ATCC | American Type Culture Collections |
| ATP | Adenosine Triphosphate |
| BaS | Bacterial strain |
| BATH | Bacterial Adhesion to Hydrocarbon |
| BC | Bacterial Cell |
| BCEFA | Bacterial Competitive exclusion from Adhesion |
| BCSH | Bacterial Cell Surface Hydrophobicity |
| BDFA | Bacterial Displacement from Adhesion |
| BH | Bacterial Hydrophobicity |
| BID | Bacterial Infection Dose |
| BIFA | Bacterial Inhibition from Adhesion |
| BiS | Bile Salt |
| BSH | Bacterial Surface Hydrophobicity |

| | |
|---|---|
| Caco-2 | Human Epithelial Colorectal Adenocarcinoma |
| cAMP | Cyclic Adenosine Monophosphate |
| CCL20 | Chemokine Ligand 20 |
| Cdx-2 | Transcription Factor |
| CE | Competitive Exclusion |
| CEFA | Competitive exclusion from Adhesion |
| CFDA-AM | 5-Carboxyfluorescein diacetate acetoxymethyl ester |
| CFLSM | Confocal Laser Scanning Microscopy |
| CFU/mL | Colony Forming Units per Millilitre |
| cGMP | Cyclic Guanosine Monophosphate |
| CN | Catalogue Number |
| DAEC | Diffusely Adherent *Escherichia coli* |
| DCC-2CML | Differentiated Caco-2 Cell Monolayers |
| D-EAEC | Diarrhoeagenic Enteroaggregative *Escherichia coli* |
| DEC | Diarrhoeagenic *Escherichia coli* |
| DFA | Displacement from Adhesion |
| DMEM | Dulbecco's Modified Eagle Medium |
| DNA | Deoxyribonucleic Acid |
| EAEC | Enteroaggregative *Escherichia coli* |
| ECM | Extracellular Matrix |
| eDNA | Environmental Deoxyribonucleic Acid |
| EDTA | Ethylenediaminetetraacetic acid |
| EGFR | Epidermal Growth Factor Receptor |
| EHEC | Enterohemorrhagic *Escherichia coli* |
| EIEC | Enteroinvasive *Escherichia coli* |
| ELISA | Enzyme-Linked Immunosorbent assay |

| | |
|---|---|
| EPEC | Enteropathogenic *Escherichia coli* |
| ETEC | Enterotoxigenic *Escherichia coli* |
| FBS | Foetal Bovine Serum |
| FIS | Factor for Inversion Stimulation |
| GALT | Gut-Associated Lymphoid Tissue |
| GIT | Gastrointestinal Tract |
| GRP | Glucose-Regulated Protein |
| HA | Hydrophobicity Ability |
| HGT | Horizontal Gene Transfer |
| HI | Humidified Incubator |
| HIV | Human Immunodeficiency Virus |
| *hlyE* | Haemolysin Encoded Gene |
| HRP | Horseradish Peroxidase |
| HT29 | Human Colon Carcinoma |
| IBD (IBS) | Inflammatory Bowel Diseases (Syndrome) |
| ICT | Intercellular Tight Junctions |
| IEC | Intestinal Epithelial Cell |
| IFA | Inhibition from Adhesion |
| IFN-α | Alpha Interferon |
| IgA | Immunoglobulin A |
| IH | Incubation Hour |
| IL-1RA | Interleukin-1-Receptor Antagonist |
| IL-1β | Interleukin-1 Beta |
| IL-8 | Interleukin-8 |
| INT | Integrated Neurocognitive Therapy |
| LAB | Lactic Acid Bacteria |

| | |
|---|---|
| LDH | Lactate Dehydrogenase |
| LEE | Locus of Enterocyte Effacement |
| LGT | Lateral Gene Transfer |
| LSD | Least Significant Difference |
| MAPK | Mitogen-Activated Protein Kinase |
| mL | Millilitre |
| MLST | Multi-Locus Sequence Typing |
| MRS | de Man, Rogosa and Sharpe |
| MUC | Mucin |
| ND-EAEC | Non-Diarrhoeagenic Enteroaggregative *Escherichia coli* |
| NF-κβ | Nuclear (Transcription) Factor kappa β |
| NHLS | National Health Laboratory Service |
| NICD | National Institute for Communicable Diseases |
| OD | Optical Density |
| PBB | Probiotic Bacteria |
| PBS | Phosphate Buffered Saline |
| PCC-2CML | Polarized Caco-2 Cell Monolayer |
| PET | Putative Enterotoxin |
| PKC | Protein Kinase C |
| PMN | Polymorphonuclear Neutrophils |
| qRT-PCR | Quantitative Real-Time Polymerase Chain Reaction |
| R | Correlation Coefficient |
| $R^2$ | Regression Coefficient |
| $R_a$ | Actual Resistance |
| $R_b$ | Background (Blank) Resistance |
| RT | Room Temperature |

| | |
|---|---|
| SBiS | Simulated Bile Salt |
| SEM | Scanning Electron Microscopy |
| SGF | Simulated Gastric Fluid |
| *shF* | *Shigella flexneri* |
| SMAC | Sorbitol-MacConkey Agar |
| STEC | Shiga toxin-producing *E. coli* |
| TBCs | Treated Bacterial Cells |
| TEM | Transmission Electron Microscopy |
| TJ | Tight Junctions |
| TLR | Toll-Like Receptors |
| TMB | Tetramethylbenzidine |
| TNF | Tumour Necrosis Factors |
| tRNA | Transport Ribonucleic Acid |
| TSA | Tryptone Soy Agar |
| TSB | Tryptone Soy Broth |
| TSP | Thrombospondin |
| TSP-1 | Thrombospondin-1 |
| TT | Treatment Time |
| UTBCs | Untreated Bacterial Cells |
| UTC | Untreated Controls |
| WHO | World Health Organisation |
| ZO | Zonal Occludins |

# CHAPTER 1.

# INTRODUCTION

Irrespective of the global success concerning the decline of reported cases over the past 30 years, acute diarrhoea (gastroenteritis) globally continues to remain the second principal cause of mortality, particularly among children below five years of age, principally as a result of infections of the gastrointestinal tract (GIT) (Florez *et al.*, 2020; Liu *et al.*, 2012; Posovszky *et al.*, 2020; Walker *et al.*, 2012). Out of the 6.9 million mortalities that were reported among children less than five years of age in the year 2011, diarrhoea was projected to account for 9.9% of mortalities (Liu *et al.*, 2012; Walker *et al.*, 2013a).

Various aetiological agents have been identified to be responsible for this clinical condition; predominantly, diarrhoeagenic *Escherichia coli* pathotypes accounted for about 15% of mortalities, mostly among children five. Diarrhoea contraction was mainly through gastrointestinal tract infection (Amisano *et al.*, 2011; Omolajaiye *et al.*, 2020; Webb and Starr, 2005; Zhou *et al.*, 2018).

The pathotypes of *E. coli* differ in their virulence attributes and mechanisms of infection, especially within the gastrointestinal tract, thereby inflicting various forms of physical damage, coupled with inflammation of the epithelium (Govindarajan *et al.*, 2020; Kaper *et al.*, 2004; Pakbin *et al.*, 2021; Shawki and Mccole, 2017; Weintraub, 2007). Among diverse diarrhoeagenic *E. coli*, more than 15% of infections are attributable to enteroaggregative *E. coli* (EAEC). Due to the considerable damage caused by these pathogens to the intestinal epithelium, the primary function of epithelial tissue, particularly the absorption of nutrients, minerals, and water, becomes compromised. This event leads to the onset of diarrhoea, which is characterised by the passage of watery stools amongst other clinical symptoms (Balestrieri *et al.*, 2020; Ciccarelli *et al.*, 2013; Ferri, 2014; Kiela and Ghishan, 2016; Singh and Fleurat, 2010). Prolonged acute diarrhoea and hence malabsorption of nutrients by the epithelium could lead to malnutrition, stunting and impairment of cognitive development, particularly among

growing children (Ciccarelli *et al.*, 2013; Farthing *et al.*, 2013; Kyle *et al.*, 2015; Walker *et al.*, 2013a). The situation accounts for frequent morbidities and mortalities among children, especially in developing countries (Liu *et al.*, 2012; Liu *et al.*, 2015).

It is increasingly becoming evident through research that probiotics, either in singles or in combinations, are effective in the prevention and treatment of acute diarrhoea (Collinson *et al.*, 2020; Guarino *et al.*, 2015; Islam, 2016; Lubbert, 2016; Plotnikova and Zakharova, 2015; Wu and Zhan, 2021). Probiotic bacteria fight diarrhoeagenic bacteria by competing for binding sites, and they also prevent the attachment of the pathogens to the epithelium of the GIT (Kudera *et al.*, 2020; Pique *et al.*, 2019; Van Zyl *et al.*, 2020). Probiotics compete with enteropathogens, including the diarrhoeagenic ones, for available nutrients and secrete certain antimicrobial substances like peroxides, organic acids and bacteriocins, lowering the luminal pH (Khaneghah *et al.*, 2020; Monika *et al.*, 2021; Prabhurajeshwar and Chandrakanth, 2019; Prabhurajeshwar and Chandrakanth, 2017; Zhang *et al.*, 2020). The selection of probiotic candidates is mainly based on their survival and stability in the gut, the bile and acidic conditions as pertains to the GIT, and their ability to colonise the mucosal lining of the intestine (Fečkaninová *et al.*, 2019; Pino *et al.*, 2019; Suvarna *et al.*, 2018). They enhance intestinal motility, upgrading and regulating genes mediating intrinsic immunity within the epithelium, which further contributes to the eradication of the diarrhoeagenic pathogens (Azad *et al.*, 2018; Do Carmo *et al.*, 2018; Fernandez and Marette, 2017; Pandey and Gokhale, 2021; Raheem *et al.*, 2021).

Despite the increasing body of knowledge regarding the valuable health benefits of probiotics (Hempel, 2014; Kim *et al.*, 2016; Strompfova *et al.*, 2017; Wang *et al.*, 2020), there is inadequate information on the application of non-dairy probiotics in the prevention of gastrointestinal inflammation (diarrhoea) as compared to their dairy counterparts. Moreover, studies with most pathogens regarding diarrhoeal diseases are mainly focused on clinical isolates, unlike food and environmental sources. Therefore, this study seeks to evaluate the effect of *Lactobacillus plantarum* FS2 and *Pediococcus pentosaceus* D39 in preventing aggregation, attachment, and inflammation of

intestinal epithelial cells (IECs) by EAEC. The output of this research could be vital to the food industry, such that it will inform the risk assessment due to contamination with these pathogens, positively impacting food safety and security assessment. Also, since probiotic bacteria in this study is not from a dairy source, it can be taken up by nutraceutical industries to produce probiotic (food) supplements for lactose intolerant individuals, amongst others.

# CHAPTER 2.

# LITERATURE REVIEW

This section will discuss the aetiological agents, prevalence, and consequences of diarrhoeal or gastroenteritis infection, especially in young children in some selected geographical locations. Among the causative agents, emphasis will be laid on enteroaggregative *Escherichia coli* (*E. coli*) (EAEC), mode of infection and transmission shall be reviewed. The review will further address the characteristics, mode of transmission and reservoirs together with various theories accounting for the virulence, epidemiology, and pathogenesis, which explains the mechanism of EAEC causing infection within the gastrointestinal tract of its host. It will continue to touch on preventive and treatment measures with much emphasis on using probiotics. In this section, the various theories explaining the different modes of probiotic action will be discussed regarding cell culture (in-vitro) models, which is a true reflection of what transpires in-vivo.

## 2.1 Diarrhoea

Diarrhoea is a clinical condition of having frequent and loose or liquid bowels more than usual each day which may last for a couple of days, leading to dehydration through loss of excess body fluid. The onset of dehydration is characterised mainly by the failure of the characteristic skin elasticity and short-tempered behaviour. The condition is due to inflammation of the epithelial layers of both the small intestines and stomach because of infection with pathogenic microbes (Anbazhagan *et al.*, 2018; Attia *et al.*, 2016; Guerrant *et al.*, 2021).

### 2.1.1 The prevalence of diarrhoea infection

Acute gastroenteritis and diarrhoea diseases account for the mortality of millions of young children annually, particularly in developing countries with so many economic consequences. It was the reason for attendance to outpatient and emergency Departments and hospitalisation even in developed countries (Freedman *et al.*, 2015; Tewell *et al.*, 2018). About 10% of 220 000 hospitalisations, over

1.5 million visits to the outpatient departments, and about 300 deaths of kids below the age of five annually, with an estimated cost of about one billion US dollars, were due to diarrhoeal infection (Chiriac *et al.*, 2017; Kumar *et al.*, 2014). In Australia, it is responsible for almost 10 000 hospitalisations, 115 000 visits for consultations with general practitioners and close to 22 000 attendance to emergency departments yearly for rotavirus-related gastrointestinal infections among children in the same age group, with a projected cost of 23 million US dollars (Aliabadi *et al.*, 2016; Davey *et al.*, 2015). 204 out of 1000 (20.1%) visits to general practitioners with children under five years were for gastroenteritis. The annual hospitalisation frequency for these children was approximately seven (0.7%) for every thousand children in the United Kingdom (Nissen *et al.*, 2019; Verstraeten *et al.*, 2017). Usually, the cost implications of diarrhoea and gastroenteritis are very high but mostly underrated since related expenses to the family and absence from work are typically disregarded. So many diarrhoeal-related diseases have caused mortalities, particularly among children below five years, mainly in most sub-Saharan African countries just as in South Asia (Ogbo *et al.*, 2017; Tambe *et al.*, 2015).

Most often, in childcare institutions, when children get infected, they go asymptomatic and can transmit the infection innocently to others. Malnourished infected children are primarily at higher risk of complications. Children from north Australia, Torres Strait Islanders and Aboriginals were reported to have higher frequencies of hospitalisations due to malnutrition, electrolyte disturbance, gastroenteritis, and comorbidity (particularly hypokalaemia) with extended hospitalisations than their foreign colleagues (Fernandez-Garrido *et al.*, 2021; Florez *et al.*, 2020).

### 2.1.2 Symptoms of diarrhoea

Diarrhoeal (gastrointestinal) infection, also called gastroenteritis, is gut inflammation involving the small intestine and the stomach. The condition is mainly characterised by abdominal pain, vomiting and then the passage of watery stools (Ciccarelli *et al.*, 2013; Galanopoulos *et al.*, 2020; Valentini *et al.*, 2013). Dehydration is the critical hazard of gastroenteritis; therefore, rehydration is vital to the

infected individual. However, gastroenteritis conditions are often self-limiting and get resolved automatically within a few days. Diarrhoea leads to reduced urination and increased heart bit and reductions in sensitivity as severity increases. It is noteworthy that it is normal for lactating babies could pass loose but non-watery stools and should not be confused with being infected (Auliailahi and Oginawati, 2020; Ciccarelli *et al.*, 2013).

The prevalence of diarrhoea among immunocompromised individuals such as infants, children of pre-school age, and the aged could be fatal (Guarino *et al.*, 2014; Liesman and Binnicker, 2016). In such situations, there is a need for the urgency of quick diagnosis and, once confirmed, to be immediately followed up with suitable and timely treatment. Faecal incontinence and overflow due to chronic constipation, which is common in children, may indicate spurious diarrhoea (Butcher, 2019; Peng and Qureshi, 2020). In young children, vomiting and running stomach are non-explicit symptoms, and so, gastroenteritis diagnosis should be questioned in children in the presence of high fever, extended symptoms together with the means of transmission; either through the respiratory channel or by faecal-oral (Koukou *et al.*, 2015; Mayindou *et al.*, 2016).

### 2.1.3 Mode of infection and transmission

Gastrointestinal (diarrhoea) infections are mainly developed through consuming (faecal) contaminated water or food or directly from another infested person. Usually, infants and toddlers stand a greater risk for gastroenteritis and subsequent diarrhoea due to a relative lack of or inadequate resistance against intestinal pathogens (Akhtar, 2015; Ngure *et al.*, 2019). This inadequate or lack of resistance is due to an immature gastrointestinal immune system coupled with the absence of previous exposure to infectious pathogens (Rhoades *et al.*, 2021; Silverman *et al.*, 2017) as well as their risky exploratory behaviours (Headey *et al.*, 2017; Ngure *et al.*, 2019). This situation leaves both their hands and feet so dirty, yet they frequently put these unclean hands and even other objects into their mouths, as often seen among toddlers under the age of two (Reid *et al.*, 2018; Williams *et al.*, 2021).

### 2.1.4 Consequences of diarrhoea infection

Early childhood diarrhoea continues to trouble many countries killing about 500 000 children annually, thus more than 1000 children daily worldwide. Most enteropathogens, including EAEC, disrupt (damage) the absorptive and barrier functions of the gut, leading to systemic and intestinal inflammation. Thus, the role of the intestinal epithelium (gut) in absorbing both macro and micronutrients and for protection becomes compromised. The situation gets worst with multiple enteric infections. Eventually, this leads to the silent pandemic of moderate to severe stunting which was estimated to affect 144 million children globally particularly during their first two essential formative years, mainly within deprived communities (Troeger *et al.*, 2018b; Vaivada *et al.*, 2020). This silent pandemic has devastating consequences for children who survive without being symptomatic but endure malnutrition coupled with multiple or repeated enteric infections during their early life. African, Asian, and Latin American children have recorded a steady decline in their height-for-age Z (HAZ) scores over their first two years of life (Rippe, 2021; Victora *et al.*, 2010). Some previous researchers illustrated the involvement of early childhood intestinal infections with growth retardation by correlating growth failure with recurrent diarrhoea and other diseases, followed by deviations in the growth curves of children (Walson and Berkley, 2018). In Santa Maria Cauque children, for example, their growth rate fell off their expected growth curve with repeated enteric infections together with other illnesses, which mostly coincides with their winning and crawling stages (Deboer *et al.*, 2017; Guerrant *et al.*, 2016). The situation further aggravated the concomitant ingestion of all items, including foods from their contaminated environments (Ngure *et al.*, 2019; Woh *et al.*, 2021). Surprisingly, malnourished children with fewer diarrhoea records experience remarkable “catch-up” with their growth, which closely follows the growth curve of healthy children. Undernourished children, on the other hand, experience severe diarrhoea fulfilling a vicious bidirectional cycle involving diarrhoea and malnutrition (Korpe *et al.*, 2016; Schorling *et al.*, 1990; Yackobovitch-Gavan *et al.*, 2018). Acute diarrhoea impairs cognitive development, especially among toddlers and young

children, as manifested by severely malnourished children with several diarrhoea episodes scoring lower marks on a series of aptitude tests (Investigators, 2018; Mokomane *et al.*, 2018a; Pinkerton *et al.*, 2016). It further contributes to a reduction in physical fitness and productivity of adults (David *et al.*, 2015; Frandemark *et al.*, 2018; Mokomane *et al.*, 2018b). Severe diarrhoea has been associated with anaemia which can be attributed to the impairment of the intestinal epithelium regarding the absorption of minerals and end products of absorption from the lumen (Ganguly *et al.*, 2015; Jayaweera *et al.*, 2019).

### 2.1.5 Causes and other aetiological agents of diarrhoea infection

The causative agents of diarrhoea include bacteria and viruses. The bacterial causative agents include *Yersinia enterocolitica*, non-typhoid *Salmonella* spp, *Campylobacter jejuni*, Shiga toxin-producing *E. coli*, enteropathogenic *E. coli*, *Shigella* spp, *S. paratyphi*, *S. typhi*, *Strongyloides stercoralis*, helminths I and *Vibrio cholera* (Benmessaoud *et al.*, 2015; Lee *et al.*, 2019a; Saeed *et al.*, 2015; Zhou *et al.*, 2018). The protozoa include *Cryptosporidium*, *Giardia lamblia*, *Entamoeba, histolytica*.

## *2.2 E. coli*

*E. coli* (the laboratory workhorse) is long known as an innocuous commensal within the gastrointestinal tract of homeothermic animals. The organism gains or/and loses specific genes to enhance its pathogenicity and environmental adaptation (Croxen *et al.*, 2013; Desvaux *et al.*, 2020; Salazar *et al.*, 2021). A broad scope of human illnesses ranging from the gastrointestinal tract to extra-intestinal locations; for instance, the nervous system, bloodstream and the urinary tract can be caused by *E. coli* (Carding *et al.*, 2015; Dale and Woodford, 2015; Gordon *et al.*, 2015). Aside from several other aetiological agents, pathogenic *E. coli* has been a significant cause of diarrhoea (Khosravi *et al.*, 2016; Schaumburg *et al.*, 2020; Zhou *et al.*, 2018).

### 2.2.1 Characteristics of *E. coli*

Commensals and pathogenic variants of *E. coli* can vary in genomic size by one million base pairs. This additional genetic material can comprise numerous genes for virulence and fitness. Comparative genomics studies of the organism indicated its genome is split between a conserved, also known as the core genome, a shared, and a flexible gene pool. Therefore, the pathogenicity and virulence of EAEC, like other pathotypes, depend on its supple gene pool. The gene pool reflects the loss and gain of genetic material at a few critical spots along its genome (Denamur *et al.*, 2021; Jang *et al.*, 2017; Lu *et al.*, 2016).

About a quarter of the genomic contents of EAEC 042 strain consists of genomic islands like those identified in Shiga toxin-producing *E. coli*, O157:H7 strain EDL933 (Povolotsky and Hengge, 2016; Segura *et al.*, 2017). Horizontal (lateral) gene transfer (HGT or LGT) occurs through the movement of DNA between prokaryotic hosts by means such as conjugation transduction which is encoded by genetic elements (Juhas, 2015b; Soucy *et al.*, 2015). The bacterial chromosome can also be invaded by genetic factors like plasmids, bacteriophages, transposons, and insertion sequences or even by auto-replication to offer novel characteristics with adaptive and competitive advantages within the new host (Croxen *et al.*, 2013; Siguier *et al.*, 2017). Most genetic materials associated with distinct virulence in pathogenic *E. coli* originated from their flexible genetic factors. The origination of several genetic factors for colonization and toxin production from the plasmids is a typical example (Morales *et al.*, 2015; Sarowska *et al.*, 2019).

Bacteriophages play a significant role in *E. coli* genome plasticity. Though most phages are faulty, some still form infectious elements. In a STEC O157:H7 isolate, 18 phages were found, including two of which have the Shiga toxin (Stx). These were capable of infecting other *E. coli* strains and thereby contributing more to the horizontal gene transfer (Juhas, 2015a; Porse *et al.*, 2017). Whereas most virulence factors are related to phages, it is possible to acquire auxiliary genes on phages (Hernandez-Doria and Sperandio, 2018; Olsen *et al.*, 2020b). For instance, transfer ribonucleic acids (tRNA) genes

have been known for rare codons and are used mainly by external genes which were introduced in STEC isolates (Hernandez-Doria and Sperandio, 2018; Javadi *et al.*, 2017; Olsen *et al.*, 2020a).

Most bacteria that undergo lateral gene transfer diversify with entirely novel characteristics through acquiring new genes. In most *Shigella* spp, for example, the rate of gene loss was reported as more than that of some pathogenic strains of *E. coli* counterparts, and this could explain its limited lifestyle and host spectrum (Hawkey *et al.*, 2020; Pasqua *et al.*, 2017). In another study, Shigella was found to have about 447 to 978 gene deletions, and the pseudogenes along its genome were far above those of pathogenic *E. coli* (Feng *et al.*, 2011; Mccutcheon, 2021). These processes of gene loss, also known as pathoadaptivity by pathogenic bacteria, may be an underestimated mode of action for pathogenesis which permits their survival within their host (Diard and Hardt, 2017; Feng *et al.*, 2011; Yue *et al.*, 2015). The act of decarboxylase and lysine discharge was reported in Shiga toxin-producing *E. coli* (STEC), Enterotoxigenic *Escherichia coli* (ETEC), and Enteropathogenic *Escherichia coli* (EPEC), in addition to EAEC. These pathological adaptive injuries may promote virulence in pathogenic EAEC strains and STEC O157:H7 (Bessone *et al.*, 2017; Langendorf *et al.*, 2015; Sinha *et al.*, 2015). Pathotype description is dependent mainly on the set(s) of genetic signatures for the phenotypic characteristics. Due to the flexibility of the genomic material of *E. coli*, with improved genomic and sequencing studies, designating specific isolates as pathotypes is mostly very difficult (Blount, 2015; Croxen *et al.*, 2013; Flament-Simon *et al.*, 2020).

### 2.2.2 *E. coli* pathotypes

Diarrhoea-causing (diarrhoeagenic) *E. coli* have been categorised into six major groups (pathotypes) based on their pathogenicity. These pathotypes include enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), enteropathogenic *E. coli* (EPEC) and enteroinvasive *E. coli* (EIEC). The rests are Shiga toxin-producing *E. coli* (STEC) and enterotoxigenic *E. coli* (ETEC). However, a seventh pathotype has been identified as adherent invasive *E. coli* (AIEC). It is associated with epidemiology, diagnosis, detection, pathogenesis, human diseases, and public health like the other

pathotypes (Aijuka *et al.*, 2018; Cabrera-Sosa and Ochoa, 2020). With different pathotypes causing diarrhoeal diseases, the location and mechanism of colonization, the clinical symptoms and outcomes vary widely, indicating the diversity of *E. coli* (Askari Badouei *et al.*, 2016; Newell and La Ragione, 2018).

## 2.3 Enteroaggregative *E. coli*

It was established that EAEC was the commonest pathogenic bacteria detected in the faecal samples of diarrhoea patients (Lääveri *et al.*, 2016; Lääveri *et al.*, 2018). This pathotype matched the adherence models of over five hundred isolates from a study (Lara *et al.*, 2017; Serichantalergs *et al.*, 2017). From the preliminary identification of EAEC, it has been isolated in global epidemic and endemic diseases in relation to diarrhoea (Lara *et al.*, 2017; Raseena Beegum *et al.*, 2019) and in quite a number of extensive surveys, it has been found to account for persistent diarrhoea particularly among young children within diarrhoeal endemic locations (Ashkenazi and Schwartz, 2020; Chissaque *et al.*, 2018; Kattula *et al.*, 2015); persistent diarrhoea among human immunodeficiency virus (HIV) and acquired immune deficiency (AIDS) patients (Ellis *et al.*, 2020; Seid *et al.*, 2018) in addition to being root cause of traveller's diarrhoea (Bamidele *et al.*, 2019; Jiang and Dupont, 2017; Van Hattem *et al.*, 2019).

### 2.3.1 Characteristics and classification of enteroaggregative *E. coli*

Selected EAEC strains were identified with the help of a probe that was subsequently discovered to hybridize a cassette transporter device for binding adenosine triphosphate (ATP) and which is capable of translocating dispersin through the bacterial cell wall (Blanton *et al.*, 2018; Dias *et al.*, 2020). EAEC strains that were positive towards this probe were identified to possess the *aggR* factor. EAEC was further categorised into two major groups; atypical (lacking *aggR*) and typical (possessing *aggR*) (Dias *et al.*, 2016a; Dias *et al.*, 2020; Vijay *et al.*, 2015).

Further classification of EAEC was based on their pattern of adherence. Some EAEC strains were found only to infect the ileum, whereas others infected both the ileum and the colon (Adefisoye and

Okoh, 2016; Dias *et al.*, 2020; Santos *et al.*, 2020; Spano *et al.*, 2017). Serotyping was also used to classify this pathotype but was found to be challenging due to the autoagglutination of several strains. A range of serotypes was identified. However, it was found that in the presence of Hep-2 cells, EAEC strains with similar serotypes were attached in different models, which is a conventional technique for classifying and detecting the diverse *E. coli* pathovars (Dias *et al.*, 2020; Ori *et al.*, 2018; Sukkua *et al.*, 2015).

The sequence type complexes of the EAEC, 126 isolates from a situation-control survey in Nigeria Using multi-locus sequence typing (MLST), shared similarities with those strains studied from other parts of the world (Guerrieri *et al.*, 2020; Okeke *et al.*, 2010). EAEC were also noted to comprise various pathogenic lineages, stressing the diversity of this pathotype worldwide. It was established that a single strain could not be characteristic of this pathotype. Therefore, scientists should accurately identify typical virulence genes of EAEC at the very onset whilst considering the modes of infection within different strains (Guerrieri *et al.*, 2020; Okeke *et al.*, 2010).

### 2.3.2 Epidemiological theories of EAEC

Several epidemics, including an outbreak in Japan due to a school lunch that made 2,697 children sick, were attributed to EAEC in 1997. This *E. coli* pathotype also accounted for the diarrhoea outbreak that struck a settlement in India and left nearly 15% of the people sick of diarrhoeal infection (Kubomura *et al.*, 2017; Modgil *et al.*, 2020a; Modgil *et al.*, 2020b). The presence of diarrhoeagenic EAEC strains in Africa was established in recent surveillance in Nigeria (Ifeanyi *et al.*, 2015; Saka *et al.*, 2019), Libya (Ahmed *et al.*, 2017) and sub-Sahara Africa (Knee *et al.*, 2018; Kotloff *et al.*, 2013). The most typical cause of bacterial diarrhoea in the outpatient departments and the emergency units of two major academic hospitals in Connecticut and Maryland in an extensive study was found to be attributed to EAEC infection (Stockmann *et al.*, 2017; Tarr *et al.*, 2021).

Germany also recorded a significant diarrhoea epidemic in 2011, which accounted for four thousand, three hundred and twenty-one (4 321) who were formerly healthy but became diseased. Over nine hundred (900) out of the 4 321 patients further acquired haemolytic uremic syndrome (HUS), with more than fifty (50) mortalities recorded (Grad *et al.*, 2012; Radosavljevic *et al.*, 2015; Rosner *et al.*, 2017). An *Stx*-encoding *E. coli* was detected as O104:H4 and found to contain virulent genes (*aggR*, *aggA*, *aap*, *set1*, and *pic*), which is characteristic of typical EAEC strains and (*stx 2*) as found in STEC (Boisen *et al.*, 2015; Estrada-Garcia and Navarro-Garcia, 2012; Kampmeier *et al.*, 2018; Kimata *et al.*, 2020).

The phage possessing *stx* was found to be strongly associated with the phage of STEC O111:NM strain (Haarmann *et al.*, 2018; Laing *et al.*, 2012). This phage was obtained possibly in the phylogenetic record of strains from the epidemic (Bai *et al.*, 2019; Kimata *et al.*, 2020). EAEC has a conventional mode of obtaining Shiga toxins, as reported from Northern Ireland, France, Japan, and the Central African Republic (Bai *et al.*, 2019; Dallman *et al.*, 2012; Newell and La Ragione, 2018; Paletta *et al.*, 2020). According to the reports, the EAEC isolates obtained from Haemolytic Uremic Syndrome (HUS) patients were described to be *stx*-positive (Bai *et al.*, 2016; Ferdous *et al.*, 2015; Kampmeier *et al.*, 2018; Senthakumaran *et al.*, 2018; Zhi *et al.*, 2021). These occurrences predict the likelihood of potential epidemics, hence the need for ongoing research to focus on the incidence and outbreaks to obtain a comprehensive picture of EAEC.

### 2.3.3 Virulence and pathogenic theories of EAEC

In a study to evaluate pathogenicity and virulence of diarrhoeagenic EAEC, non-*Stx* variants of EAEC 042 strain were employed as a classical strain because of its ability to elicit diarrhoea in most volunteers (Cox *et al.*, 2020; Valeri, 2015). Nevertheless, the genes for the expression of adhesins, toxins and other proteins concerning virulence were mostly found to be different among the various strains analysed (Dias *et al.*, 2020; Estrada-Garcia and Navarro-Garcia, 2012; Petro *et al.*, 2020; Sarowska *et al.*, 2019). Also, it was reported that there is a wide diversity regarding their specific location of

infection in the gastrointestinal tract (Mathew *et al.*, 2019; Nascimento *et al.*, 2021; Rogawski *et al.*, 2017). EAEC 042 strain, for instance, was sequestered from the jejunum of infected individuals, whereas the same strain firmly attached to colonic, ileal and jejunal mucosae (Anand *et al.*, 2016; Menge, 2020; Rossi *et al.*, 2018; Segura *et al.*, 2021). In another study involving five diverse non-Shiga (*Stx*) toxin, EAEC isolates from pre-schoolers, it was noteworthy that each isolate exhibited a varying attraction for the colonic, ileal and jejunal mucosal layers (Stavroulaki, 2020). Regardless of the heterogeneous nature of the various non-*Stx* EAEC strains, a general three distinct models have been found to describe and harmonise their mechanism of pathogenesis in general. These models commence with (a) adhesion to the epithelial membrane of the gut, (b) metabolic synthesis and secretion of toxins such as cytotoxins and enterotoxins, and finally, (c) inflammation of the intestinal mucosa (Estrada-Garcia and Navarro-Garcia, 2012; Gioia-Di Chiacchio *et al.*, 2018; Jenkins, 2018; Panel on Biological Hazards, 2015).

#### *2.3.3.1 Adhesion model of EAEC*

This phase is the first stage of EAEC pathogenesis, during which the bacteria secure attachment to the intestinal mucosa with the aid of its two adaptive features: fimbrial and afimbrial adhesins together with fimbrial (aggregative adhesive fimbrial [AAF]) structures (Boll *et al.*, 2017; Jenkins, 2018; Rajan *et al.*, 2018). The afimbrial adhesins, also known as outer membrane proteins, are known to be associated with its aggregative adherence as was reported to vary from one strain to another (Aijuka *et al.*, 2018; Boisen *et al.*, 2020; Dias *et al.*, 2020; Estrada-Garcia and Navarro-Garcia, 2012; Hebbelstrup Jensen *et al.*, 2017; Rajan *et al.*, 2018).

EAEC form biofilms on the intestinal mucosal layer within the GIT. Within these biofilms, the bacterial aggregates secure attachment to the membranes of the enterocytes to create a distinctive aggregative adherence design called “stacked brick” (Figure 2.1) (Aijuka and Buys, 2019; Aijuka *et al.*, 2018; Gupta *et al.*, 2016; Jønsson *et al.*, 2015; Pakbin *et al.*, 2021). Different EAEC strains were demonstrated to have diverse aggregative adherence fimbriae patterns with scanning electron

microscopy (Aijuka *et al.*, 2018; Dias *et al.*, 2016b; Elias and Navarro-Garcia, 2016; Gomes *et al.*, 2016; Jønsson *et al.*, 2015).

Four different essential physical subunits were identified, which exist as the following alternatives: *AggA* (AAF/I), *AafA* (AAF/II), *Agg3A* (AAF/III), and *Agg4A* (AAF/IV) as possessed by any EAEC strain (Havt *et al.*, 2017; Jønsson *et al.*, 2015; Jonsson *et al.*, 2017; Nezarieh *et al.*, 2015; Serichantalergs *et al.*, 2017). These factors are encrypted on the *pAA* plasmid for virulence, which doubles for encoding *AggR*, a transcription factor responsible for regulating the biogenesis of aggregative adhesive fimbriae (Priya *et al.*, 2017; Toffel *et al.*, 2019).

EAEC strains have been reported to be able to encode variant fimbrial structures like type IV pili and variants of the aggregative adhesive fimbriae as was reported with EAEC strain C1096 (Hebbelstrup Jensen *et al.*, 2017; Jønsson *et al.*, 2015; Schüroff *et al.*, 2021; Soria-Bustos *et al.*, 2022).

#### *2.1.1.1 Toxin production model of EAEC*

The toxin production phase follows the adhesion phase of EAEC to the intestinal mucosa during its virulence mechanism on the target host. This phase is associated with several putative virulence factors, as illustrated, and studied by various researchers (Boisen *et al.*, 2012; Elias and Navarro-Garcia, 2016; Estrada-Garcia and Navarro-Garcia, 2012; Ikumapayi *et al.*, 2017; Lima *et al.*, 2013).

EAEC produce putative toxins (Figure 2.1), leading to vesiculation within microvilli, causing enlargement of crypt openings and increased extrusion of the enterocytes (Dos Santos *et al.*, 2016; Elias and Navarro-Garcia, 2016; Govindarajan *et al.*, 2020; Harrington *et al.*, 2006; Pakbin *et al.*, 2021). These toxins include Shigella enterotoxin (*ShET1*), which triggers the discharge of intestinal cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), which are also encoded by *Shigella flexneri* (Ghosh, 2018; Gill and Hecht, 2018; Gomes *et al.*, 2016; Pakbin *et al.*, 2021). Other toxins include plasmid-encoded toxin (*Pet*), also known as an auto transporter identified to be responsible for regulating the cytoskeleton of the bacteria (Navarro-Garcia and Elias,

2011; Patzi-Vargas *et al.*, 2015; Sanchez-Villamil *et al.*, 2019b), protein involved in colonization (*Pic*), is a mucinase common amongst numerous diverse pathogenic *Shigella strains* and *E. coli*

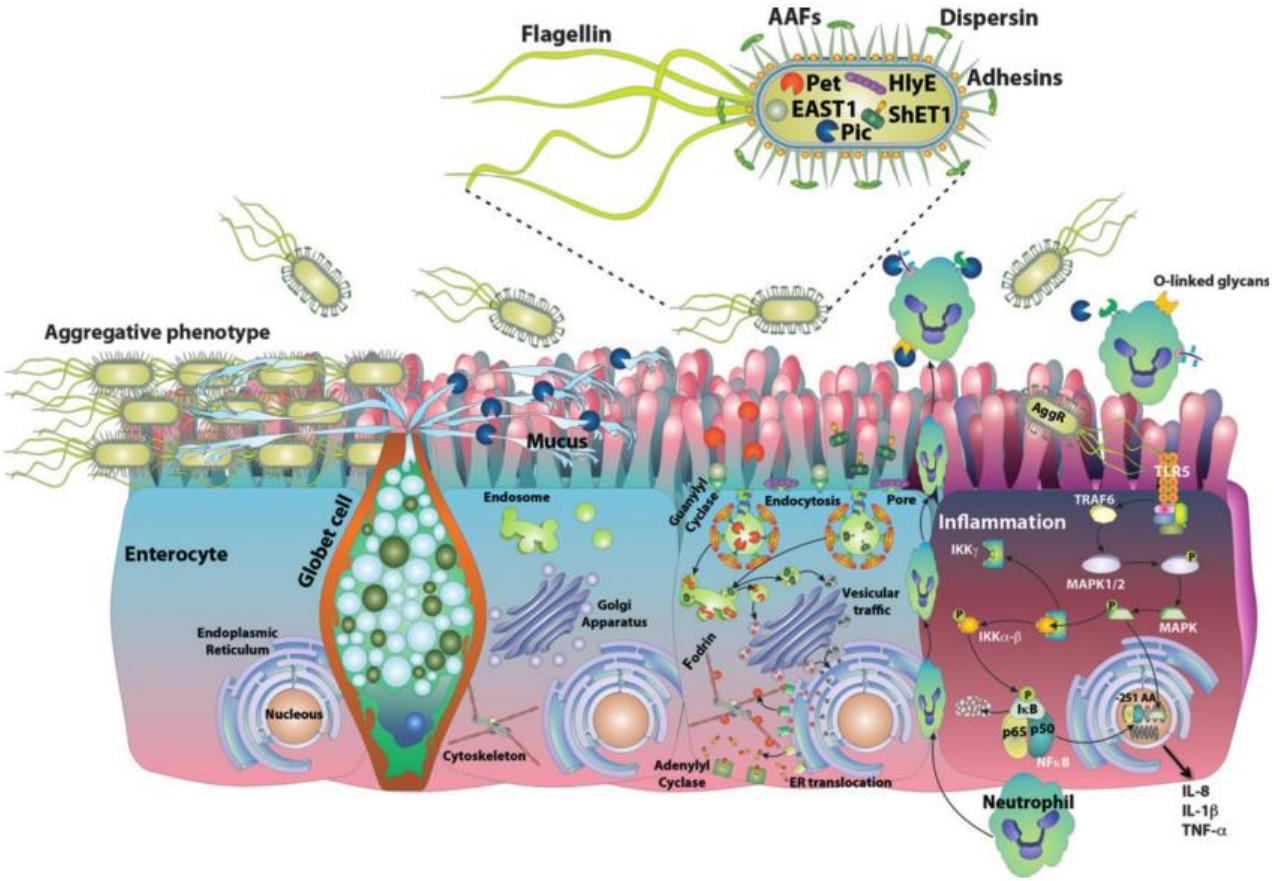


Figure 2.1: Schematic illustration of the virulence factors and pathogenic mechanisms of EAEC and their destructive effects on the intestinal mucosa

This illustration shows the adhesion, toxin production and inflammation models of EAEC with some of their destructive actions with targets of their virulence factors (EAST1, ShET1, and HylE) and their roles as established with other familiar pathogens (Adapted from Estrada-Garcia, T. and Navarro-Garcia, F. 2012).

(Dias *et al.*, 2020; Ellis *et al.*, 2020; Lara *et al.*, 2017; Liu *et al.*, 2020a; Rajan *et al.*, 2020), and a heat-stable enterotoxin 1 (EAST-1) which is encrypted by quite a lot of other pathogens beside EAEC (Ellis *et al.*, 2020; Nyholm *et al.*, 2015; Ochi *et al.*, 2017). They also produce putative enterotoxin (*Pet* toxins) and protein C (*EspC*), which can split fodrin, an actin-binding protein. These toxins exhibit

different methods of invading the host cell (Sanchez-Villamil *et al.*, 2019a; Serapio-Palacios and Navarro-Garcia, 2016; Tapader *et al.*, 2019).

Some of the toxins produced are uncharacterised and are detected from metabolic profiling and genome sequencing consisting of a possible haemolysin encoded (*hlyE*) gene (Aijuka *et al.*, 2018; Clark and Maresso, 2021; Panel on Biological Hazards, 2015); in addition to dispersin or anti-aggregation protein (*aap* formerly called *aspU*) (Shah *et al.*, 2016; Shahbazi *et al.*, 2015). EAEC 042 strain was demonstrated to bear the *Shigella flexneri* (*shF*) gene, which was projected to share similarities with some proteins of *Staphylococcus epidermidis, IcaB.* This protein was known to play a significant role in the modification of exopolysaccharide towards the formation of bacterial biofilm (Borgersen *et al.*, 2018; Grad *et al.*, 2012; Kaoukab-Raji *et al.*, 2020; Rodrigues *et al.*, 2019a; Rodrigues *et al.*, 2019b). AAF also mediates biofilm formation in EAEC (Blanton *et al.*, 2018; Ellis *et al.*, 2020; Jønsson *et al.*, 2015; Lara *et al.*, 2017; Nagy *et al.*, 2016). Categorizing and describing toxins encoded by various EAEC strains is still under investigation. The different virulence genes concerned with the aggregation and toxin production phases of EAEC pathogenesis have been reported to be regulated by transcriptional activator of aggregative adherence (*AggR*) (Dias *et al.*, 2020; Elias and Navarro-Garcia, 2016; Muller *et al.*, 2016; Schuroff *et al.*, 2021). *AggR* gene belonged to the family of *AraC* regulators and was found to be responsible for positively regulating EAEC. It has been found to regulate itself positively but negatively moderate by the global repressor of transcription in enterobacteria (H-NS) factor with factor for inversion stimulation (FIS), a c-binding protein (Abdelwahab *et al.*, 2021; Huttener *et al.*, 2018; Prieto *et al.*, 2021; Santiago *et al.*, 2017).

#### *2.1.1.2 Mucosal inflammatory model of EAEC*

The intensity of mucosal inflammation that marks the final phase of EAEC pathogenesis on its host is affected by multiple factors (Figure 2.1 and Figure 2.2). These include the enteropathogen and the host intrinsic immune system (Elias and Navarro-Garcia, 2016; Ellis *et al.*, 2020; Estrada-Garcia and Navarro-Garcia, 2012; Govindarajan *et al.*, 2020). The adhesion of EAEC to the intestinal epithelia

cells (IECs) during gastrointestinal tract infection triggers the secretion of chemokine ligand 20 (CCL20) and interleukin 8 (IL-8), which in turn stimulates the immune action of neutrophils. It then arouses inflammatory diarrhoea (Edwards *et al.*, 2011). Research into the proteomics of EAEC-T8 strain revealed a significant interaction among four main membrane proteins, including epidermal growth factor receptor (EGFR), fibronectin, glucose-regulated protein (GRP)-96, and thrombospondin-1 (TSP-1) of INT-407 cells with the plasmid-encoded cell wall proteins of EAEC-T8. These proteins were reported to be engaged in the adhesion of these strains to the cells (Boll *et al.*, 2017; Gill and Hecht, 2018; Konar *et al.*, 2012; Melendez-Avalos *et al.*, 2020; Yáñez *et al.*, 2016). These plasmalemma proteins have substantial homology with Hsp90, a protein which stimulates communicating pathways due to the attachment of other pathogenic bacteria. These pathways lead to the stimulation of immune / inflammatory responses through nuclear (transcription) factor β (NF-kappa β or NF-κβ) and mitogen-activated protein kinase (MAPK) (Nickerson *et al.*, 2021; Paradis *et al.*, 2021; Sahu *et al.*, 2022; Sanchez-Villamil and Navarro-Garcia, 2015; Shinoda *et al.*, 2015; Yáñez *et al.*, 2016).

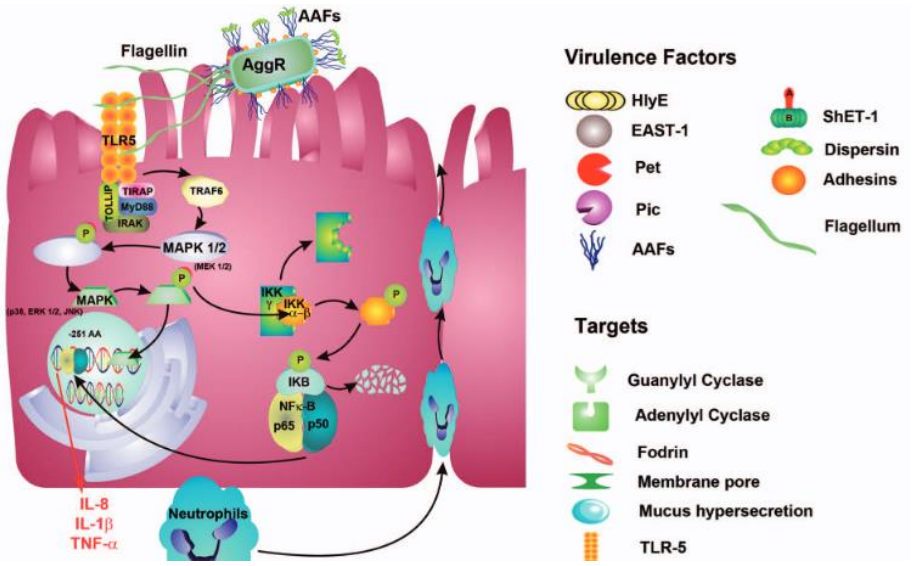


Figure 2.2: Schematic representation of induction of mucosal inflammation by EAEC

This figure shows some virulence factors and targets (Adapted from Navarro-Garcia and Elias, 2011)

In another study involving the EAEC-T8 strain, it was similarly found that the pathogen interacts with Thrombospondin-1 (TSP-1), an extracellular matrix glycoprotein secreted in high doses in swollen and injured tissues.

The secretion of the TSP-1 is a strong indication of a molecular connection between the host tissue cells and Gram-positive pathogens. Similarly, a few EAEC were demonstrated to interact with TSP1, to extracellular matrix (ECM) glycoprotein secreted in copious amounts in inflamed and injured tissues and has been linked to a molecular connection involving the host tissue cells and Gram-positive pathogens (Boll *et al.*, 2017; Bose and Mukherjee, 2020; Buchholz *et al.*, 2011; Konar *et al.*, 2012; Rajan *et al.*, 2020).

Aggregative adhesive fimbriae type II (AAF/II) elicits IL-8 secretion from the basolateral side of differentiated human colon epithelial (T84) monolayers (cells) (Buchholz *et al.*, 2011; Elias and Navarro-Garcia, 2016; Ellis *et al.*, 2020; Rajan *et al.*, 2018; Valeri, 2015; Werneburg and Thanassi, 2018). The aggregative adhesive fimbriae (AAF) have also been recognized to facilitate the infiltration of polymorphonuclear neutrophils (PMN) *in vitro* and *in vivo* due to EAEC invasion. This process activates eicosanoid based PMN chemoattractant production from the host cells (Boll *et al.*, 2017; Boll *et al.*, 2012; Pakbin *et al.*, 2021; Yáñez *et al.*, 2016). The secreted eicosanoid through the intestinal mucosa lining regulates PMNs by creating a chemotactic gradient (Boll *et al.*, 2012; Sanchez-Villamil and Navarro-Garcia, 2015). The incidence of many of the putative virulence factors was found to correlate with an increase in the levels of faecal leukocytes, occult blood, faecal cytokines, lactoferrin, alpha interferon (IFN-α), interleukins [interleukin-1-receptor antagonist (IL-1RA), interleukin 1 beta (IL-1β), and IL-8] (Boll *et al.*, 2012; Cutone *et al.*, 2020; Estrada-Garcia and Navarro-Garcia, 2012; Sanchez-Villamil and Navarro-Garcia, 2015).

## 2.2 Inflammation and inflammatory cytokines

Inflammation generally refers to the process during which the body of any living being fights harmful activities or substances, including toxins, injuries, infections, and toxins, to heal itself (Medzhitov, 2021; Otvos and Ostorhazi, 2015). This process happens by secreting specific chemicals that trigger a response from one's immune system. The reaction involves the secretion of antibodies and other specialised cytokines (a signalling molecule) secreted from resistant and different cell types (Table 2.1) to promote inflammation (Brusini *et al.*, 2020; Sage *et al.*, 2019). Thus, cytokines regulate host responses to immune reactions, infection, inflammation, and trauma. Inflammatory cytokines [principally produced by T helper (Th) cells] and macrophages are engaged in the upregulation of inflammatory reactions (Fang and Zhu, 2020; Kunkl *et al.*, 2020; Tatiya-Aphiradee *et al.*, 2018). The colonisation (invasion) of the intestinal epithelium by pathogens like the EAEC leads to the production and secretion of some inflammatory cytokines [chemokines, interferons, interleukins (IL), and tumour necrosis factors (TNF)] to stimulate, recruit, and increase immune cells. (Broggi and Granucci, 2015; Pujari and Banerjee, 2021; Shapouri-Moghaddam *et al.*, 2018; Spiering, 2015; Zhao *et al.*, 2021a).

The anti-inflammatory cytokines constitute a series of immunoregulatory molecules that regulate one's proinflammatory cytokine response. Anti-inflammatory cytokines operate together with specific cytokine inhibitors and soluble cytokine receptors to control the individual's immune response (Yahfoufi *et al.*, 2018; Zeinali *et al.*, 2017). Interestingly, some cytokines worsen the disease condition and are known as pro-inflammatory cytokines, contrary to anti-inflammatory cytokines that help in the ameliorating inflammation whilst promoting one's healing (Al-Azemi *et al.*, 2017; Dinarello, 2000; Liang *et al.*, 2015). The principal pro-inflammatory cytokines include IL-1, IL-6, and TNF-α, signalling type I cytokine receptors (CCR1) that are structurally different from other cytokine receptors. They are essential for modulating cell-mediated immune responses and play a critical role in modulating the immune system. Other inflammatory (adaptive immunity), anti-inflammatory and pro-inflammatory cytokines, their primary sources, receptors, target cells, principal sources and

Table 2.1: Summary of selected cytokines and their functions

| Cytokine | Main Sources | Receptor | Target Cell | Major Function |
|---|---|---|---|---|
| Erythropoietin | Endothelium | EpoR | Stem cells | Red blood cell production |
| **Class: Adaptive immunity** | | | | |
| GM-CSF | T cells, macrophages, fibroblasts | CD116, CDw131 | Stem cells | Granulocytes production, growth and differentiation of eosinophil and monocytes |
| IL-2 | Th1 cells | CD25 | Activated B, NK, and T cells | Multiplication and activation of B, NK, and T cells function |
| IL-3 | T cells | CD123, CDw131 | Stem cells | Proliferation and differentiation of hematopoietic precursor |
| IL-4 | Th Cells | CD124 | Macrophages, B and T cells | Enhancement of MHC class II expression, proliferation of B and cytotoxic T cells, stimulation of IgE and IgG production |
| IL-5 | Th2 Cells and mast cells | CDw125, 131 | Eosinophils and B-cells | Stimulation of IgA and IgM production and B-cell proliferation and maturation |
| IL-7 | BM stromal cells, epithelial cells | CD127 | Stem cells | B and T cell growth factor |
| IL-9 | T cells | IL-9R, CD132 | T cell | Growth and multiplication |
| M-CSF | Fibroblasts, endothelium | CD115 | Stem cells | Production and activation of monocytes |
| **Class: Anti-inflammatory** | | | | |
| IL-10 | T cells, B cells, macrophages | CDw210 | B cells, macrophages | Inhibition of cytokine production and mononuclear cell function |
| IL-12 | T cells, macrophages, monocytes | CD212 | Macrophages, NK, and tumour cells | Activation of NK and phagocytic cells, cachexia, endotoxic shock, and tumour cytotoxicity |
| IL-22 | Activated T-cells and NK cells | IL-22R | Epithelial and stromal cells | Proliferation and stimulation of cell survival |

| | | | | |
|---|---|---|---|---|
| IL-37 (1L-1F7) | B-cells, NK cells, and monocytes | CD218a (IL-18Ra) and potentially SIGGR | | Believed to negatively regulate cellular endoplasmic environment by interacting with SMAD3 that is activated downstream of TGFβ activity. |
| IL-38 (IL-1F10) | B cells and macrophages | IL-1R1 | | Unknown |
| TGF-β | T cells and B cells | TGF-βR1, 2, 3 | Activated B and T cells | Inhibition of B and T cells proliferation, haematopoiesis whilst promoting wound healing |
| **Class: Pro-inflammatory** | | | | |
| G-CSF | Fibroblasts, endothelium | CD114 | Stem cells in BM | Granulocyte production |
| IL-1 | Macrophages, B cells, DCs | CD121a | B, NK, and T cells | Differentiation, pro-inflammatory, proliferation, pyrogenic, and BM cell proliferation |
| IL-6 | Th Cells, macrophages, fibroblasts | CD126, 130 | B and plasma cells | B-cell differentiation |
| IL-8 | Macrophages | IL-8R | Neutrophils | Chemotaxis for neutrophils and T cells |
| IL-11 | BM stromal cells | IL-11Ra, CD130 | B cells | Differentiation and induction of acute phase proteins |
| IL-17 | Th17 cells | IL-17R | Monocytes, neutrophils | Recruitment of monocytes and neutrophils to the site of infection. Activation of IL-17 which in turn activates downstream of many cytokines and chemokine such as IL-1, IL-6, IL-8, IL-21, MCP-1 and TNF-β |
| IL-18 | Macrophages, dendritic cells, and epithelial cells | CD218a (IL-18Ra) | Monocytes and T cells | Recruitment of monocytes and T lymphocytes. Synergist with IL-12 in the induction of IFN-ɣ production and inhibition of angiogenesis. |
| IFN-α | Macrophages, neutrophils, and some somatic cells | CD118 (IFNAR1, IFNAR2) | Various | Anti-viral |
| IFN-β | Fibroblasts | CD118 (IFNAR1, IFNAR2) | Various | Anti-proliferative and anti-viral |

| | | | | |
|---|---|---|---|---|
| IFN-γ | T Cells and NK cells | CDw119 (IFNG R1) | Various | Anti-viral, upregulation of neutrophil and macrophage activation, monocyte function, MHC-I and -II expression on cells |
| TNF-α | Macrophages | CD120a,b | Macrophages | Phagocyte activation and endotoxic shock |
| TNF-β | T Cells | CD120a,b | Phagocytes, tumour cells | Chemotactic, oncostatic, phagocytosis and induction of other cytokines |

IL; interleukin, TNF; tumour necrosis factor, IFN; interferon, G-CSF; granulocyte colony stimulating factor, GM-CSF; granulocyte macrophage colony stimulating factor, M-CSF; macrophage colony stimulating factor, TGF; transforming growth factor, CD; cluster of differentiation; CDw; cluster of differentiation designated by only one monoclonal antibody, BM; bone marrow, DC; dendritic cells.

**Adapted from (ThermoFisher Scientific, 2022).**

functions (Table 2.1) (Conti *et al.*, 2018; Guan and Zhang, 2017).

### 2.2.1 Transmission and reservoirs of EAEC

EAEC accounts for traveller's diarrhoea transmission frequently occurring by ingesting contaminated water and foods such as salads (Aijuka *et al.*, 2018; Gomez-Aldapa *et al.*, 2016; Jenkins, 2018; Panel on Biological Hazards, 2015; Yang *et al.*, 2017). For example, desserts and salads in Mexico mainly were contaminated with EAEC (Bari and Yeasmin, 2018; Leung *et al.*, 2019; Toe *et al.*, 2018). Food vendors and handlers could also be EAEC transferors, underlining the importance of sanitary food handling practices to prevent the transmission of EAEC (Jenkins, 2018; Panel on Biological Hazards, 2015; Syahrul *et al.*, 2020; Waturangi *et al.*, 2019).

People bearing AA instead of AT or TT genotype at the 251$^{st}$ location along the gene for expressing IL- 8 produced an abnormally more considerable amount of faecal IL-8 in response to EAEC invasion, so they were reported to be correlated with an elevated rate of infection with EAEC (Czepiel *et al.*, 2018; Elias and Navarro-Garcia, 2016; Govindarajan *et al.*, 2020; Li *et al.*, 2018). Reports illustrated that animals like piglets, horses and calves were found to be significant carriers of only atypical EAEC. This implies that typical EAEC as human pathogens exclude animals (Etcheverría *et al.*, 2016; Panel on Biological Hazards, 2015; Rivas *et al.*, 2015; Smith and Fratamico, 2018). However, due to the severally reported heterogeneity of EAEC isolates, it might be too early to exclude animals as carriers, particularly for under-characterized EAEC strains.

### 2.2.2 Treatments and preventive measures against diarrhoea infection

The inadequacy of comprehensive pharmacological interventions like relevant drugs and vaccines, amongst others, motivated the scientific community to explore other fields involving the brain-gut axis concept.

## 2.3 Probiotics

According to the World Health Organization (WHO), probiotics are living microorganisms (like yeasts and bacteria); when consumed in adequate quantities, they exert some beneficial effects on the general health of the host (Cremon *et al.*, 2018; Martin and Langella, 2019; Sánchez *et al.*, 2017; Swanson *et al.*, 2020). The various mechanisms through which probiotics exercise their beneficial effects on their host have been intensively studied (Ayala *et al.*, 2017; Galdeano *et al.*, 2019; Plaza-Diaz *et al.*, 2019; Sánchez *et al.*, 2017; Teame *et al.*, 2020). Some of these mechanisms include competition for adhesion sites of the pathogen, improvement of the multiplicity and gut microbiota stability, production and secretion of antimicrobial agents and immuno-modulation (Ashaolu, 2020; Bajaj *et al.*, 2021; Kuebutornye *et al.*, 2020; Monteagudo-Mera *et al.*, 2019; Ribeiro *et al.*, 2020; Teame *et al.*, 2020). Some *Bacillus spp.* have been reported to inhibit avian pathogenic *E. coli* (APEC) in addition to other microbes (Fancher *et al.*, 2020; Hu *et al.*, 2020; Kathayat *et al.*, 2021).

Pathogen inhibition by lactobacillus may offer substantial human health benefits through defence against pathogen infection as a natural blockade against exposure to pathogens in the gastrointestinal tract. Thus, probiotic bacteria must exist in adequate mass in aggregates to impact their beneficial effects on the host. Probiotics that can co-aggregate with other bacteria, such as pathogens, may have significant advantages over non-co-aggregating microbes, which can be removed easily from the gut environment.

The microbiota has been essential within the brain-gut axis construct due to the advent of bacterial flora as therapeutic agents with the use of antibiotics (Angelucci *et al.*, 2019; Dinan and Cryan, 2017; Ghaisas *et al.*, 2016; Petra *et al.*, 2015; Sherwin *et al.*, 2016) and probiotics (Lee *et al.*, 2019b; Mangiola *et al.*, 2016; Sinagra *et al.*, 2020) being the principal means of health intervention. Despite the numerous health benefits probiotic bacteria offer, their exact mechanisms of action were formally not clearly understood. However, contemporary developments in probiotics significantly facilitate a

more transparent understanding of their mode of action (Bajaj *et al.*, 2021; Plaza-Diaz *et al.*, 2019; Reid, 2016; Yong *et al.*, 2020) based on the following theories.

### 2.3.1 Pathogen colonization preventive / resistance theory

The critical requirement of any enteric pathogen is to colonize and trigger its virulence and pathogenicity on its host, and this principally depends on its ability to first secure attachment to the intestinal epithelium (Caballero-Flores *et al.*, 2021; Kitamoto *et al.*, 2016; Pickard *et al.*, 2017; Woodward *et al.*, 2019). Their adhesion ability is closely related to their surface characteristics (Khojah *et al.*, 2022; Monteagudo-Mera *et al.*, 2019; Nivoliez *et al.*, 2015a; Rokana *et al.*, 2018). In the same way, for a lactic acid bacterium to be qualified as a suitable probiotic bacteria candidate, it must be able to competitively secure attachment in numerous colonies or numbers along with the epithelial layer of the gut (Behbahani *et al.*, 2019; Nivoliez *et al.*, 2015b; Reuben *et al.*, 2019). The fundamental concepts that explain the mechanisms of probiotic action and their defensive role against enteric pathogens have been studied extensively. The abilities of probiotic bacteria to aggregate among themselves (auto-aggregate) and to co-exist (co-aggregate) with their enteric pathogenic counterparts to form a barrier along epithelium, as illustrated by (Figure 2.3). This significantly empowers the intestinal epithelium (Montoro *et al.*, 2016; Sahoo *et al.*, 2015; Tomičić *et al.*, 2022).

Although some probiotic strains can secrete factors that affect the luminal microbiota, others can competitively exclude luminal pathogens from getting attached to the gut epithelium by getting themselves to adhere to the mucosal lining on the epithelial tissues. This action also excludes subsequent effects of such enteric pathogens. Some probiotic bacteria are equipped with unique characteristics such as cell membrane hydrophobic properties, which allow them to bind to host cell or tissue surfaces like apical microvilli membranes as found in the intestinal epithelium (Khojah *et al.*, 2022; Krausova *et al.*, 2019; Monteagudo-Mera *et al.*, 2019; Yadav *et al.*, 2017). Thus, probiotic surface hydrophobicity and adhesion are dependent on their thick surface-layer protein, which also enables them to mitigate the enteropathogenic effects (Alp and Kuleasan, 2019; Celebioglu and

Svensson, 2018; Monteagudo-Mera *et al.*, 2019; Wang *et al.*, 2017). Additionally, probiotics and their non-viable forms can occupy receptor binding sites (Figure 2.3) of the epithelium to prevent pathogen adhesion and subsequent invasion of the host GIT (Lukic *et al.*, 2017; Monteagudo-Mera *et al.*, 2019; Pique *et al.*, 2019; Qureshi *et al.*, 2019; Singh *et al.*, 2017).

Another mechanism of probiotic action is by competitive exclusion of enteropathogens (Campana *et al.*, 2017; Kuebutornye *et al.*, 2020; Monteagudo-Mera *et al.*, 2019; Singh *et al.*, 2017; Wan *et al.*, 2019). With this tactic, the probiotics exert their beneficial effects on the host by engaging in keen competition with their pathogenic counterparts for the limited niche in the microbiome within the host's colon, thereby limiting sites for attachment and subsequent replication of the pathogens in their host (Figure 2.3). This method of probiotic action is also known as resistance to colonization. *Saccharomyces boulardii*, for example, was found to indirectly prevent the adhesion of enterohaemorrhagic *E. coli* O157:H7 serotype to the intestinal epithelium by directly attaching themselves to the pathogen (Cordonnier *et al.*, 2017; Thevenot *et al.*, 2015; Xue and Zhu, 2021).

### 2.3.2 Gut microbiota stability and diversity improvement theory

The gut of a normal foetus is deficient in microbial ecology, and complex microbiota starts to develop only during and after the birthing process (Moore and Townsend, 2019; Rodriguez *et al.*, 2015; Walker *et al.*, 2017). The equilibrium between the intestinal epithelium and the microbiome is very sensitive to the newly born. The gut-associated lymphoid tissue (GALT) is significant in establishing intestinal homeostasis (Delgobo *et al.*, 2019; Gomes *et al.*, 2018; Kurashima and Kiyono, 2017; Ohland and Jobin, 2015). The GALT empowers the enteric microbiome to regulate the functions of the gut, including blood circulation, secretion, intestinal permeability, visceral sensations, mucosal immunity and motility of the brain-gut axis (Bercik, 2020; Jacobs *et al.*, 2021; Martin *et al.*, 2018). The beneficial effects of probiotics on the GIT under diverse conditions have severally been subjected to meta-analyses and multiple randomized controlled clinical trials (Dong *et al.*, 2016; Huang *et al.*, 2017; Jiang *et al.*, 2020; Liu *et al.*, 2017; Taylor *et al.*, 2017; Zhang *et al.*, 2016).

Probiotics have demonstrated various capabilities to colonize the GIT briefly and then step up their concentration within the shortest possible time by so doing establishing an equilibrium in the gut microbiome to the benefit of their host (Heimer *et al.*, 2022; Sánchez *et al.*, 2017; Singhi and Kumar, 2016; Williams and Angurana, 2019). In this way, they form the front line of defence in opposition to the antagonistic effects of enteric pathogenic bacteria by exerting their antimicrobial effects directly (Cox and Dalloul, 2015; La Fata *et al.*, 2018; Mathipa and Thantsha, 2017; Wan *et al.*, 2016b).

### 2.3.3 Mucosal and epithelial barrier maintenance theory

Bioactive peptide factors secreted by lactic acid bacteria have been shown to enhance epithelial barrier function both in vivo and in vitro (Ghosh *et al.*, 2019; Ren *et al.*, 2018; Tagliazucchi *et al.*, 2019). The intestinal barrier function in rats after acute psychological stress was reported to improve upon pre-treatment with a mixture of probiotic bacteria consisting of *L. helveticus* and *L. rhamnosus* by inhibiting pathogenic bacterial translocation (Rodino-Janeiro *et al.*, 2015). Similarly, *L. plantarum*, 299V strain was reported to impede *E. coli* from causing intestinal permeability due to invasion (Kazmierczak-Siedlecka *et al.*, 2020; Nordstrom *et al.*, 2021; Seddik *et al.*, 2017). In a survey using human intestinal epithelial (HT-29) cells, the production and secretion of mucin (MUC2 and MUC3) were reported to be boosted by this very strain (Figure 2.3) cells (Nordstrom *et al.*, 2021).

In a parallel study involving a rat colon, an increment was recorded in the expression and secretion of mucin protein and MUC2 gene with mixtures of different probiotic bacteria (Barouei *et al.*, 2015; Liu *et al.*, 2020c). The adhesion of *E. coli* as an enteric pathogen to intestinal mucosal surfaces was hindered due to the enhancement of the mucus layer over the gut epithelium, which served as antibacterial protection against the pathogen (Cai *et al.*, 2020; Mathipa and Thantsha, 2017; Sassone-Corsi and Raffatellu, 2015). The mucus layer enhances the clearance of the pathogen from the gut lumen (Kim *et al.*, 2017; Mcdonald *et al.*, 2020; Pickard *et al.*, 2017). In reaction to various toxic stimuli, including pathogenic bacteria, antibacterial peptides, such as trefoil factors, are secreted from

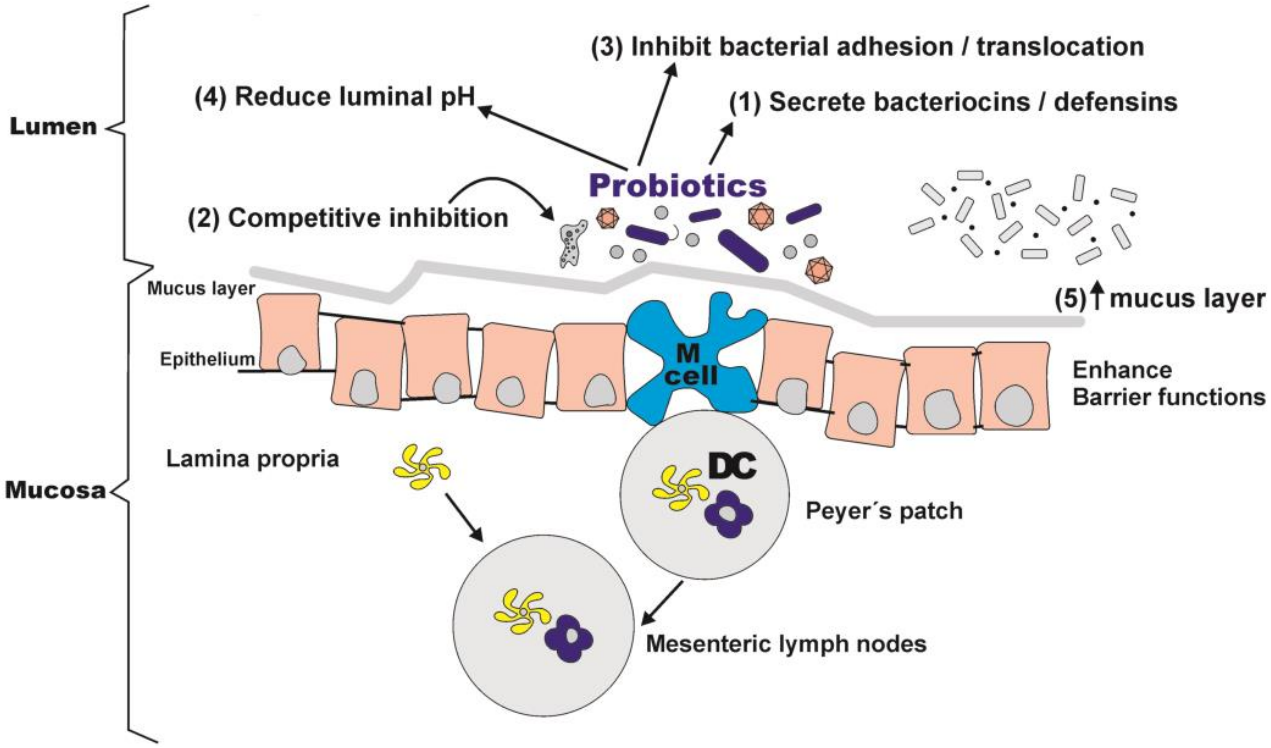


Figure 2.3: A schematic representation of the exclusion (inhibition) of enteric bacteria and enhancement of barrier function by probiotic bacteria

The antimicrobial activities of probiotics include the (1) secretion of bacteriocins/defensins, (2) competitive inhibition of pathogenic bacteria, (3) exclusion (inhibition) of bacterial attachment or translocation, and (4) reduction of luminal pH. Probiotic bacteria can also enhance intestinal barrier function by (5) increasing mucus production. (Adapted from Ng, *et. al*., 2009).

mucin-producing cells. The adhesion of enteric pathogens to the intestinal epithelial layer was significantly reduced if not completely prevented due to these factors associated with mucins (Chatterjee *et al.*, 2018; Nishiyama *et al.*, 2016).

Antimicrobial cationic peptides also called defensins (or cryptdins, in mice), were secreted by crypt (Paneth) cells close to the base of the distal section within the small intestine. The production and secretion of defensins from the intestinal mucosa could be boosted by some probiotic bacteria explaining the positive and beneficial effects of probiotics in chronic infectious enteritis (Holly and

Smith, 2018; Nakamura *et al.*, 2016; Yokoi *et al.*, 2019). The secretion and expression of human β-defensin mRNA and protein in intestinal (Caco2) each of the probiotics stimulated epithelial cells cultivated in tissue culture, *E. coli* (strain Nissle 1917) and *Lactobacillus fermentum* in a dose- and time-dependent manner (Bhat *et al.*, 2019b; Wan *et al.*, 2016b). Besides the synthesis and secretion of antibacterial substances, probiotics also exert a direct effect on the intestinal epithelial barrier and integrity for improvement (Camilleri, 2021; Liu *et al.*, 2020b; Wan *et al.*, 2016a; Wan *et al.*, 2019). They accomplish this action by improving the transepithelial electrical resistance (TEER) of tight junctions in the apical junction complexes between adjoining polarized epithelial cells. A robust epithelial barrier function is crucial for maintaining gut immunological stability. It is regulated by junctional complex networks like the epithelial tight junctions, which ensure paracellular space between adjoining epithelial cells (Bhat *et al.*, 2019c; Ren *et al.*, 2020). Tight junction-associated genes and proteins (Claudin-1, Occludin and ZO-1) were boosted by substances like oxyresveratrol (an isomer of hydroxylated resveratrol isolated from plants) (Liu *et al.*, 2020b; Llewellyn and Foey, 2017). Some of these substances are noted to activate protein kinase C (PKC) whilst increasing the expression levels of MAPK and transcription factor (Cdx-2) genes and proteins (Jo *et al.*, 2017; Paradis *et al.*, 2021; Zhao *et al.*, 2021b). The MAPK contribute to the reduction in programmed apoptosis (cell death), which also enhances barrier resistance to damaging substances such as proinflammatory cytokines (Fan *et al.*, 2019; Fong *et al.*, 2020; Gorska *et al.*, 2019; Wu *et al.*, 2019b). This mechanism helps maintain the gut's epithelial barrier integrity and resist detrimental agents, such as enteric pathogenic bacteria, by reducing disruptions within the intestinal epithelium barrier.

### 2.3.4 Antimicrobial and metabolic effects theory

Some strains of probiotic bacteria secrete bacteriocins which are antimicrobial substances (Figure 2.3). The bacteriocins with other antimicrobial substances secreted by probiotics impede the multiplication and virulence of enteric pathogenic bacteria. Isogenic mutants of probiotics were found to be less effective when tested on a mouse model with *Listeria monocytogenes* since they lack the gene coding

for the synthesis and secretion of bacteriocins (Desiderato *et al.*, 2021; Umu *et al.*, 2016; Yin *et al.*, 2018).

The pH reduction of the luminal microenvironment due to the production of lactic acids by most probiotic bacteria as a result of their intrinsic metabolic activities within the gut, as demonstrated by co-culturing of *Lactobacillus species* (Figure 2.3) with pathogens, lead to significant declines in the growth of the virulent pathogenic bacteria (Aldunate *et al.*, 2015; Ballan *et al.*, 2020; Martínez *et al.*, 2021; Peredo-Lovillo *et al.*, 2020).

### 2.3.5 Signal transduction and immunomodulation theory

The inherent and flexible sections of the host immune system are affected by probiotics through interactions between the bacterial and epithelial cells (Javanshir *et al.*, 2021; Owaga *et al.*, 2015; Watters *et al.*, 2016). For example, some strains of probiotics have been reported to exhibit the capability to boost the maturation of β cells into plasma cells and fuel polymeric immunoglobulin A (IgA) synthesis and secretion (Abokor *et al.*, 2021; Hand and Reboldi, 2021; Ma *et al.*, 2018). This IgA constrains pathogens' growth by reducing their mobility and ability to access the epithelium and the microvilli surface by covering the gut epithelium's mucus layer.

Probiotics further engage in inhibiting the stimulation of pro-inflammatory nuclear transcription factor kappa β (NF-κβ), which subsequently decreases the secretion of a potent neutrophil chemo-attractant known as chemokine interleukin (IL)-8 (Alagón Fernández Del Campo *et al.*, 2019; Gupta *et al.*, 2021; Kim *et al.*, 2015; Šefcová *et al.*, 2019). The relocation of NF-κβ from the cytosol to the nucleus has been reported to be carried out by probiotics and non-pathogenic microorganisms through the inhibition of phosphorylation and then degradation via ubiquitination of the NF-κβ inhibition enzyme, I-κβ (Javanshir *et al.*, 2021; Merchak and Gaultier, 2020; Tavares *et al.*, 2020; Yousefi *et al.*, 2019). Even though some probiotics can trigger the nuclear transcription factor, other pieces of evidence contrarily indicate that other probiotics are capable of activating the NF-κβ leading to elevated amounts of pro-inflammatory cytokines such as interleukin 6 (IL-6) (Chondrou *et al.*, 2020; Mendes *et al.*,

2017; Šefcová *et al.*, 2019). These variations also bolster the effects of probiotics on their host vary from strain to strain.

Some strains of probiotics have been demonstrated to affect the immune responses of a few T cells. In a study involving the transfer of CD4+CD45RBhi T lymphocytes from healthy mice into acute immunodeficient (SCID) mice, a probiotic yeast, *S. boulardii*, for example, was reported to minimise intestinal inflammation (Dowdell and Colgan, 2021; Glassner *et al.*, 2020; Martín *et al.*, 2017; Nikolakis *et al.*, 2022). This observation could be explained by the influence of lactic acids and secretions such as bacteriocins and other antimicrobial substances from the probiotics from the healthy mice on the immune system of the immune-deficient mice, significantly reducing the inflammation of the intestine. Together with T helper-1 cells, these processes can lead to the synthesis of proinflammatory cytokines and interferon-γ from mesenteric lymph nodes of the inflamed colon.

The secretion of pro-inflammatory (e.g., IL-12) and anti-inflammatory (e.g., IL-10) cytokines by immune cells have been reported to vary with the diversity of probiotic bacteria strains (Citar *et al.*, 2015; Deutsch *et al.*, 2017; Junjua *et al.*, 2016; Ochangco *et al.*, 2016). Thus, different probiotic bacteria strains have diverse effects on these secretions. The ability of the probiotic strains to have positive impacts on curing various diseases has been found to depend on the differences in the profiles of such secretions, as reported in an experimental model of hapten-induced colitis mice (Dargahi *et al.*, 2019; Khan *et al.*, 2022; Martín *et al.*, 2017; Owaga *et al.*, 2015).

In randomisation of human volunteers with irritable bowel syndrome (IBS) in a survey with *Bifidobacterium infantis*, 35624 treatment, significant improvements were reported regarding the twenty-one (21) clinical symptoms besides the stabilization of the ratio of IL-10 to IL-12 secretion from mononuclear cells of peripheral blood (Horvat *et al.*, 2021; Javanshir *et al.*, 2021; Riedel, 2018; Salami, 2021). *Lactobacillus salivarius* (strain UCC4331), on the other hand, recorded only a marginal effect on clinical symptoms of the abdomen with no significant impact on its very low IL-10 to IL-12 ratio for patients with IBS (Cristofori *et al.*, 2021; Leylabadlo *et al.*, 2022; Vitetta *et al.*, 2015).

Numerous diversities of these observations with different probiotic bacteria strains emphasise that the host's benefits will grossly depend on the kind of probiotic bacteria ingested. These observations further suggest that various probiotics should be prescribed to address various health-related issues.

### 2.3.6 Innate / adaptive immunomodulation theory

Probiotics have been demonstrated in a recent novel study (Banfi *et al.*, 2021; Colombel *et al.*, 2019; Curro *et al.*, 2017; Wasilewski *et al.*, 2015), their ability to stimulate explicit cannabinoid and opioid receptors within the gut. Such probiotic bacteria strains might be helpful, especially in treating patients with gut pain and even those with persistent inflammatory bowel syndrome (IBS).

### 2.3.7 Quorum sensing theory

Quorum sensing is a critical procedure through which unicellular organisms such as eukaryotes and, for that matter, most microorganisms communicate with one another (Allocati *et al.*, 2015; Folcik *et al.*, 2020; Mukherjee and Bassler, 2019). Some strains of probiotics have been postulated to be capable of influencing gene expression of microbial pathogens, and by so doing, they significantly reduce their virulence potential (Gunaratnam *et al.*, 2021; Rana *et al.*, 2020; Zhou *et al.*, 2016). Factors secreted by the *Lactobacillus acidophilus* La-5 strain have been reported to significantly affect the virulence gene expression of the O157:H7 serotype of enterohaemorrhagic *E. coli* (Bondue *et al.*, 2016, 2019; Lee *et al.*, 2021; Najarian *et al.*, 2019; Thevenot *et al.*, 2015). In another study, a pathogenic *E. coli* was reported to secrete significantly lower amounts of autoinducer-2 molecules in the presence of some probiotic bacteria strains. This preventive action is critical for interceding intimate bacterial binding to host cell surfaces which are referred to as attaching and effacing lesion using extra reduction in the gene expression within the pathogenicity island of the locus of enterocyte effacement (LEE) (Cordonnier *et al.*, 2017; Surendran Nair *et al.*, 2017; Tarsillo and Priefer, 2020; Thevenot *et al.*, 2015; Tran *et al.*, 2018; Xue and Zhu, 2021).

The mechanisms of action and efficacy of probiotics have been reported to vary from one probiotic strain to another and are never the same (Dimidi *et al.*, 2020; Do Carmo *et al.*, 2018; Dudek-Wicher *et al.*, 2020; Kleerebezem *et al.*, 2019; Plaza-Diaz *et al.*, 2019; Reid, 2016; Sanders *et al.*, 2018). Unquestionably, this implies that some probiotics will possess several activities and, therefore will be able to influence multiple phases during their intervention through the virulence and pathogenesis of EAEC. Thus, the efficacy of probiotic bacteria is dose and strain dependent using several potential modes of action that are specifically characteristic to the LAB or their collections being sought for an intervention (Ji *et al.*, 2019; Miglioranza Scavuzzi *et al.*, 2015; Tremblay *et al.*, 2021).

### 2.4 Cell culture model

Studies involving microbial interactions have been studied at in vitro levels, and it is mainly based on cell culture models, which involve the use of quite a wide a range of cell lines over the years (Dutta *et al.*, 2017; Kampfer *et al.*, 2020; Mirabelli *et al.*, 2019; Niu and Wang, 2015; Vandussen *et al.*, 2015). For example, several studies involving the endocytosis of *L. monocytogenes* were centred around different human cell lines (Hurley *et al.*, 2016; Karthikeyan *et al.*, 2019; Kim *et al.*, 2021; Su *et al.*, 2019). Some of the most preferred cell lines include human epithelial colorectal adenocarcinoma (Caco-2) cells and the human colon carcinoma (HT29) cells which are continuous and heterogeneous in nature (Chang *et al.*, 2020a; Elbakush *et al.*, 2018; Kuehl *et al.*, 2015; Lamason and Welch, 2017; Walsham *et al.*, 2016b; Wolfe and Dutton, 2015). Bacterial adhesion to these cell lines mostly depends on the strain and is not different to specific levels within any species. For example, *Lactobacillus rhamnosus*, GG strain, for example, was found to have a comparatively high level (9.7 %) of attachment to Caco-2 cells (Guerin *et al.*, 2018; Iglesias *et al.*, 2017; Okochi *et al.*, 2017; Sophatha *et al.*, 2020). However, strains of *L. casei L. acidophilus*, *L. rhamnosus* and *L. paracasei* of dairy origin recorded adhesion from 3 to 14% to Caco-2 cells (Chondrou *et al.*, 2018; Chondrou *et al.*, 2020; Rocha-Mendoza *et al.*, 2020; Živković *et al.*, 2016). These same strains recorded much higher levels (20–40%) of attachment to HT29 and MTX cells which are known for mucus production within the

gastrointestinal tract (Davoodabadi *et al.*, 2015b; Krausova *et al.*, 2019; Miljkovic *et al.*, 2019; Torres-Maravilla *et al.*, 2016; Živković *et al.*, 2016).

Usually, bacterial adhesion to epithelial cells is calculated by working out the difference between the initial number of bacterial cells before adhesion assay and the number of bacterial cells that could not get attach to the epithelial cells by the means of visualising the cells by radioactive labelling or even traditional plating (Gonzalez-Aspajo *et al.*, 2015; Li *et al.*, 2020; Ortega *et al.*, 2017); Giemsa staining (Grilli *et al.*, 2019; Ortiz *et al.*, 2021; Sharma and Kanwar, 2017) or Gram-staining of the non-attached bacterial cells (Garcia-Gonzalez *et al.*, 2018; He *et al.*, 2020; Prakasita *et al.*, 2019; Song *et al.*, 2019; Wang *et al.*, 2018a). In recent times, this assay could also be carried out by staining the bacteria with fluorescent dyes which are capable of binding specifically to their nucleic acids (Huang *et al.*, 2019; Kurutos *et al.*, 2020; Pandit *et al.*, 2020; Rosenberg *et al.*, 2019).

## 2.5 Conclusion

EAEC is an emerging diarrheal pathogen associated with acute and persistent diarrhoea not only among developing countries but within developed countries too. Different strains vary widely in their colonisation location, virulence, and mechanisms of pathogenesis. Their colonisation of the gut may lead to intestinal inflammation, and persistent destruction of the mucosal and epithelial layers due to the production of various toxins (e.g., cytotoxins). Eventually, this results in malnutrition due to the impairment of the fundamental structure and functionality of the intestinal epithelium regarding the provision of a physical barrier against enteric pathogens, unhealthy foreign substances, and nutrient absorption. Microbial processes like the formation/presence of bacterial biofilm along the mucosal lining may also present a barrier against nutrient absorption from the lumen. Malnutrition and gut infection may continue and empower each other, operating in a vicious cycle. The pathophysiologic mechanism which drives toddlers along this path is possibly multifactorial and therefore, presents numerous opportunities for intervention against this vital challenge, worldwide.

Cumulative evidence establishes that most probiotic bacteria have several modes of action linked to their effects within the gastrointestinal tract, adaptive and inherent immune responses, and the mucosal barrier integrity of the host. Nevertheless, such effects widely vary from one probiotic to the other in most instances. Even within a given probiotic bacteria species, different strains can demonstrate different impacts due to diversity in their mechanisms of action. Therefore, strain consideration and selection based on the merits of their properties is vital in achieving target results. Amalgamations of probiotics might deliver excellent and superior results to achieve more desirable objectives, especially during critical interventions.

# CHAPTER 3.

# HYPOTHESES AND OBJECTIVES

## 3.1 Hypotheses

1. The selected strains of both EAEC and LAB will demonstrate hydrophobic, auto- and co-aggregation properties, whilst the two potential probiotic LAB strains, *Lactobacillus plantarum* FS2 and *Pediococcus pentosaceus* D39 will also show varying levels of antimicrobial properties against the selected EAEC strains.

   Aggregation between bacterial cells is dependent on the occurrence of electrostatic and chemical interactions between cell membrane molecules, which can associate among themselves or bind to a distinctly different receptor (Giaouris *et al.*, 2015; Nwoko and Okeke, 2021; Zaman *et al.*, 2019). EAEC, like most other *E. coli* pathotypes, possesses specialised cell-surface organelles, including bundle-forming pili (fimbriae) and flagella, in addition to specific surface proteins constituting aggregation effectors that will mediate localised adherence to epithelial cells. These will lead to the formation of microcolonies strengthened by pilus retraction and stabilised by other adhesins like Escherichia common pilus (Govindarajan *et al.*, 2020; Rossi *et al.*, 2018; Scaletsky and Fagundes-Neto, 2016). These retractable pili usually facilitate bacterial adhesion to host cells by attaching pilus tip proteins to specialised receptors (Ageorges *et al.*, 2020; Farfán and Girón, 2016; Werneburg and Thanassi, 2018). auto-aggregation is facilitated by lateral bundling interactions amongst the major structural subunits of various pili (Farfán and Girón, 2016; Olilo *et al.*, 2016). Besides fimbrial and afimbrial adhesins, specific secreted macromolecules such as polysaccharides and extracellular DNA can indirectly facilitate aggregation between bacterial cells of the same or different strains (Giaouris *et al.*, 2015; Serra and Hengge, 2021).

The cell surface of LAB cells, on the other hand, is smooth with a compact layer of globular proteins known as the S-layer coated by polymeric surface constituents, which confer some degree of roughness to the bacterial cell wall (Martinez *et al.*, 2020; Murphy *et al.*, 2017). The cell walls of most LAB further contain specialised surface proteins and (lipo)teichoic acids, which confer various degrees of hydrophobic properties to the bacterial cell surface contrary to polysaccharide constituents which present hydrophilic properties (Albedwawi *et al.*, 2021; Mora-Villalobos *et al.*, 2020; Shen *et al.*, 2019; Wang *et al.*, 2015a). This nature of the LAB cell wall enables them to aggregate among themselves (auto-aggregate) and between different strains (co-aggregate). Lactic acid bacteria are well noted to produce several antimicrobial substances, including lactic acids, organic acids, acetic acid, ammonia, ethanol, hydrogen peroxide, bacteriocins and bacteriocin-like substances etc. These substances are antagonistic against most enteric pathogens, including EAEC (Agriopoulou *et al.*, 2020; Islam *et al.*, 2020; Özogul and Hamed, 2018; Voidarou *et al.*, 2020).

2. The two good probiotic bacteria, *Lactobacillus plantarum* FS2 and *Pediococcus pentosaceus* D39 will autoaggregate, co-aggregate and competitively exclude the selected EAEC strains from securing attachment to the Caco-2 cells. Once excluded, they (the selected EAEC strains) will not be able to cause inflammation of Caco-2 cells.

   In the presence of enteric pathogens, including non-Shiga toxin-producing *E. coli* O157, *Lactobacillus plantarum* FS2 and *Pediococcus pentosaceus* D39 demonstrated high aggregative abilities together with increased levels of adhesion to Caco-2 cells (Fayemi, 2015b). Additionally, the two potential probiotic bacteria independently and in combination demonstrated significantly high levels of bacteriostatic and antimicrobial properties, as they inhibited the growth of the enteric pathogen [non- Shiga toxin-producing *E. coli* (O157)] during the fermentation of *ogi*, a non-alcoholic cereal beverage (Fayemi, 2015b). *Lactobacillus plantarum* strains competed with the pathogens for limited luminal substrates and engaged in

the aggressive synthesis of certain antimicrobial substances like organic acids, peroxides and bacteriocins, which lower the luminal pH (Carvalho *et al.*, 2021; Surendran Nair *et al.*, 2017; Yang *et al.*, 2018a). Therefore, it is expected that these two lactic acid bacteria strains will competitively exclude, displace, and inhibit EAEC from adhesion to the Caco-2 cells, preventing inflammation of the epithelial cells.

3. The two potential probiotics, *L. plantarum* FS2 and *Pediococcus pentosaceus* D39, isolated from the West-African fermented cereal, *ogi*, will protect and maintain the epithelial barrier integrity and function from the ravaging effects of the selected D-EAEC strains isolated from the unpasteurised fresh milk.

Probiotics promote human health by inhibiting the growth of pathogens by competing for limited nutrients that are otherwise available for utilisation by pathogens. They also synthesise low molecular weight compounds including alcohols (methanol, ethanol, propanol etc.), organic acids (like acetic, butyric, isobutyric lactic, propanoic, propionic acids) and large molecular weight antimicrobial compounds known as bacteriocins (bifidocin B, enterocin, epidermine, lactacin B, lactacin F, mersacidin, nisin, pediocin, plantaricin, sakacin, subtilin etc.) which are naturally antimicrobial against most Gram-negative bacteria including pathogens (Abdi *et al.*, 2021; Gaspar *et al.*, 2018; Hernández-González *et al.*, 2021; Li *et al.*, 2022). Probiotics including *Lactobacillus rhamnosus* strain GG and *L. plantarum* inhibited the attachment of enteropathogenic *Escherichia coli* (Monteagudo-Mera *et al.*, 2019; Tuo *et al.*, 2018; Wang *et al.*, 2018c). Probiotics confer beneficial health-promoting properties on their host by adhering to the intestinal epithelium through toll-like receptors (TLRs) followed by mediation of the immune stimulation. The intestinal epithelium demonstrates several physical adaptations for separating the host connective tissue from the external environment. This physical barrier comprises a single layer of epithelial cells connected by intercellular tight junctions and a mucous layer (Hollander and Kaunitz, 2020; Seo *et al.*, 2021; Suzuki, 2020).

The intestinal epithelium is reinforced by biochemical modifications like glycocalyx from the secretion and apical adhesion of a densely glycosylated mucin-rich layer by Goblet cells. Collectively, these constitute a viscous and relatively impermeable layer covering and protecting the apical surface of the intestinal epithelium from the ravaging effects of enteropathogens (Allaire *et al.*, 2018a; Allaire *et al.*, 2018b; Faderl *et al.*, 2015). Probiotics have been demonstrated to improve the intestinal barrier by multiplying the Goblet cells to reinforce the mucus layer (Camilleri, 2021; Galdeano *et al.*, 2019; Hiippala *et al.*, 2018). Furthermore, numerous *Lactobacillus* species were demonstrated to boost mucin expression in human IECs (Davoren *et al.*, 2019; Garcia-Gonzalez *et al.*, 2018; Ren *et al.*, 2018). For example, the probiotic formula, VSL#3 contains some Lactobacillus species increased MUC2, MUC3, and MUC5AC expression in HT29 cells (Galdeano *et al.*, 2019; La Fata *et al.*, 2018; Liu *et al.*, 2020c; Wan *et al.*, 2016b). MUC 2 expression was also increased in the same cell line by *L. acidophilus* A4, which was independent of the bacterial adhesion to the cell monolayers (Galdeano *et al.*, 2019; Simon *et al.*, 2021; Suez *et al.*, 2019; Wan *et al.*, 2019). Thus, probiotics are indispensable for preventing intestinal inflammation and maintaining epithelial barrier integrity and function, as demonstrated by various *in vitro* and animal models.

4. The two promising probiotics, *L. plantarum* FS2 and *Pediococcus pentosaceus* D39 from the West-African fermented cereal, *ogi,* will mitigate against the cytotoxicity of epithelial host cells and protect the epithelial barrier to prevent gut permeability from the pathogenetic effects of the selected diarrhoeagenic enteroaggregative *E. coli* (D-EAEC) isolates from the unpasteurised fresh milk.

   The intestinal epithelium has developed mechanisms to protect itself from uncontrolled inflammatory responses. These mechanisms include limiting its direct contact with luminal bacteria and preventing bacterial translocation into underlying tissue. When disrupted, this barrier can lose its immune tolerance to microflora leading to improper inflammatory response

as manifested in Crohn's disease and inflammatory bowel diseases (IBD) (Kiesler *et al.*, 2015; Li *et al.*, 2021; Sartor and Wu, 2017; Silva *et al.*, 2016). The antimicrobial peptides, the epithelial apical junctional complex, and the secretory Immunoglobulin A (IgA) constitute the intestinal epithelial barrier defences (Camilleri, 2019; Halpern and Denning, 2015; Luissint *et al.*, 2016; Wan *et al.*, 2016b; Wang *et al.*, 2015b). Consumption of commensal (non-pathogenic) bacterial species such as probiotics can improve intestinal barrier function by offering inherent defence against enteropathogens, limiting paracellular and transepithelial permeabilities. They can also improve the intestinal mucosa's physical barrier functionality, which may collectively protect against infections, counteract chronic inflammation, and sustain mucosal integrity.

## 3.2 Objectives

1. To characterise cell surface characteristics (auto-aggregation, co-aggregation, and hydrophobicity abilities) and the antimicrobial properties of LAB isolates from *ogi*, a traditionally fermented West-African cereal, against selected diarrhoeagenic enteroaggregative *Escherichia coli* from unpasteurised fresh milk samples with the aim of developing fermented functional foods towards human clinical interventions such as the prevention and treatment of gastrointestinal infections.

2. To evaluate the effects of cereal-based LAB isolated from traditional African fermented food, *ogi*, on the adhesion of selected EAEC strains isolated from unpasteurised fresh milk samples.

3. To evaluate the abilities of *L. plantarum* FS2 and *Pediococcus pentosaceus* D39 isolated from a West-African fermented cereal, *ogi*, to maintain the epithelial barrier integrity and function from the destructive effects of selected diarrhoeagenic enteroaggregative *E. coli* (D-EAEC) strains from unpasteurised fresh milk.

4. To evaluate the abilities of *L. plantarum* FS2 and *Pediococcus pentosaceus* D39 isolated from a West-African fermented cereal, *ogi* to mitigate cytotoxicity and epithelial barrier permeability due to the pathogenetic effects of selected diarrhoeagenic enteroaggregative *E. coli* (D-EAEC) strains from unpasteurised fresh milk.

## RESEARCH CHAPTERS

The focus of the research chapters covered by this thesis are entitled as follows:

1. In vitro evaluation of aggregation, hydrophobicity, and antagonistic abilities of potential probiotic bacteria from *ogi* against enteroaggregative *Escherichia coli*

2. Presumptive probiotic bacteria from traditional fermented African food challenge the adhesion of enteroaggregative *E. coli*

3. Immunomodulatory activities of potential probiotics, *Lactobacillus plantarum* and *Pediococcus pentosaceus* in enteroaggregative *Escherichia coli* (EAEC)-challenged Caco-2 cells

4. Potential probiotics, *Lactobacillus plantarum* (FS2) and *Pediococcus pentosaceus* (D39) inhibit enteroaggregative *E. coli* impaired Caco-2 cells viability and permeability

# CHAPTER 4.

## RESEARCH CHAPTER 1

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**In vitro evaluation of aggregation, hydrophobicity, and antagonistic abilities of potential probiotic bacteria from *ogi* against enteroaggregative *Escherichia coli***

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This chapter was previously submitted to *LWT Food Science and Technology*.

# In vitro evaluation of aggregation, hydrophobicity, and antagonistic abilities of potential probiotic bacteria from *ogi* against enteroaggregative *Escherichia coli*

## 4.1 Abstract

Probiotic bacteria undergo specialised activities, including aggregation, cell surface hydrophobicity, adhesion to epithelial cells, and production of certain antimicrobial substances against pathogens. These activities enable them to exert their beneficial effects on their host. Selected lactic acid bacteria (LAB) and enteroaggregative *E. coli* (EAEC) previously isolated from traditionally fermented West-African cereal, *ogi* and unpasteurised fresh milk, respectively, were obtained studied for their abilities to aggregate and adhere to hydrocarbons. The LAB was further evaluated for their abilities to inhibit the EAEC. The LAB (*Lactobacillus plantarum* FS2 and *Pediococcus pentosaceus* D39) and EAEC demonstrated satisfactory cell surface properties and aggregation abilities with their EAEC counterparts. However, their auto-aggregation and co-aggregation were strain specific. Also, their adhesion to hydrocarbons and the exclusion of EAEC by LAB depended on the two bacteria involved and their incubation time. Neither LAB nor EAEC shows a correlation between auto-aggregation and adhesion, a requirement for colonisation and infection of the gastrointestinal tract. Likewise, there was no correlation between auto-aggregation and adhesion to Caco-2 cells and between hydrophobicity and auto-aggregation abilities for the two combatting bacteria. Therefore, the two LAB from traditionally fermented West African cereal, *ogi*, demonstrated promising properties worth considering for further applications in fermented functional foods and food products to improve human health.

## 4.2 Introduction

Humans' quest for quality life and their preference for naturally preserved and health-promoting foods whilst confronting the rising cost of healthcare are some of the motives for engaging in aggressive research and development activities into functional foods. Functional foods include foods containing bioactive compounds and probiotics. "Probiotics are microorganisms which, when administered in

adequate quantities, confer health benefits on their host" (Cremon *et al.*, 2018; Gasbarrini *et al.*, 2016; Santacroce *et al.*, 2019). *ogi* (*Akamu*) is a traditionally fermented Nigerian food from sorghum or millet. The grains are steeped (about three days), wet-milled, and sieved, followed by semi-solid-state fermentation (roughly three days). It is then cooked into a creamy pudding (pap) and served with *moin moin* (steamed cowpea flour) or *akara* (deep-fried cowpea flour) (Fayemi, 2015a). Like other traditionally fermented African foods, including asana, kenkey etc., *ogi* are a rich source of several lactic acid bacteria (LAB), some of which are beneficial to their hosts. Consumption of non-cooked fermented foods delivers several logs of these valuable LAB into the gut.

The contributions of functional foods to promoting human health cannot be overemphasised. In 1994, the World Health Organisation considered probiotics as an essential bioagent for boosting one's immune system in an era of increasing resistance to most prescribed antibiotics (Yeşilyurt *et al.*, 2021). Probiotics have been associated with most fermented foods, which have become traditionally accepted within their producing communities over the years (Lei *et al.*, 2006; Vasiljevic and Shah, 2008). Spontaneously fermented foods have attracted diverse research with the primary focus on identifying the predominant microbes involved with the process. Some of these studies have further recognised and characterised microorganisms for their probiotic potential. There is a need for more information on the probiotic prospects of these LAB, considering the position of spontaneously fermented foods in Africa. This information will facilitate the exploration and exploitation of this knowledge to produce safe, consistent, and shelf-stable foods and food products with health-promoting properties.

The process by which two or more genetically unrelated bacteria cells physically attach to one another using specific cellular or extracellular surface molecules is referred to as co-aggregation (Rickard *et al.*, 2003). The ability of probiotic bacteria (PBB) to co-aggregate with enteric pathogens is a requirement to form an intestinal barrier to inhibit enteric pathogens from colonising the gut (Boris *et al.*, 1997; Del Re *et al.*, 2000; Schachtsiek *et al.*, 2004). Bacterial auto-aggregation and adhesion were reported to be influenced by the hydrophobicity of the cell membranes (Del Re *et al.*, 2000; Pérez *et*

*al.*, 1998; Wadstroum *et al.*, 1987). Studies with bifidobacterial species reported a relationship between its adhesion and auto-aggregation to epithelial cells (Pérez *et al.*, 1998). Previous reports indicate that bacterial aggregation and adhesion were found to be dependent on several factors, including cell wall properties and the proteinaceous nature of the plasmalemma (Bibiloni *et al.*, 2001; Canzi *et al.*, 2005; Mukai and Arihara, 1994). Some predominant microorganisms from the human gut microbiota include *Bifidobacterium bifidum*, *Lactobacillus plantarum*, *Pediococcus pentosaceus*, *Lactobacillus acidophilus* etc. (Krishnamoorthy *et al.*, 2020; Mowat and Agace, 2014).

Several studies reported LAB's adhesion and aggregation abilities mainly from dairy sources and their effects on various enteric pathogens, focusing less on potential PBB from alternative (non-dairy) sources. Additionally, quantifying the antagonistic impacts of beneficial LAB was mainly limited to determining inhibition zones with less attention to other *in vitro* experimental procedures. This study was aimed to characterise cell surface characteristics [auto-aggregation, co-aggregation, and hydrophobicity abilities (HAs)] and the antimicrobial properties of LAB isolates from *ogi*, a traditionally fermented West-African food, against selected diarrhoeagenic enteroaggregative *Escherichia coli* from unpasteurised fresh milk samples with the aim of developing fermented functional foods towards human clinical interventions such as the prevention and treatment of gastrointestinal infections. These interventions would enhance human health towards economic growth development, especially within vulnerable communities.

## 4.3 Materials and methods

### 4.3.1 Bacterial strains (BaSs) and culturing conditions

Four selected diarrhoeagenic (3591-87, K2, K3, K16) and a non-diarrhoeagenic *E. coli* (ND-EAEC), N23 strains (Table 4.1); isolated from unpasteurised fresh milk (except for 3591-87 being a positive clinical reference control) were used in this study (Aijuka, 2018; Ntuli *et al.*, 2017). Two LAB, *Lactobacillus plantarum* FS2 and *Pediococcus pentosaceus* D39, with some potential probiotic

characteristics, were obtained out of the several isolates from a previous study involving traditional non-alcoholic fermentation of maise for the production of a West African gruel (*ogi*) from an earlier study (Fayemi and Buys, 2017; Fayemi *et al.*, 2017). The other LAB (probiotics) used in this study and their culturing conditions were described (Table 4.1).

Table 4.1: Sources and characteristics of selected enteroaggregative *E. coli* (EAEC) and lactic acid bacteria (LAB) strains used in this study

| Bacteria strain | Characteristic | Source |
|---|---|---|
| EAEC 3591-87 | Clinical and diarrhoeagenic (positive reference strain) | [a]NICD of NHLS |
| EAEC K2 | Diarrhoeagenic | [b]Unpasteurised fresh milk |
| EAEC K3 | Diarrhoeagenic | [b]Unpasteurised fresh milk |
| EAEC K16 | Diarrhoeagenic | [b]Unpasteurised fresh milk |
| EAEC N23 | Non-Diarrhoeagenic | [b]Unpasteurised fresh milk |
| *Lactobacillus acidophilus ATCC* 4356 | Reference probiotic bacteria | [c]ATCC Collections |
| *Bifidobacterium bifidum ATCC* 11863 | Reference probiotic bacteria | [c]ATCC Collections |
| *Lactobacillus plantarum* FS2 | Promising probiotic characteristics | [d]Traditional fermented food (*ogi*) |
| *Pediococcus pentosaceus* D39 | Promising probiotic characteristics | [d]Traditional fermented food (*ogi*) |

[a]National Institute for Communicable Diseases (NICD), a division of the National Health Laboratory Service (NHLS), Johannesburg, the Republic of South Africa.

[b]Previously isolated by Aijuka *et al.* (2018) and Ntuli *et al.* (2017).

[c]American Type Culture Collection (ATCC, USA).

[d]Previously isolated by Fayemi and Buys (2017) Fayemi and Buys (2017).

Equation 4.1: Bacterial auto-aggregation

$$Autoaggregation = (1 - A_t/A_0) \times 100$$

### 4.3.2 Cell culture

Human colon adenocarcinoma (Caco-2) cells supplied by American Type Culture Collections (ATCC, Maryland, USA) were obtained and cultured under the same conditions as previously described (Agbemavor and Buys, 2021).

### 4.3.3 Auto-aggregation

The four LAB and the five EAEC strains were investigated for their auto-aggregation based on their sedimentation characteristics. The effect of BaS and treatment time on auto-aggregation was carried out as outlined (Del Re et al., 2000; Kos et al., 2003) with certain modifications. Briefly, 18 h old bacterial cultures were harvested and washed three times with phosphate-buffered saline (PBS) by centrifugation (5 000 g, 10 min, 25 ℃). The bacterial cells (BCs) were re-suspended in PBS (≈ 1.5 x 108 CFU/mL) for both EAEC and LAB. The suspensions of each BaS were plated (100 µL/well) using a 96-well plate. The initial absorbance (A0) of the bacterial suspensions was quickly measured at 600 nm. The setup was incubated (37 ℃, 1, 2, 4 and 5 h), and the final absorbance readings (At) were measured at different times accordingly, and the auto-aggregation of each bacterial suspension was calculated (Equation 4.1) (Kos et al., 2003).

#### *4.3.3.1 Effect of simulated gastric fluid and bile salt conditioning on auto-aggregation*

The auto-aggregation abilities (A-AAs) of EAEC and LAB cells were evaluated using simulated gastric fluid (SGF), prepared according to previous protocols (Falah *et al.*, 2019; Ruiz *et al.*, 2013) with a few modifications. Briefly, the pH of distilled water was adjusted to 1.5, 2.5, 4.5, 6.5 and 7.0 by adding 1 M HCl in drops and then used to prepare PBS, followed by sterilisation (cold filtration with a syringe). Standardised bacterial suspensions were prepared using the pH-adjusted PBS described (section 4.3.1).

Bile salt (BiS) solutions (0, 1.0 and 2.0 %) were used to prepare PBS to determine the effect of BiS on the auto-aggregation of the BCs. The pH of these solutions was adjusted to 6.5 using 1 M HCl followed

by cold, sterile filtration. The sterile PBS was used to standardise BCs described earlier (section 4.3.1). The BCs were incubated (37 ℃, 5 h), and their respective absorbance readings at 600 nm were taken to calculate their auto-aggregations as described (section 4.3.1).

### 4.3.4 Co-aggregation

For co-aggregation determination, the BCs were prepared as described (section 4.3.3), except that pure EAEC suspensions were mixed with equal volumes of LAB (Table 4.2). As a control set-up, equal volumes of PBS were added to each BaSs, followed by mixing and incubation (37 ℃, 1 and 5 h). 100 µl prepared bacterial suspensions were taken carefully from the top of the test tube without agitation, mixed, and plated as described (section 4.3.3). The absorbance readings for the mixture [*A(x+y)*] and the control (*Ax* and *Ay*) of the individual bacterial suspensions were taken (1 and 5 h; Table 4.2). The co-aggregation was calculated using the mathematical Equation 4.2 (Handley *et al.*, 1987).

Equation 4.2: Bacterial co-aggregation

$$Coaggregation\ (\%) = [[(A_x - A_y)/2 - A(x+y)]/[(A_x - A_y)/2]] \times 100$$

### 4.3.5 Antimicrobial effect of LAB against EAEC

This assay was carried out as illustrated (Michail and Abernathy, 2002b) using 18 h old cultured bacterial cells. EAEC ($1.0 \times 10^8$ CFU/mL) was homogenised and incubated in the presence or absence of equal volumes of LAB ($1.0 \times 10^9$ CFU/mL) as indicated (Table 4.2). The setups were incubated (37 °C; 1 and 5 h). The viable EAEC were enumerated by plating as described (section 4.3.1). EAEC colonies were countered in colony-forming units (CFU/mL).

### 4.3.6 Bacterial cell surface hydrophobicity (BCSH)

BCSH was determined by measuring the adhesion of BCs to hydrocarbon solvents as described (Kos *et al.*, 2003) with some modifications (Bellon-Fontaine *et al.*, 1996; Collado *et al.*, 2008b; Rosenberg *et al.*, 1980). BCs were harvested by centrifugation (8 000 g; 10 min; 4 ℃), washed twice and resuspended in PBS (pH; 7.2; 3.0 x $10^8$ CFU/mL).

Table 4.2: Enteroaggregative *E. coli* (EAEC) and lactic acid bacteria (LAB) treatment template for Caco-2 monolayers infection

| LAB strains | EAEC Strains | | | | |
|---|---|---|---|---|---|
| Control | 3591-87 | K2 | K3 | K16 | N23 |
| 4356 | 3591-87 + 4356 | K2 + 4356 | K3 + 4356 | K16 + 4356 | N23 + 4356 |
| 11863 | 3591-87 + 11863 | K2 + 11863 | K3 + 11863 | K16 + 11863 | N23 + 11863 |
| FS2 | 3591-87 + FS2 | K2 + FS2 | K3 + FS2 | K16 + FS2 | N23 + FS2 |
| D39 | 3591-87 + D39 | K2 + D39 | K3 + D39 | K16 + D39 | N23 + D39 |

The EAEC constitutes diarrhoeagenic and non-diarrhoeagenic ones. The diarrhoeagenic EAEC strains include 3591-87, K2, K3 and K16, denoted by 3591-87, K2, K3 and K16, respectively. The lactic acid bacteria include *L. acidophilus* ATCC 4356 and *B. bifidum ATCC* 11863, *Lactobacillus plantarum* FS2 and *Pediococcus pentosaceus* D39 represented by 4356, 11863, FS2 and D39.

The initial absorbance of the BCs was measured as $A_0$ (600 nm). An equal volume of the solvent (chloroform, ethyl acetate and xylene) was added to the standardised bacterial suspension and mixed thoroughly (4 min). After incubating the mixture (room temperature, 1 h), the aqueous phase was separated, and its absorbance ($A_f$) was measured at (600 nm). BCSH was calculated and reported as the percentage of bacterial adhesion to hydrocarbons (BATH), as shown by Equation 4.3.

Equation 4.3: Bacterial surface hydrophobicity

$$BSH\ (\%) = [(A_0 - A_f)/A_0] \times 100$$

### 4.3.7 Bacterial adhesion Assay

The human colon adenocarcinoma (Caco-2) cell line was used as an IEC model to evaluate the abilities of EAEC and LAB cells to adhere to the intestinal epithelium (Nueno-Palop and Narbad, 2011). This assay was carried out as outlined (Agbemavor and Buys, 2021).

### 4.3.8 Data analysis

All experiments were independently run-in triplicates. The strain and incubation time effects on auto-aggregation for the pure BaSs were compared using a two-way analysis of variance (ANOVA) tool-pack of Statgraphics Centurion XVI (Statpoint Technologies Inc, 2013). Contrarily, the effect of BaSs on co-aggregation between the EAEC and LAB strains and the antimicrobial effects of LAB against the EAEC strains were analysed using a one-way ANOVA. Results were compared at a 95 % confidence level and mean values with $p \leq 0.05$ were considered statistically significant. Multiple range tests were conducted using Fisher's least significant difference.

## 4.4 Results and discussions

This section involves the four major experiments: a comparative study of the auto-aggregation of the BaSs, the co-aggregation of EAEC with LAB strains and the direct bactericidal effects of LAB against EAEC and hydrophobicity abilities (HAs) concerning auto-aggregation and adhesion to Caco-2 monolayers.

### 4.4.1 Auto-aggregative properties of EAEC and LAB

We evaluated different EAEC and LAB strains for their auto-aggregation and co-aggregation abilities. As expected, the A-AAs of all the bacteria strains showed time-dependent effects. However, the LAB strains showed higher ($P < 0.05$) auto-aggregation scores (A-ASs) than their diarrhoeagenic enteroaggregative *Escherichia coli* (D-EAEC) counterparts at any given incubation (treatment) time (Table 4.3). Accordingly, the magnitude of increment in auto-aggregation with time was more pronounced among the LAB strains than their EAEC counterparts. Moreover, it was quite interesting to note that the non-diarrhoeagenic EAEC (ND-EAEC) had higher ($P < 0.05$) auto-aggregation values (3.6 – 26.2 %) than the D-EAEC (1.3 – 12.4%) throughout the various treatment times. Generally, the final A-AAs illustrated by the LAB (24.4 – 46.9 %) were much higher than their D-EAEC counterparts

Table 4.3: The effects of bacterial strains and treatment (incubation) time on the auto-aggregation of enteroaggregative *E. coli* and probiotic bacteria strains

| Treatment time (h) | Auto-aggregation (%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 3591-87 | K2 | K3 | K16 | N23 | ATCC 4356 | ATCC 11863 | FS2 | D39 |
| 1 | $1.5^{ab} \pm 0.2$ | $1.7^{abc} \pm 0.2$ | $1.3^{a} \pm 0.2$ | $2.3^{a-e} \pm 0.4$ | $3.6^{b-f} \pm 0.6$ | $5.5^{f-j} \pm 1.2$ | $4.0^{d-g} \pm 0.6$ | $12.0^{nop} \pm 2.3$ | $8.7^{lm} \pm 2.0$ |
| 2 | $2.8^{a-e} \pm 0.5$ | $2.5^{a-e} \pm 0.4$ | $2.3^{a-d} \pm 0.3$ | $3.8^{c-f} \pm 0.5$ | $7.9^{klm} \pm 1.2$ | $15.1^{q} \pm 1.2$ | $11.7^{no} \pm 1.8$ | $22.4^{st} \pm 3.6$ | $13.9^{pq} \pm 1.1$ |
| 3 | $3.2^{a-e} \pm 0.3$ | $3.6^{b-f} \pm 0.3$ | $3.9^{d-g} \pm 0.6$ | $6.3^{hk} \pm 0.6$ | $19.1^{r} \pm 3.1$ | $23.8^{st} \pm 3.6$ | $15.6^{q} \pm 3.0$ | $33.5^{w} \pm 3.6$ | $29.4^{v} \pm 3.5$ |
| 4 | $4.1^{d-h} \pm 0.5$ | $5.9^{g-j} \pm 0.6$ | $6.4^{ijk} \pm 1.1$ | $9.9^{mn} \pm 1.1$ | $23.6^{st} \pm 4.5$ | $28.4^{v} \pm 5.2$ | $22.0^{s} \pm 4.1$ | $40.9^{y} \pm 5.6$ | $36.6^{x} \pm 6.2$ |
| 5 | $4.6^{e-i} \pm 0.7$ | $6.5^{ijk} \pm 1.2$ | $7.2^{jkl} \pm 1.0$ | $12.4^{op} \pm 2.1$ | $26.2^{u} \pm 2.4$ | $34.7^{wx} \pm 4.0$ | $24.4^{tu} \pm 3.4$ | $46.9^{z} \pm 6.0$ | $41.5^{y} \pm 4.9$ |

The table illustrates the effects of bacterial strains and treatment (incubation) time on auto-aggregation of selected EAEC and probiotic bacteria strains. The EAEC strains were diarrhoeagenic and non-diarrhoeagenic. The diarrhoeagenic EAEC strains include 3591-87, K2, K3, and K16. The non-diarrhoeagenic EAEC strain was N23. The lactic acid bacteria include *L. acidophilus* ATCC 4356 and *B. bifidum*; ATCC 11863, *L. plantarum* FS2 and *P. pentosaceus* D39. Each set of value is a mean of three independent replicates (n=27) with its corresponding standard deviation. Mean values with different superscripts (a-z) indicate significant differences ($p \leq 0.05$) according to Fisher's LSD test.

(4.6 to 12.4 %). Surprisingly, the ND-EAEC N23 amongst the EAEC strains demonstrated the highest auto-aggregation ability (A-AA, 26.2 %).

In comparison, the auto-aggregation results obtained for the LAB in this study generally agreed with that of Collado *et al.* (2008a). Contrarily, LAB from separate studies demonstrated exceptionally higher A-AAs (51.2 – 78.2 %) and (96.7 to 98.6 %) as previously reported (Zakaria Gomaa, 2013) and (Balakrishna, 2013; Malik *et al.*, 2003), respectively. De Souza *et al.* (2019) also reported similarly higher A-ASs (61.0 to 96.2 %) for *L. fermentum* and *L. casei*. Interestingly, our results for EAEC strains were higher than previous findings (Collado *et al.*, 2007c). Obligatory heterofermentative LAB, including *Lactobacillus gasseri, Lactobacillus acidophilus*, and *Lactobacillus crispatus* scored higher auto-aggregation values (Pisano *et al.*, 2008). Previous studies equally reported the linear relation between the auto-aggregation and incubation time (Balakrishna, 2013; Nuramkhaan *et al.*, 2019; Trunk *et al.*, 2018b; Wang *et al.*, 2018d).

Auto-aggregation refers to the physical interaction of BCs among themselves, leading to their settlement at the bottom of a stationary liquid suspension as buffered peptone water or PBS (Chaudhary and Saharan, 2018; Krausova *et al.*, 2019). The ability of bacteria to form cellular aggregates through auto-aggregation (cellular aggregation within the same BaS) or co-aggregation (cellular aggregation between genetically different BaSs) plays a very significant role in their persistence within the GIT. However, bacterial aggregation can antagonise potential pathogens (Gut *et al.*, 2019; Khusro *et al.*, 2018; Tatsaporn and Kornkanok, 2020). PBB need to form large biomass via aggregation to confer desirable health benefits on their host. Contradicting opinions have been reported about the significance of co-aggregation. Some researchers reported that pathogens need to co-aggregate with probiotics at lower levels for the prevention of biofilm formation and also to minimise the colonisation of the GIT (Gut *et al.*, 2019). Alternatively, other researchers believe co-aggregation of pathogens with PBB is advantageous in promoting the elimination of pathogens from the GIT (De Souza *et al.*, 2019; Todorov and Dicks, 2008).

### 4.4.2 Effect of simulated gastric fluid conditioning on auto-aggregation

The auto-aggregation of the D-EAEC were significantly ($P < 0.05$) lower than that of the ND-EAEC N23 and their LAB counterparts (Figure 4.1). The A-ASs of D-EAEC strains were generally not

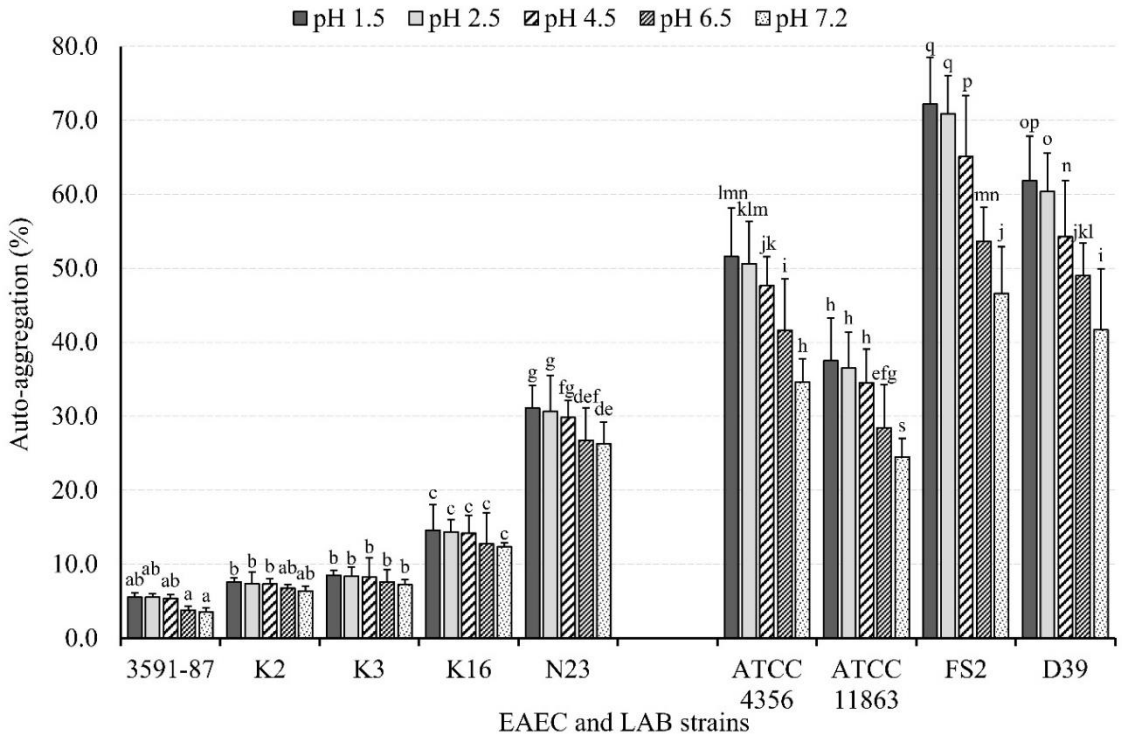


Figure 4.1: Effect of simulated gastric fluid conditioning on auto-aggregation of bacterial cells after 5 hours of treatment

The enteroaggregative *E. coli* (EAEC) include diarrhoeagenic (3591-87, K2, K3, and K16) and a non-diarrhoeagenic (N23) strain. *L. acidophilus* ATCC 4356 and *B. bifidum ATCC* 11863; *L. plantarum* FS2 and *P. pentosaceus* D39 are the lactic acid bacteria (LAB) strains. Each bar is a mean of three independent replicates (n=12) with its corresponding standard deviation. Bars with different letters (a-q) indicate significant differences ($P \leq 0.05$) according to Fisher's Least Significant Difference (LSD) test.

affected by the treatment with the gastric fluids at different pH levels. The ND-EAEC and the LAB showed a general decrease in their A-ASs with pH. The A-AA of ND-EAEC N23 was higher ($P < 0.05$) than all their diarrhoeagenic counterparts across the different acidity levels (Figure 4.1). On the

other hand, it recorded lower A-ASs than all the LAB except for *B. bifidum ATCC* 11863. The auto-aggregation of ND-EAEC N23 increased by 0.4, 3.5, 4.3 and 4.8 from 26.3 % in the presence of SGF at pH 1.5, 2.5, 4.5, 6.5, and 7.2, respectively. However, there was no difference between pH 6.5 and 7.2 regarding its AA-As which were higher at the higher acidities (pH 1.5, 2.5 and 4.5) than at lower acidities (pH 6.5 and 7.2).

Around the neutral pH (7.2), the A-AA of *L. acidophilus ATCC* 4356 was 34.6 % which increased by 7.0, 13.0, 16.0 and 16.9 % in the presence of SGFs at pH 6.5, 4.5, 2.5, and 1.5, respectively (Figure 4.1). Similarly, *B. bifidum ATCC* 11863 also demonstrated increments of 3.9, 10.0, 12.0 and 13.0 % from 24.5 % (pH 7.2) to 28.4, 34.5, 36.5, and 37.5 % at pH levels of 6.5, 4.5, 2.5, and 1.5, respectively. Decreasing the SGF pH from the neutral pH (7.2) to 6.5, 4.5, 2.5, and 1.5 increased the auto-aggregation of *L. plantarum* FS2 (46.6 to 53.6, 65.1, 70.9 and 72.2 %, respectively, Figure 4.1). The A-ASs of *P. pentosaceus* D39 also increased from 41.7 to 49.0, 54.3, 60.4 and 61.8 % from the neutral pH (7.2) to 6.5, 4.5, 2.5, and 1.5, respectively. Thus, except for *P. pentosaceus* D39, there was no difference in the A-AAs between pH 2.5 and 1.5 for the other three LAB.

### 4.4.3 Effect of simulated bile salt (SBiS) conditioning on auto-aggregation of BCs

Both EAEC and LAB strains demonstrated some reductions with an increase in BiS concentration (Figure 4.2). The auto-aggregation of EAEC 3591-87 decreased by 0.3 and 0.6 % with 1 and 2 % concentrated bile salts respectively; K2, (0.6 and 1.3 %); K3 (0.4 and 0.8 %); K16 (0.7 and 1.3 %) and N23 (1.0 and 2.2 %) with 1 and 2 % BiS, respectively. However, the BiS concentrations did not affect the A-ASs of all the EAEC, regardless of their diarrhoeagenic status. Among the LAB, differences in BiS concentration did not affect the auto-aggregation of the two reference PBB (Figure 4.2). Contrarily, the auto-aggregation of *L. plantarum* FS2 (58.6 %) was significantly reduced ($P < 0.05$) by 3.3 and 3.8 % in the presence of 1 and 2 % concentrated BiS. However, the auto-aggregation of *P. pentosaceus* D39 (50.5 %) suffered a significant reduction (3.5 %) when treated with 2 % concentrated BiS.

### 4.4.4 Bacterial cell surface hydrophobicity (BCSH)

Hydrophobicity was determined by analysing bacterial adhesion to hydrocarbon (BATH). Both EAEC and LAB strains showed varying affinities to the three hydrocarbons, namely chloroform, ethyl ester,

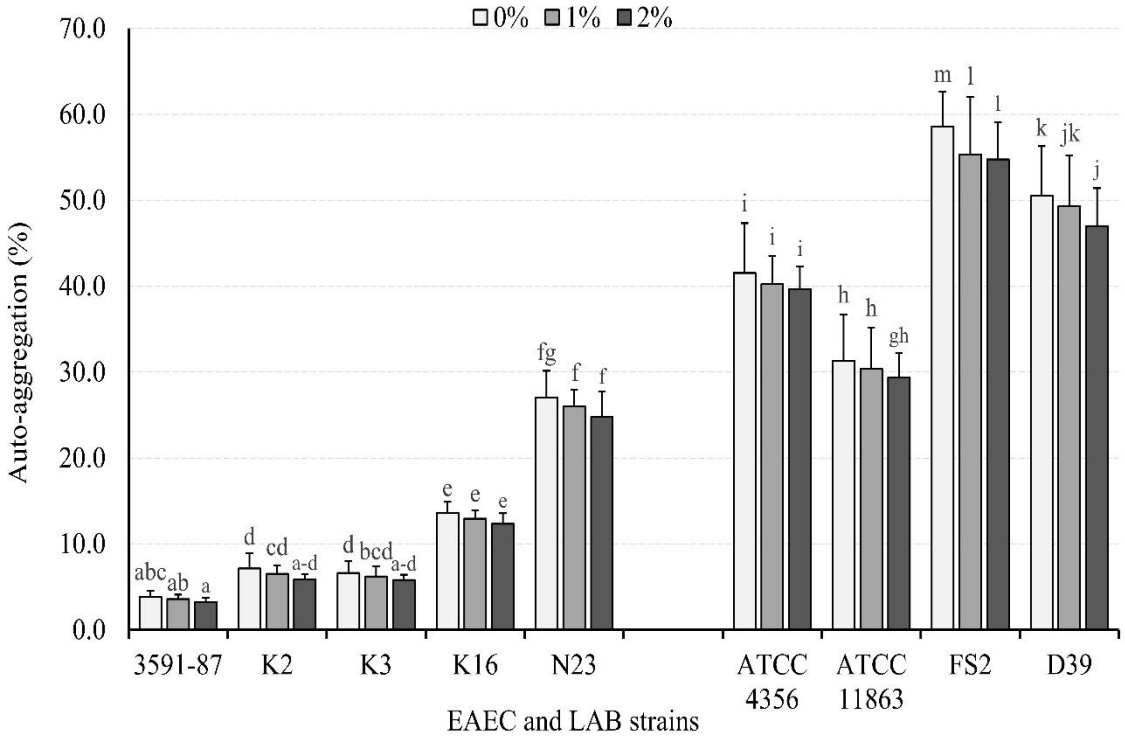


Figure 4.2: Effect of simulated bile salt conditioning on auto-aggregation of bacterial strains after 5 hours of treatment

The enteroaggregative *E. coli* (EAEC) include diarrhoeagenic (3591-87, K2, K3, and K16) and a non-diarrhoeagenic (N23) strain. *L. acidophilus* ATCC 4356 and *B. bifidum;* ATCC *L. plantarum* FS2 and *P. pentosaceus* D39 are the lactic acid bacteria (LAB) strains. Each bar is a mean of three independent replicates (n=12) with its corresponding standard deviation. Bars with different letters (a-m) indicate significant differences ($P \leq 0.05$) according to Fisher's LSD test.

and xylene. The hydrophobicity of EAEC and LAB based on xylene ranged from 32.7 (EAEC N23) to 50.7 % (EAEC K2) and 34.4 % (*L. acidophilus ATCC* 4356) to 49.3 % (*B. bifidum ATCC* 11863),

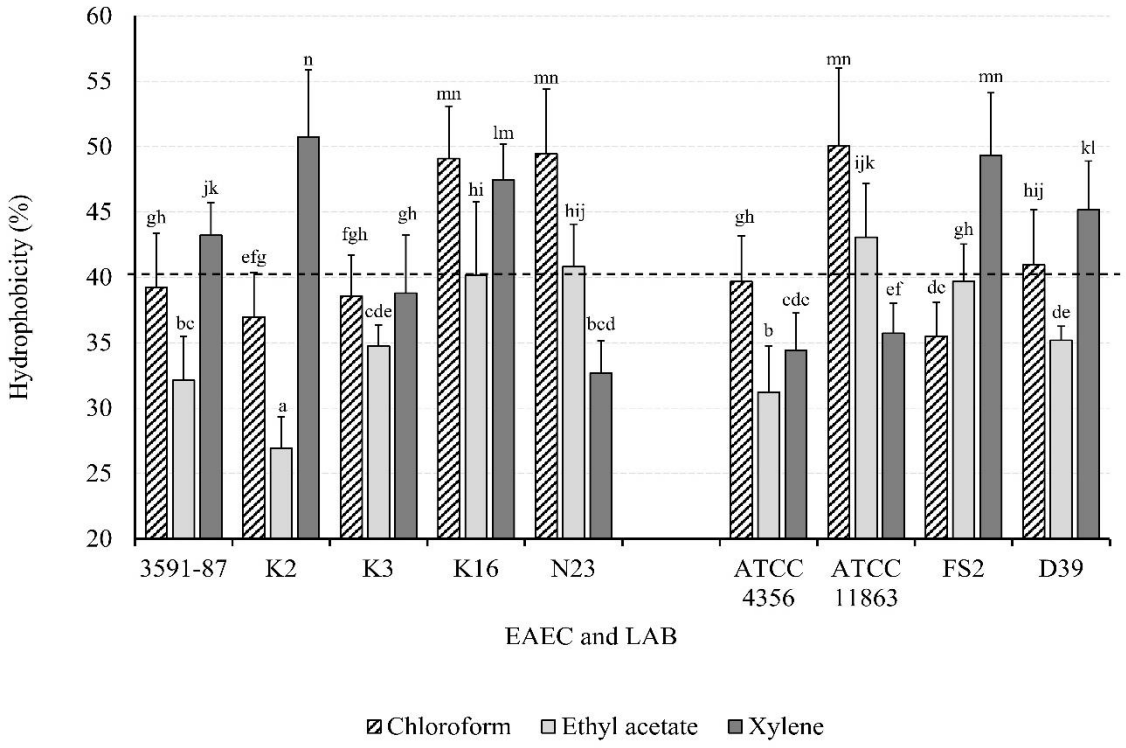


Figure 4.3: Bacterial cell surface hydrophobicity of bacterial strains by adhesion to different hydrocarbon solvents

The enteroaggregative *E. coli* (EAEC) include diarrhoeagenic (3591-87, K2, K3, and K16) and a non-diarrhoeagenic (N23) strain. *L. acidophilus* ATCC 4356 and *B. bifidum;* ATCC *L. plantarum* FS2 and *P. pentosaceus* D39 are the lactic acid bacteria (LAB) strains. Each bar is a mean of three independent replicates (n=12) with its corresponding standard deviation. Bars with different letters (a-n) indicate significant differences ($P \leq 0.05$) according to Fisher's LSD test.

respectively (Figure 4.3). The affinities of EAEC and LAB to hydrocarbon (xylene) were significantly different ($P < 0.05$) with the BaSs. These results suggest that the HAs of both EAEC and LAB were strain dependent. Additionally, except for EAEC K3, N23, and the two reference LAB, all the other EAEC and LAB strains had their HAs above the critical threshold (40 %) (Bellon-Fontaine *et al.*, 1996). Our results showed that the HAs of LAB in this study agreed with previous findings (Adebajo *et al.*, 2018; Fayemi, 2015a; Gandomi *et al.*, 2019; Poddar *et al.*, 2019). Contrarily, the EAEC's HAs

were higher than the reported results for *E. coli* (Khare and Verma, 2020; Yilmaz and Guvensen, 2016). Interestingly, higher HAs were reported allowing BCs to tolerate stress inflicted by hyperosmotic medium (Haddaji *et al.*, 2017; Lopez-Buesa *et al.*, 1998). The different ($P < 0.05$) with the BaSs. These results suggest that the HAs of both EAEC and LAB were strain dependent. Additionally, except for EAEC K3, N23, and the two reference LAB, all the other EAEC and LAB strains had their HAs above the critical threshold (40 %) (Bellon-Fontaine *et al.*, 1996). Our results showed that the HAs of LAB in this study agreed with previous findings (Adebajo *et al.*, 2018; Fayemi, 2015a; Gandomi *et al.*, 2019; Poddar *et al.*, 2019). Contrarily, our study's HAs for the EAEC were higher than the reported results for *E. coli* (Khare and Verma, 2020; Yilmaz and Guvensen, 2016). Interestingly, higher HAs were reported allowing BCs to tolerate stress inflicted by hyperosmotic medium (Haddaji *et al.*, 2017; Lopez-Buesa *et al.*, 1998). The nature of surface molecules conferring hydrophobicity on BCs is yet to be fully elucidated. However, lipoteichoic and other protein molecules that constitute their plasmalemma have been suggested to be responsible for the cell surface affinity of Gram-positive bacteria to hydrocarbons (Miörner *et al.*, 1983; Whitnack and Beachey, 1985). The adhesion of LAB to the epithelial cells may also be mediated by lipoteichoic acid through certain negative charges within their cell membranes. Most bacterial cell surfaces promote electrostatic binding with the different surfaces (Mohamadzadeh *et al.*, 2011).

This study used three solvents (chloroform, ethyl ester and xylene) as the hydrocarbons. Chloroform (acidic and electron-accepting) and ethyl ester (basic and electron-donating) are both monopolar solvents, whilst xylene is an apolar (non-polar) solvent. Among the three, it is the adhesion of cells to xylene that truly represents BCSH. Bacterial affinity to chloroform and ethyl ester indicates their abilities to donate and accept electrons, respectively, from these organic solvents (Akhtar *et al.*, 2017; Bellon-Fontaine *et al.*, 1996; Bouarab-Chibane *et al.*, 2019). Our results demonstrate that the capabilities of the BCs to offer and receive electrons closely followed their affinity (adhesion) to xylene were mostly higher than 40 %. Bacteria cells with outstanding hydrophobic abilities must score at least

40 % for their BATH. These results suggest that both EAEC and LAB could be adherent bacteria with the potential to adhere to the intestinal epithelium (Collado *et al.*, 2008b; Kos *et al.*, 2003). BCSH is a prominent factor in determining their adhesion to the intestinal mucosa (Aijuka *et al.*, 2019; Collado *et al.*, 2008b; Schillinger *et al.*, 2005). The adhesion of PBB and pathogens to the gut depends on a multiplicity of other factors aside from their hydrophobic properties (Collado *et al.*, 2008b; Schillinger *et al.*, 2005).

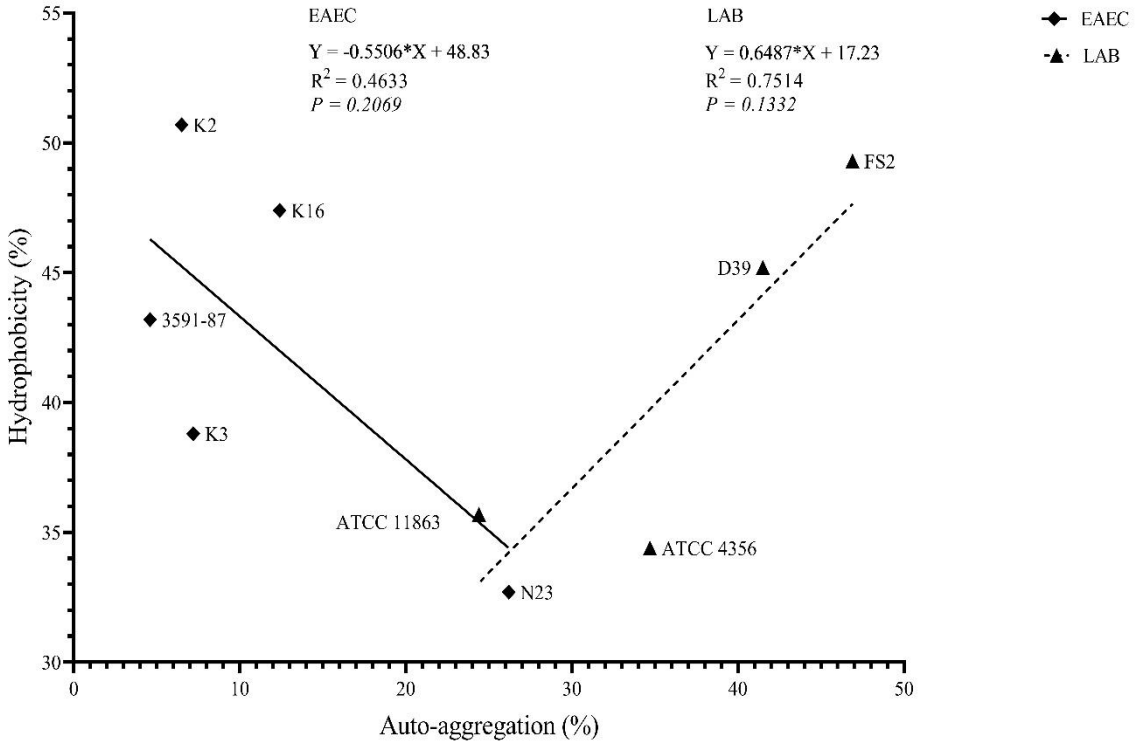


Figure 4.4: Relationship between bacterial adhesion to Caco-2 monolayers and auto-aggregation

The enteroaggregative *E. coli* (EAEC) include diarrhoeagenic (3591-87, K2, K3, and K16) and a non-diarrhoeagenic strain (N23). *L. acidophilus* ATCC 4356 and *B. bifidum;* ATCC *L. plantarum* FS2 and *P. pentosaceus* D39 are the lactic acid bacteria (LAB) strains. Each data point is a mean of three independent replicates (n=12).

Our study shows that EAEC demonstrated higher HAs with lower auto-aggregation levels. Thus, the higher their HAs, the lower their A-AAs and vice versa (Figure 4.4). Regardless of this linearity, our results demonstrated no correlation [correlation coefficient, (R) = 0.6807; *P=0.2069*] between these

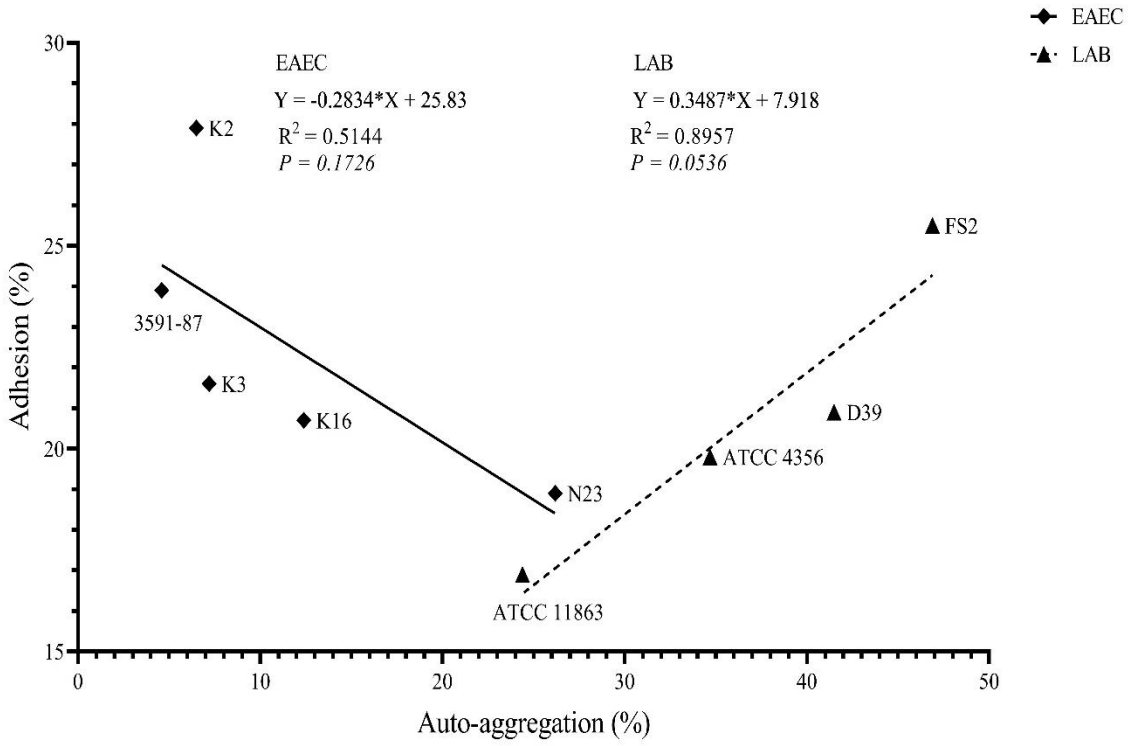


Figure 4.5: Relationship between bacterial adhesion to Caco-2 monolayers and auto-aggregation

The enteroaggregative *E. coli* (EAEC) include diarrhoeagenic (3591-87, K2, K3, and K16) and a non-diarrhoeagenic strain (N23). *L. acidophilus* ATCC 4356 and *B. bifidum;* ATCC *L. plantarum* FS2 and *P. pentosaceus* D39 are the lactic acid bacteria (LAB) strains. Each data point is a mean of three independent replicates (n=12).

two variables for the tested EAEC strains (Figure 4.4). This observation contradicts LAB, where strains with lower A-AAs demonstrated lower HAs. Like the EAEC strains, the LAB showed no correlation (R = 0.8668; *P=0.1332*) between the two variables. Our results agree with *Enterococcus faecalis* and *L. fermentum,* as reported by (Li *et al.*, 2015). Contrarily, our findings disagreed with previous reports (Rahman *et al.*, 2008). Our results further indicate that hydrophobicity and A-AAs for both bacteria seemed to be two independent traits, both of which were essential for adhesion.

### 4.4.5 Bacterial adhesion to Caco-2 cells

The tested EAEC and LAB adhered to the Caco-2 cells (Figure 4.5 and Figure 4.6). EAEC and LAB adhesion to the Caco-2 monolayers varied significantly ($P < 0.05$), ranging from 18.9 to 27.9 % and 16.9 to 25.5 %, depending on the strain. The D-EAEC showed higher adhesion capacities (20.7 – 23.9 %) than the ND-EAEC N23 (18.9 %). Except for *B. bifidum ATCC* 11863 (16.9 %), all the LAB

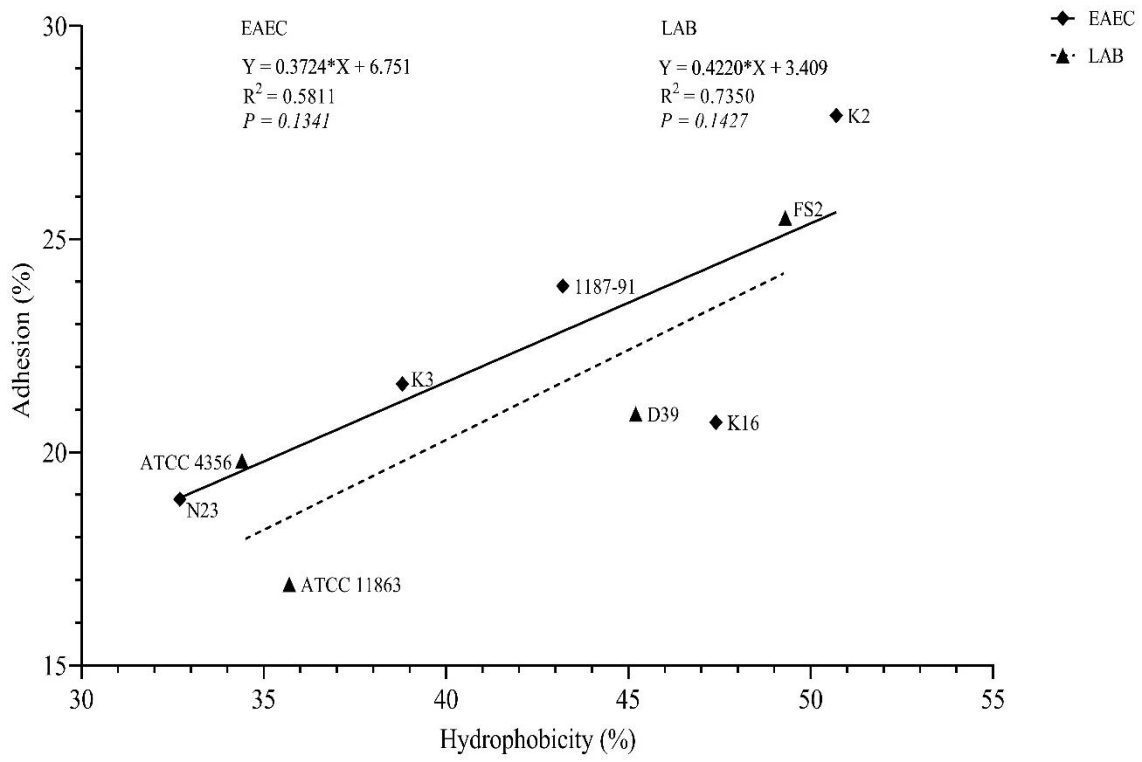


Figure 4.6: Relationship between bacterial adhesion to Caco-2 monolayers and hydrophobicity.

The enteroaggregative *E. coli* (EAEC) include diarrhoeagenic (3591-87, K2, K3, and K16) and a non-diarrhoeagenic (N23) strain. *L. acidophilus* ATCC 4356 and *B. bifidum;* ATCC *L. plantarum* FS2 and *P. pentosaceus* D39 are the lactic acid bacteria (LAB) strains. Each data point is a mean of three independent replicates (n=12).

exhibited higher adhesion affinities (19.8 – 25.5 %) to the differentiated Caco-2 cell monolayers. Interestingly, despite some linearity, there was no correlation between adhesion and A-AAs of EAEC

(R = 0.7172; *P=0.1726*) and LAB (R = 0.9464; *P=0.0536*) to the Caco-2 monolayers (Figure 4.5). Similarly, no correlation was detected between adhesion and HAs for EAEC (R = 0.7623; *P=0.1341*) and LAB (R = 0.8573; *P=0.1427*; Figure 4.6).

The current adhesion results agree with previous findings (Agbemavor and Buys, 2021), which demonstrated that the LAB strains from this study might have some probiotic potential. However, the lack of correlation indicates that the adhesions of both EAEC and LAB were not dependent on the auto-aggregation. These findings suggested that apart from the auto-aggregation, other factors could contribute to the adhesion of the two distinct bacteria to the intestinal epithelium. The lack of correlation results confirmed previous findings (Gandomi *et al.*, 2019; García-Cayuela *et al.*, 2014; Li *et al.*, 2015; Mackenzie *et al.*, 2010). Interestingly, however, our findings disagreed with the (Chaffanel *et al.*, 2018; Devi *et al.*, 2015; Grajek *et al.*, 2016; Ma *et al.*, 2020). Our findings agreed with previous findings which also reported a lack of correlation between adhesion and HAs (Collado *et al.*, 2008b; Li *et al.*, 2015) but contradicted other findings (Grajek *et al.*, 2016; Ma *et al.*, 2020).

One principal requirement for selecting potential PBB is their ability to adhere to the intestinal epithelium to colonise and remain within the gut (Borah *et al.*, 2016). This competence enables them to resist accidental removal from the lumen by natural physiological processes like peristalsis. Bacterial adhesion to the gut epithelium was influenced by several factors, including BCSH, hydrophilic and hydrophobic constituents, among other macromolecules (Borah *et al.*, 2016; Gandomi *et al.*, 2019). Additionally, BCs with high HAs are noted to form strong interactions with the intestinal epithelium (Gandomi *et al.*, 2019). Glycoproteins on microbial cell walls contribute to higher HAs, whereas hydrophilic molecules such as polysaccharides decrease this affinity (Kos *et al.*, 2003). BCSH is an essential factor in predicting bacterial adhesion to the intestinal epithelium. This property is dependent on the structure and composition of the microbial cell membrane, especially with the presence of hydrophobic proteins (Pan *et al.*, 2006). Unfortunately, our results declined from these theories.

### 4.4.6 Co-aggregation of LAB with EAEC

Our results demonstrated that all the tested LAB aggregated with the EAEC strains (Figure 4.7). However, the co-aggregation degree was found to depend on the specific pairing between the EAEC and the LAB strains and the incubation time. The co-aggregation scores between the selected pathogens and LAB ranged from 2.7 (*B. bifidum ATCC* 11863 and EAEC K2) to 35.2 % (*L. fermentum*, FS2 and EAEC K16) after the fifth IH. Interestingly, most of the BaSs demonstrating high A-ASs in this study also showed high degrees of co-aggregation.

The co-aggregation between LAB and enteropathogens was previously reported (Collado *et al.*, 2007c; Collado *et al.*, 2008b; García-Cayuela *et al.*, 2014). Our findings from this study generally agreed with previously reported results (Balakrishna, 2013; Janković *et al.*, 2012; Malik *et al.*, 2003). However, the co-aggregation abilities between the selected LAB and EAEC strains in our study were lower than other reported values (De Souza *et al.*, 2019; García-Cayuela *et al.*, 2014) whilst higher than others (Collado *et al.*, 2007c). As reported in previous studies, none of our bacterial treatments exhibited co-aggregation above 40 % (García-Cayuela *et al.*, 2014).

The co-aggregation of LAB with other pathogens such as EAEC could be advantageous because it facilitates the elimination of the pathogens from the GIT (Todorov and Dicks, 2008). The results for the co-aggregation between EAEC and LAB confirm the hypothesis that auto-aggregation could be related to co-aggregation. They further suggest that bacterial aggregative phenotype could be taken as one of the criteria for screening LAB for co-aggregative characteristics for mitigating pathogens.

Co-aggregation plays a significant role in several ecological niches, including the human gut. It has been postulated that the co-aggregation abilities of lactobacilli interfere with the virulence of pathogens and can therefore prevent the colonization of the human gut by food-borne pathogens. Additionally, the microenvironment around the pathogens could be controlled by lactobacillus strains during co-aggregation due to the production and secretion of inhibitory substances (Gao *et al.*, 2019; Manzoor *et al.*, 2016; Scatassa *et al.*, 2017). Co-aggregation was reported to be a precise process between two or

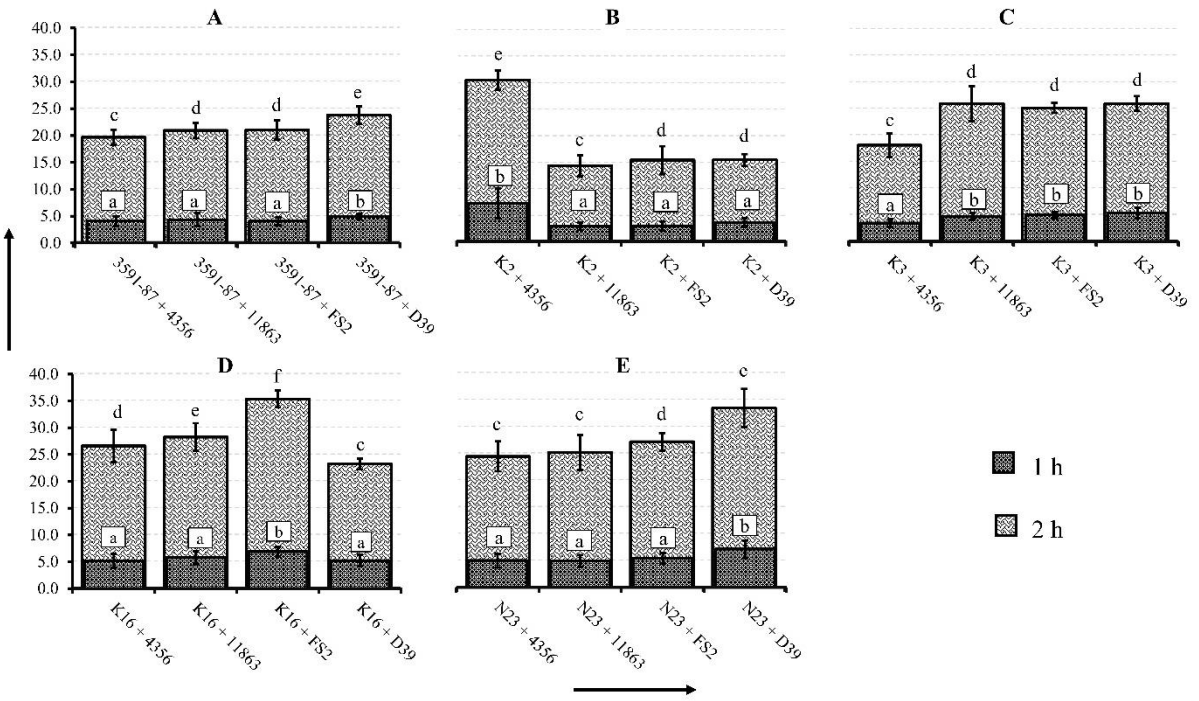


Figure 4.7: The effect of treatment (incubation) time on the co-aggregation of enteroaggregative *E. coli* (EAEC) and lactic acid bacteria (LAB)

The figure illustrates the effects of bacterial treatment (incubation) time on co-aggregation of selected EAEC and probiotic bacteria strains. The co-aggregation was done after 1 and 5 hours of incubation. The EAEC strains were diarrhoeagenic (3591-87, K2, K3, and K16) and non-diarrhoeagenic (N23). The LAB include *L. acidophilus* ATCC 4356 and *B. bifidum;* ATCC 11863; *Lactobacillus plantarum* FS2; and *Pediococcus pentosaceus* D39. Each bar represents the mean of three independent replicates (n=27) with its corresponding standard deviation. Mean values with different superscripts (a-f) indicate significant differences ($p \leq 0.05$) according to Fisher's LSD test.

more microorganisms involving interactions between carbohydrates from the cell wall of one bacterium and a complementary lectin from the cell surface of another (Kolenbrander and Andersen, 1986). Thus, differences in these surface proteins and polysaccharides may vary with different

bacterial genera, species, and strains, leading to variations in their co-aggregation abilities between/among themselves. Our results further show that even though bacterial properties such as auto-aggregation, co-aggregation, adhesion, and BCSH are phenotypic traits, the contribution of other factors cannot be overlooked. BC surface hydrophobicity and aggregation abilities may not be the only factors accounting for adhesion. Some of these complex mechanisms enable interaction between enteric microbes and their host. Subsequently, the enteric microbes confer beneficial effects on their host (García-Cayuela *et al.*, 2014).

### 4.4.7 Antimicrobial effects of LAB against EAEC

This study evaluated the antagonistic effect of the two potential probiotic LAB (*L. plantarum* FS2 and *P. pentosaceus* D39) from *ogi* and the two reference PBB, *L. acidophilus ATCC* 4356 and *B. bifidum ATCC* 11863 on the selected diarrhoeagenic EAEC from unpasteurized fresh milk samples. Our results (Figure 4.8) show that all the LAB demonstrated varying competencies for inhibiting the growth of the selected EAEC irrespective of the incubation (treatment) time. These competencies depended on the specific strains of the EAEC, the LAB involved, and the treatment (exposure) time (duration). Whilst a few LAB significantly ($P < 0.05$) inhibited some of the EAEC strains, a few of the pathogens defied the antimicrobial effects of the former. The two potential PBB and their positive reference controls were previously found to inhibit the adhesion of the selected EAEC to Caco-2 monolayers in varying capacities (Agbemavor and Buys, 2021). These findings suggest that the protective effect of the LAB in reducing the adhesion of EAEC may be caused by the inhibition of their adherence through a competitive exclusion mechanism. Whilst most LAB successfully inhibited the EAEC, LAB defiance by EAEC cannot be ignored. *Pediococcus pentosaceus* D39 inhibited all the EAEC strains for the first IH, whereas it only inhibited D-EAEC K2, K16 and ND-EAEC N23 after the fifth IH. Surprisingly, the reference clinical D-EAEC 3591-87 and K3 defied this LAB after the fifth IH. No specific scientific reason was found to explain these results. *Lactobacillus plantarum* FS2 had the highest antagonistic effect against the selected EAEC, except for EAEC K2 (Figure 4.8). Our

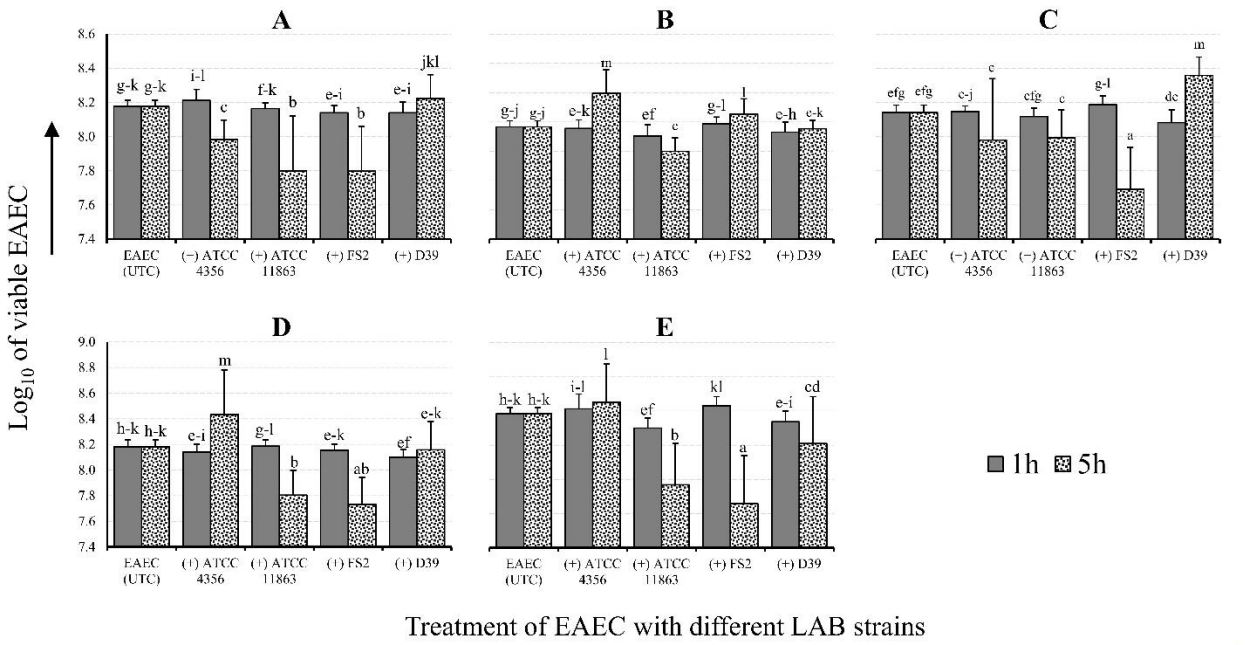


Figure 4.8: Direct bactericidal effect of lactic acid bacteria (LAB) against enteroaggregative *E. coli* (EAEC)

The figure illustrates the direct bactericidal (log reduction) effect of [with (+) or without (-)] probiotic bacteria and treatment (incubation) time against selected EAEC. The EAEC strains were diarrhoeagenic and non-diarrhoeagenic types. The diarrhoeagenic EAEC strains include 3591-87 (A), K2 (B), K3 (C), and K16 (D). The non-diarrhoeagenic EAEC strain was N23 (E). The lactic acid bacteria include *L. acidophilus* ATCC 4356 and *B. bifidum*; ATCC 11863; *Lactobacillus plantarum* FS2; and *Pediococcus pentosaceus* D39. Each bar is a mean of three independent replicates (n=18) with the corresponding standard deviation. Mean values with different superscripts (a-m) indicate a significant difference ($p \leq 0.05$) according to Fisher's LSD test.

findings disagreed with Michail and Abernathy (2002a), where *L. plantarum* did not affect enteropathogenic *E. coli*. The deviation in results could be due to the longer treatment time of our EAEC.

Soon after birth, the GIT of infants becomes colonised with different bacterial collections. Facultative anaerobes dominate initially, followed by LAB and coliforms (Berg, 1996). Maintenance of the gut microflora is critical for intestinal integrity and function. Several gastrointestinal pathological conditions could set in when this balance is interrupted, as previously reported for antibiotic-associated diarrhoea with *Clostridium difficile* colitis (Salminen *et al.*, 1995). Healthy gut microflora is typically associated with probiotics. Identifying PBB requires that they should be safe for human use with proven efficacy whilst stable under gastric acids and BiSs. Moreover, such bacteria should be capable of attaching to the intestinal mucosa (Salminen *et al.*, 1995).

PBB are noted for demonstrating antimicrobial properties through several mechanisms. Based on these properties, they are clinically exploited for prophylactic and therapeutic mitigations against recurrent antibiotic-associated diarrhoea (Vanderhoof *et al.*, 1999); *Clostridium difficile* diarrhoea (Gorbach *et al.*, 1987); viral-induced diarrhoea (Majamaa *et al.*, 1995); and inflammatory bowel syndrome (Caradonna *et al.*, 2000; Gupta *et al.*, 2000). Several mechanisms through which PBB exerts beneficial effects on their host have been reported. Firstly, they regulate and improve the immune response of their host. Secondly, they are indispensable in restoring microbial equilibrium for example, under situations where the equilibrium of the gut microbial ecology is destabilised (Oksanen *et al.*, 1990; Vanderhoof *et al.*, 1999). Thirdly, they produce certain antimicrobial substances such as hydrogen peroxides and bacteriocins which inhibit (even kill) most of such pathogens (Gorbach, 1990; Silva *et al.*, 1987). They have also been noted for eliminating enteropathogens through competition and elimination from adhesion (Agbemavor and Buys, 2021).

## 4.5 Conclusion

In conclusion, our study indicates that the presumptive PBB isolated from *ogi* demonstrated varying abilities to auto-aggregate and aggregate with diarrhoeagenic and non-diarrhoeagenic EAEC strains. These findings further suggest that apart from dairy sources, LAB from traditionally fermented cereal foods such as *ogi* has excellent potential to mitigate enteric pathogens like EAEC by exerting their

bactericidal actions against them. Therefore, these LAB isolates from fermented *ogi* might play significant roles in alleviating gastrointestinal infection to prevent diarrhoea. Traditionally fermented cereals could be considered as alternative commercial sources of probiotics with great potential for prophylactic and therapeutic management of gastrointestinal disorders.

# CHAPTER 5.

## RESEARCH CHAPTER 2

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**Presumptive probiotic bacteria from traditional fermented African food challenge the adhesion of enteroaggregative *E. coli***

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This chapter was accepted and published in the *Journal of Food Safety* (2021).

# Presumptive probiotic bacteria from traditional fermented African food challenge the adhesion of enteroaggregative *E. coli*

## 5.1 Abstract

The colonisation of the intestinal tract with the potential to exclude, displace and inhibit enteric pathogens is principally dependent on the adhesion ability of probiotics. Therefore, probiotic efficacy is mainly determined by their adhesion ability. The current study reports the antagonistic effect of four lactic acid bacteria (LAB) on the adhesion profile of four diarrhoeagenic with one non-diarrhoeagenic enteroaggregative *E. coli* (EAEC). All the bacterial strains investigated adhered to the Caco-2 cells. All the LAB tested competitively eliminated, displaced, and excluded at least three (non-) diarrhoeagenic EAEC strains from adhesion ($P < 0.05$). *Lactobacillus plantarum* FS2 exhibited the highest adhesion to the Caco-2 cells, competitive exclusion, displacement, and exclusion against most of the EAEC. Additionally, the competence to competitively exclude, displace and inhibit the EAEC from adhesion depending on the pathogens and the LAB strains tested signifies the participation of several mechanisms. Contrary to all the EAEC strains, gastrointestinal stress factors such as low pH (2.5) did not affect ($P > 0.05$) the adhesion of the LAB. Unlike the gastrointestinal acidic conditions, bile salt conditioning (at pH 6.5) did not affect ($P > 0.05$) the adhesion of both EAEC and LAB. In conclusion, all the LAB tested showed specific anti-adherence effects, including competitive exclusion, displacement, and exclusion against the selected EAEC. The results indicated that all the LAB, especially *Lactobacillus plantarum* FS2, had an excellent ability to exert antagonistic effects against the selected EAEC to prevent gastrointestinal infection.

## 5.2 Introduction

Gastrointestinal infection (diarrhoea) remains one of the leading causes of morbidity and mortality among children below school-going age (Asare *et al.*, 2020; Shrestha *et al.*, 2020). In 2011, the disease was estimated to be responsible for 9.9 % of the 6.9 million mortalities among children below five

years, and it is widespread in both developed and developing countries (Liu *et al.*, 2012; Walker *et al.*, 2013b). The prevalence of extended acute diarrhoea, particularly among children below school-going age, could be disastrous. Once the child becomes infested, the primary physiological function of the gut like absorption becomes compromised, leading to malabsorption and passage of watery stools. The individual becomes malnourished, leading to stunted growth, retardation in cognitive development, hospitalization and even death could set in (Abe *et al.*, 2000; Ciccarelli *et al.*, 2013; Ferri, 2014; Okumura *et al.*, 2004; Singh and Fleurat, 2010).

There are several causative agents for gastrointestinal infection, one of which is diarrhoeagenic *E. coli* (DEC). DEC accounted for about 15% of mortalities among children below five years (Amisano *et al.*, 2011; Krishnamoorthy *et al.*, 2020; Webb and Starr, 2005). This *E. coli* was characterized to exist in six different pathotypes. These pathotypes include enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), enterohaemorrhagic *E. coli* (EHEC), and enteropathogenic *E. coli* (EPEC). The rest are enterotoxigenic *E. coli* (ETEC) and diffusely adherent *E.S coli* (DAEC). Their characterization was based on their virulence, infection, and pathogenicity modes within the host. It also includes their epidemiological significance in childhood diarrhoea (Nataro and Kaper, 1998).

EAEC has gained progressive recognition as an emerging diarrhoeagenic pathogen over the years, accounting for 15 % of diarrhoea cases in both developing and developed countries (Aijuka, 2018; Ellis *et al.*, 2020; Fedor *et al.*, 2019; Laaveri *et al.*, 2018). The pathogen colonizes the gastrointestinal tract (GIT) by adhesion shortly after an individual consumes contaminated food or water. The attachment process is aided through its cell wall and associated structures such as adhesins, pili, flagella, fimbriae and afrimbriae. These surface structures have been reported to vary widely both morphologically and functionally. Based on these variations, this pathotype exhibits so much diversity in virulence and pathogenicity (De Mello Santos *et al.*, 2020; Ellis *et al.*, 2020; Petro *et al.*, 2020).

However, a group of lactic acid bacteria known as probiotic bacteria (PBB) offer their host several beneficial effects either in isolation or in combination for the prevention and treatment of even severe diarrhoea (Allen, 2010; Sazawal *et al.*, 2010; Szajewska *et al.*, 2006). PBB affect enteropathogens such as DEC through several mechanisms, one of which is by competing for binding sites within the gut, thereby preventing attachment to the epithelium (Isolauri, 2003). Additionally, they vigorously compete with the enteric pathogens for limited available substrates within the gut and secretion of certain antimicrobial substances such as bacteriocins, organic acids and peroxides, which generally reduce luminal pH (Isolauri, 2003; Sazawal *et al.*, 2006). Subsequently, the high demand for these beneficial PBB is principally dependent on their ability to survive and remain stable within the gut, besides their competence to colonize the intestinal mucosal lining irrespective of the prevailing acidic and bile salt conditions (Isolauri *et al.*, 2002; Ruiz *et al.*, 2013). In the presence of PBB, enteropathogens, including DEC, become progressively eradicated from the gut (Neish *et al.*, 2000). Many lactobacilli can displace and even inhibit DEC from the intestinal epithelium (Collado *et al.*, 2007b; Collado *et al.*, 2005).

Several studies have illustrated the abundance and diversity of PBB in fermented cereal foods (Diaz *et al.*, 2019; Fayemi and Buys, 2017; Oguntoyinbo and Narbad, 2015; Salmerón *et al.*, 2015). Additionally, the consumption of fermented foods has been associated with numerous health benefits (Lindner *et al.*, 2013; Marco *et al.*, 2017; Şanlier *et al.*, 2019). Regardless of the increasing body of scientific knowledge about the beneficial health effects of probiotics, there is limited literature on the application of cereal-based PBB in the management of diarrhoeal-related diseases as compared to their dairy counterparts. This study aimed to evaluate the effects of cereal-based LAB isolated from traditional African fermented food, *ogi*, on the adhesion of selected EAEC strains isolated from unpasteurized fresh milk samples.

## 5.3 Materials and methods

The characteristics and sources of bacterial cultures; selected EAEC and LAB with promising probiotic properties used for the current studies are presented in Table 5.1.

### 5.3.1 Bacterial strains and growth conditions

Selected frozen (-80 ℃) LAB and EAEC strains were revived for this study by culturing them independently in de Man, Rogosa and Sharpe (MRS) broth and tryptone soy broth (TSB), respectively, followed by incubation (37 ºC, 18 h). They were then plated on MRS agar (Oxoid, UK) and tryptone soy agar (TSA, Oxoid), respectively, for renewal and enumeration of the pure colonies. The EAEC cells were plated on Sorbitol-MacConkey agar (SMAC, Oxoid) for enumeration in the presence of PBB cells. Both bacteria were incubated (37 ºC, 18 h).

### 5.3.2 Culturing of Caco-2 Cells

We obtained the human colonic epithelial cell line, colorectal adenocarcinoma (Caco-2), from American Type Culture Collections (ATCC, Maryland, USA). The cell line was routinely grown (passage 30-40) using Dulbecco's Modified Eagle Medium [DMEM (Gibco, ThermoFisher, USA)] containing 4 500 mg/L D-glucose, non-essential amino acids (NEAA), and 110 mg/L of sodium pyruvate. Additionally, we supplemented the media with 1% L-glutamine, 10% (v/v) gamma-irradiated, heat-inactivated foetal bovine serum [FBS (Gibco)], 1% penicillin-streptomycin. The cells were cultivated consistently in T75 (75 $cm^2$) cell culture flasks [with catalogue number (CN), “658940” (Greiner Bio-One GmbH, Frickhausen, Germany)] and sub-cultured (60 to 70 % confluence) into a ratio of 1:3 followed by incubation (37 ℃, 5% $CO_2$) in a $CO_2$ humidified incubator (Healforce, HF 212UV, China). For adhesion assays, the cells were seeded at 2.65 x $10^4$ cells/$cm^2$ in 24-well cell culture plates [catalogue number (CN), “662160” (Greiner Bio-One)].

Table 5.1. Bacterial strains, sources, and culturing conditions for selected enteroaggregative *E. coli* (EAEC) and lactic acid bacteria (LAB) strains

| Bacteria strain | Characteristic | Source |
|---|---|---|
| [a]EAEC 3591-87 | Clinical and diarrhoeagenic (positive reference strain) | [c]NICD of NHLS |
| [a]EAEC K2 | Diarrhoeagenic | [d]Unpasteurised fresh milk |
| [a]EAEC N23 | Non-Diarrhoeagenic | [d]Unpasteurised fresh milk |
| [a]*B. bifidum ATCC* 11863 | Reference probiotic bacteria | [e]ATCC Collections |
| [a] *L. plantarum* FS2 | Promising probiotic characteristics | [e]Traditional fermented food (ogi) |
| [a]*P. pentosaceus* D39 | Promising probiotic characteristics | [f]Traditional fermented food (*ogi*) |

[a]These strains were revived and cultured in tryptone soy broth periodically plated on tryptone soy agar but sorbitol McConkey agar for enumeration and incubated statically (37 ℃, 18 h).

[b]These LAB were revived and cultured in de Mann Rogosa and Sharpe (MRS) broth, plated on MRS agar for enumeration and incubated statically (37 ℃, 18 h).

[c]This EAEC strain was obtained from the National Institute for Communicable Diseases (NICD), a division of the National Health Laboratory Service (NHLS), Johannesburg, the Republic of South Africa.

[d]These strains were obtained as isolates from previous studies (Aijuka *et al.*, 2018; Ntuli *et al.*, 2017).

[e]Obtained from American Type Culture Collection (ATCC, USA).

[f]Obtained from previously isolated by Fayemi and Buys (2017) Fayemi and Buys (2017).

### 5.3.3 Invitro bacterial adhesion to Caco-2 cells

This assay was performed following protocols adopted by (Collado *et al.*, 2007a; Collado *et al.*, 2005; Pazhoohan *et al.*, 2020a; Rajan *et al.*, 2018) with a few modifications. Briefly, the differentiated Caco-2 monolayers

Table 5.2: Enteroaggregative *E. coli* (EAEC) and lactic acid bacteria (LAB) treatment template for Caco-2 monolayers infection

| LAB Bacteria Strains | EAEC Strains | | |
|---|---|---|---|
| | 3591-87 | K2 | N23 |
| 11863 | 3591-87 + 11863 | K2 + 11863 | N23 + 11863 |
| FS2 | 3591-87 + FS2 | K2 + FS2 | N23 + FS2 |
| D39 | 3591-87 + D39 | K2 + D39 | N23 + D39 |

Note: The EAEC strains include 3591-87, K2, K3, K16, and N23 and the LAB include *L. acidophilus* ATCC 4356 and *Bifidobacterium bifidum ATCC* 11863, *L. plantarum* FS2, and *P. pentosaceus* D39, respectively.

were prepared by replacing the growth medium with DMEM (without serum and antibiotic) after washing the monolayers twice with phosphate-buffered saline (PBS) for at least 2 h before the bacterial infection. The enterocytes were infected independently with 18 h old pure EAEC and LAB cultures at 8.0 x $10^7$ and 8.0 x $10^8$ bacterial cells/$cm^2$, as shown (Table 5.2). The infected monolayers were incubated (37 ℃, 5 % $CO_2$, 2 h), after which non-attached bacterial cells were withdrawn. The monolayers were then washed with PBS and trypsinized with 0.5 % trypsin-ethylenediaminetetraacetic acid [trypsin-EDTA, [ThermoFisher Scientific)]. We countered the attached bacterial cells by plating the EAEC and LAB on sorbitol McConkey and MRS agar plates, respectively. Bacterial adhesion was calculated as a percentage of the number of bacterial cells recovered after incubation relative to the number of cells in the suspension added to the Caco-2 monolayers.

### 5.3.4 Competitive exclusion of EAEC from adhesion

Competitive exclusion (CE) of EAEC from adhesion assay was determined to evaluate the ability of the LAB to exclude the selected EAEC from adhering to the enterocytes. This assay was carried out by adopting procedures described by Collado *et al.* (2007a) with minor modifications. Briefly, equal volumes of EAEC (1.5 x $10^8$ cells/mL) and LAB (1.5 x $10^9$ cells/mL) were mixed as outlined in Table 5.2 to form bacterial mixtures leading to final concentrations of 8.0 x $10^7$ and 8.0 x $10^8$ cells/$cm^2$ for

EAEC and LAB strains, respectively. The monolayers were simultaneously infected with the bacterial mixtures (Table 5.2) followed by incubation (37 ℃, 5 % $CO_2$ for 2 h). The Caco-2 monolayers were trypsinized to dislodge the attached EAEC cells, which were then plated, incubated, and counted as previously described. CE of EAEC from adhesion was calculated as the difference between pathogen adhesion in the absence and presence of LAB. Experiments were carried out in triplicate with four replicates within each.

### 5.3.5 Displacement of EAEC from adhesion

EAEC displacement from adhesion (DFA) was carried out to evaluate the aptitude of LAB to dislodge already adhered EAEC cells from the differentiated Caco-2 monolayers. This assay was conducted as previously described (Collado *et al.*, 2005). Briefly, the monolayers were first infected with EAEC ($8.0 \times 10^7$ cells/$cm^2$) as illustrated (Table 5.2), followed by incubation (37 ℃, 5 % $CO_2$, 1 h). This procedure was followed by treating the previously infected monolayers with the LAB strains (8.0 × 108 cells/cm2) and then incubating as described earlier. The attached EAEC was enumerated by plating and incubation as described earlier. The displacement of EAEC from adhesion was calculated as the difference between the adhesion of the pathogen with and without LAB. Experiments were carried out in triplicate with four replicates within each.

### 5.3.6 EAEC inhibition from adhesion assay

Contrary to the displacement assay, the exclusion of EAEC from adhesion assay was to test the competence of already attached LAB strains to exclude EAEC from adhering to the enterocytes. This procedure was carried out as earlier described (Collado *et al.*, 2005). Briefly, Caco-2 monolayers were first treated with LAB strains ($8.0 \times 10^8$ cells/$cm^2$) as illustrated in Table 5.2 and incubated (37 ℃, 5 % $CO_2$, 1 h). The previously LAB-treated enterocytes were then infected with EAEC ($8.0 \times 10^7$ cells/$cm^2$) and incubated as described earlier. The attached EAEC cells were estimated by plating and

incubation as previously described, and the exclusion of EAEC by LAB was calculated as described earlier.

### 5.3.7 Gastric acid and bile salt stress response of EAEC and LAB for adhesion to the Caco-2 monolayers

The adhesion competence of EAEC and LAB cells were evaluated using simulated gastric juice, prepared based on procedures followed by (Falah *et al.*, 2019; Ruiz *et al.*, 2013) with a few modifications. Briefly, the pH of the cell culture medium without antibiotics and FBS was adjusted using 1 M HCl (previously sterile filtered) to pH values of 2.5, 4.5 and 6.5. Bile salt (sterile filtered) was prepared and added to the cell culture medium at pH 6.5 to a final concentration and pH of 1 % (w/v). In preparing the cells for the adhesion assay, the pH-adjusted cell culture media were used to replace the previous one containing the FBS and antibiotic at least 2 h before the infection of the monolayers with the two bacteria.

### 5.3.8 Scanning electron microscopy

Scanning electron microscopy (SEM) was carried out to examine the attachment of EAEC and LAB cells to the Caco-2 monolayers. The Caco-2 cells were cultured and incubated as described earlier, except that they were seeded on glass coverslips [CN, "CB00120RA120MNT0" (ThermoFisher Scientific)] in preparation for the SEM. They were allowed to differentiate fully into enterocytes 15 days post-confluence. The monolayers were infected with the EAEC and LAB ($8.0 \times 10^7$ cells $cm^{-2}$) each, as illustrated in Table 5.2 and incubated (37 °C, 5 % $CO_2$, 2 h). The non-attached bacterial cells were withdrawn, after which the monolayers were washed twice with PBS. Protocols outlined by Atassi *et al.* (2006) were followed for the preparation and viewing of the infected and uninfected monolayers using the Zeiss Ultra PLUS FEG SEM (Carl Zeiss S.A.S, Oberkochen, Germany) at an electron acceleration voltage of 1.00 kV. Different fields of interest were captured at different optical magnifications. This experiment was independently duplicated for the same bacterial treatments.

The Caco-2 cells used for the study ranged from passages 30 to 40. Though using Caco-2 cells from different passages for a given study is acceptable, the range should be within 10. Using Caco-2 cells from various passages could bring some minor deviations in the results. The study involving the effect of simulated gastric fluid conditioning at pH 2.5 and 4.5 and SiBS conditioning at pH 6.5 could have been done together. However, this study was divided based on the three pH regimes due to the excess work overload.

### 5.3.9 Statistical analysis

Except for the SEM assay, all experiments were independently carried out in triplicates with four replicates within each to cater for intra-assay variation. Statistical analysis was performed using Statgraphics Centurion XVI (Statpoint Technologies Inc, 2013). The data were appropriately subjected to one-way and two-way variance analysis (ANOVA). Mean values with $P < 0.05$ were statistically significant (95 % confidence level). A multiple range test was conducted using the least significant difference for means separation.

## 5.4 Results and discussion

### 5.4.1 Comparative adhesion of EAEC and LAB to the Enterocytes

The adhesion scores (ASs) of the EAEC and LAB were significantly different ($P < 0.05$) from one another. The ASs of the EAEC strains ranged from 18.9 to 27.9 % (Figure 5.1). The diarrhoeagenic EAEC (D-EAEC) strains exhibited higher adhesion affinities to the epithelial monolayers than the non-diarrhoeagenic EAEC N23. EAEC K2 had the highest adhesion (27.9 %) to the monolayers than even the diarrhoeagenic clinical EAEC positive reference strain (23.9 %). This result implies that consuming food or water contaminated with EAEC K2 may potentially inflict gastrointestinal infection (diarrhoea). Except for the D-EAEC K2, all the other EAEC strains had comparatively lower adhesion capacities to the enterocytes. Additionally, gastrointestinal infection depends on the pathogen's infection dose and the adhesion capacity of the causative agent (Nataro and Kaper, 1998). Generally,

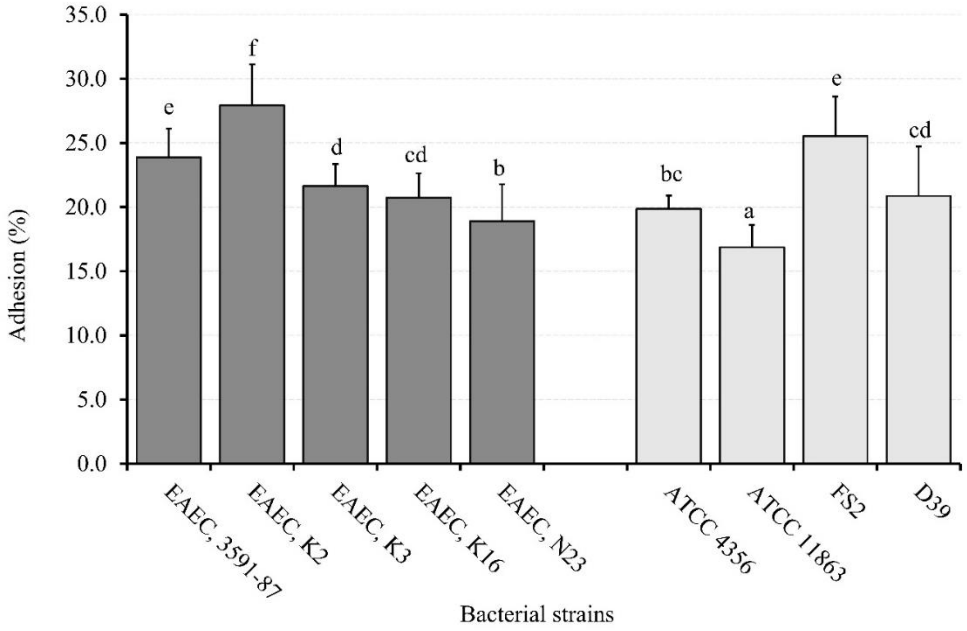


Figure 5.1: Adherence of enteroaggregative *E. coli* (EAEC) and lactic acid bacteria (LAB) strains to differentiated epithelial (Caco-2) monolayers

Results were expressed as per cent adhesion (mean ± standard deviation) for each bacterial strain. Bars with different letters significantly differ ($P < 0.05$) from one another (n=12).

EAEC strains: 3591-87; K2; K3; K16; and N23.

LAB strains: *L. acidophilus* ATCC 4356, *B. bifidum* ATCC 11863, *L. plantarum* FS2 and *P. pentosaceus* D39, respectively.

the EAEC strains showed significantly higher ($P < 0.05$) adhesion affinities to the enterocytes than their LAB counterparts, except for *L. plantarum* FS2 (25.5 %) and *P. pentosaceus* D39 (20.87 %). There was no difference between the adhesion of the diarrhoeagenic and clinical EAEC reference strain, 3591-87 (23.87 %) and *L. plantarum* FS2 (25.5 %) to the differentiated Caco-2 monolayers. Despite being an established probiotic bacterium, *B. bifidum ATCC* 11863 among the LAB demonstrated the most negligible adhesion affinity (16.9 %) to the differentiated enterocytes. The higher EAEC AS could be due to specialized extracellular surface microstructures such as fimbriae, flagella, or pili. These features offer the EAEC cells extra adhesion capability (Haiko and Westerlund-

Wikström, 2013; Harrington *et al.*, 2005; Nataro *et al.*, 1992; Nataro *et al.*, 1998). Their cell walls and extracellular surface microstructures were reported to be coded by specialized proteins, which vary in structure and function from one EAEC strain to another (Nataro, 2005; Nataro *et al.*, 1992). The differences in these adaptive features could account for the diversity in their adhesion patterns and abilities within the gut. The LAB, on the other hand, only had adherent cell membranes which lack those extracellular surface structures, as in the case of the EAEC cells. These results trends confirmed the findings from a previous study (Collado *et al.*, 2007b).

According to our results, *L. plantarum* FS2, isolated from *ogi*, had the highest AS to the differentiated Caco-2 monolayers even than the two-positive reference PBB, *L. acidophilus* ATCC 4356 and *B. bifidum ATCC* 11863 by 5.7 and 8.7 %, respectively (Figure 5.1). These results suggest that the *L. plantarum* FS2 may have the highest potential of alleviating gastrointestinal infection compared to the other LAB in this study. The reason is that *L. plantarum* FS2 recorded the highest adhesion to the Caco-2 cells. This further implies that in the presence of pathogens, this LAB strain had the highest potential of engaging the adhesion sites of the enterocytes and hence rendering fewer sites available for the attachment of the former like the diarrhoeagenic EAEC in our study (Boirivant and Strober, 2007). The adhesion capacities recorded by the LAB from this study were generally higher than findings from previous studies (Abdulkareem *et al.*, 2018; Collado *et al.*, 2007a).

### 5.4.2 Competitive exclusion of EAEC from adhesion

The competitive exclusion assay evaluated the competence of the selected LAB to eliminate EAEC from adhering to the enterocytes. The results (Figure 5.2) indicate no difference between the ASs of D-EAEC 3591-87 (20.0 %) and D-EAEC K3 (19.1 %) and between D-EAEC K16 and non-diarrhoeagenic EAEC (ND-EAEC) in the absence of LAB. Similarly, there was no difference between the ASs of *L. acidophilus* ATCC 4356 (16.8 %) and *P. pentosaceus* D39 (17.3 %) in the differentiated Caco-2 monolayers. *L. plantarum* FS2 and *B. bifidum ATCC* 11863 recorded the highest (22.6 %) and

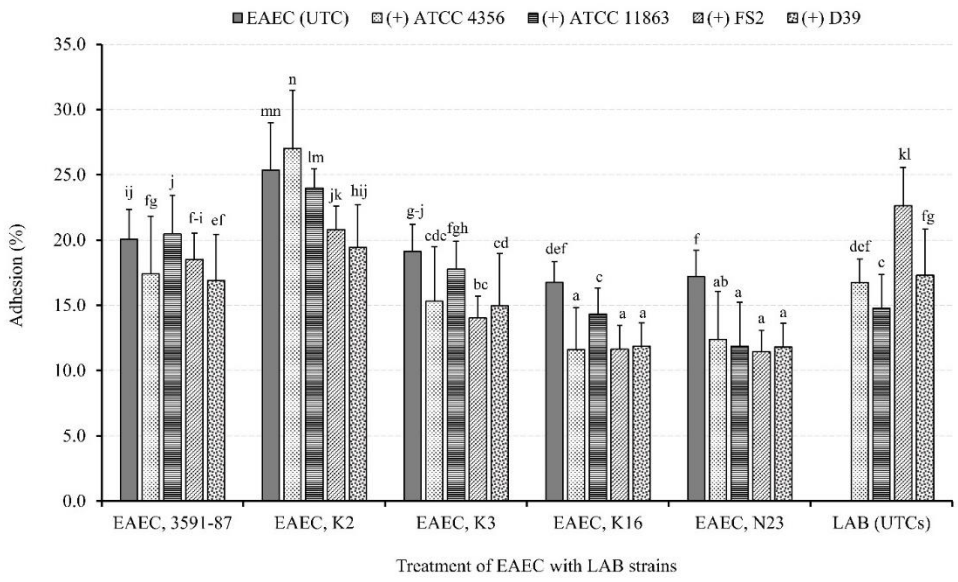


Figure 5.2: Competitive exclusion of enteroaggregative *E. coli* (EAEC) by lactic acid bacteria (LAB) from adhesion to Caco-2 monolayers.

Results were expressed as per cent adhesion (mean ± standard deviation) for all treated and untreated EAEC controls [EAEC (UTC)] as well as untreated LAB controls [LAB (UTC)]. Bars with different letters significantly differ ($P < 0.05$) from one another (n=12).

EAEC strains: 3591-87; K2; K3; K16; and N23.

LAB strains: *L. acidophilus* ATCC 4356, *B. bifidum* ATCC 11863, *L. plantarum* FS2 and *P. pentosaceus* D39, respectively.

the lowest adhesion (14.8 %) scores, respectively, to the differentiated monolayers. Accordingly, *L. plantarum*, D39 and *B. bifidum ATCC* 11863 were expected to exhibit the highest and lowest competitive exclusion, respectively, against the adhesion of the EAEC to the monolayers. Similarly, the competence between *L. acidophilus* ATCC 4356 and *P. pentosaceus* D39 in excluding EAEC 3591-87 from adhesion should not differ. The adhesion sites within the gut are limited, and the LAB with a higher adhesion potential can prevent any existing enteric pathogen from securing adhesion sites along the intestinal mucosa than one with less adhesion capacity (Monteagudo-Mera *et al.*, 2019). However, these expectations were not necessarily the case in some cases in our current study.

Generally, all the LAB tested significantly ($P < 0.05$) excluded (reduced) the adhesion of EAEC strains to the enterocytes (Figure 5.2). However, the results show strain-to-strain specificity between this study's selected EAEC and LAB strains. *P. pentosaceus* D39 had the highest competitive exclusion effect against the clinical diarrhoeagenic positive EAEC reference strain, 3591-87. In the presence of *P. pentosaceus* D39, the adhesion of EAEC 3591-87 was reduced by 3.2 %. This trend was followed by the presence of *L. acidophilus* ATCC 4356 (2.7 %) and *L. plantarum* FS2 (1.5 %). Interestingly, the reference diarrhoeagenic EAEC 3591-87 defied the presence of *B. bifidum ATCC* 11863 by increasing its adhesion to the monolayers by 0.42 %. A similar increase in the AS of D-EAEC K2 was recorded with the presence of *L. acidophilus* ATCC 4356 (1.2 %). Just like D-EAEC 3591-87, *P. pentosaceus* D39 excluded D-EAEC K2 by 5.9 %, followed by *L. plantarum* FS2 (4.6 %) and then finally by *B. bifidum ATCC* 11863 (1.4 %). Despite the lower AS ($P < 0.05$) of *B. bifidum ATCC* 11863 (14.8 %) compared to *L. acidophilus* ATCC 4356 (16.75 %), this EAEC defied the presence of the LAB by gaining an increase in its adhesion by 1.2 %. Results from the current studies generally agreed with reports (Collado *et al.*, 2007a). Contrarily, our results were generally lower than previous reports when the adhesion medium was intestinal pig mucus (Collado *et al.*, 2007b).

One of the probiotic's mechanisms of action against enteric pathogens is antagonism (Monteagudo-Mera *et al.*, 2019). This antipathogenic characteristic of PBB is multifactorial, including competitive exclusion (direct) and synthesis of antimicrobial substances (indirect). Probiotics with higher adhesion capabilities can block the attachment of pathogens through competition for binding sites within the host. This feature is strain dependent and used as a means for screening potential PBB for research and clinical interventions (Lau and Chye, 2018; Tuo *et al.*, 2018; Walsham *et al.*, 2016a). For instance, in colonized patients, *Lactobacillus rhamnosus* GG was effective against vancomycin-resistant enterococci (Manley *et al.*, 2007; Szachta *et al.*, 2011).

The competition of probiotics with enteric pathogens for adhesion sites within the host has been reported by several studies. This competition was mainly due to the possession of adhesins by both

microorganisms. Additionally, the competition for adhesion sites has been reported to depend on the probiotic strains and the pathogens involved as well as the nature of the adhesion medium (Chen *et al.*, 2007; Gueimonde *et al.*, 2007). This study evaluated four LAB strains with promising probiotic characteristics for their ability to competitively exclude five selected EAEC strains using Caco-2 as an experimental model. The competitive exclusion of EAEC from adhesion to the Caco-2 monolayers by the selected LAB illustrated a high variability and showed that the exclusion was a strain-specific property. Even though the chosen LAB showed different competitive exclusion capacities against the selected EAEC strains, each LAB effectively excluded at least four EAEC strains from adhesion. In most cases, *Lactobacillus plantarum* FS2 had the highest exclusion capacity against the selected EAEC strains to the Caco-2 monolayers indicate that their competitive exclusion abilities of the LAB depend on their adhesion capacity to the Caco-2 monolayers, which agrees with previous findings (Gueimonde *et al.*, 2007; Singh *et al.*, 2017). Several other workers equally reported the competitive exclusion of pathogens by PBB (Bernet *et al.*, 1993; Collado *et al.*, 2005; Forestier *et al.*, 2001; Lee *et al.*, 2003; Pham *et al.*, 2009; Weizman *et al.*, 2005; Wine *et al.*, 2009; Zhang *et al.*, 2013) Meanwhile, the competitive exclusion ability of the LAB strains against the pathogens did not correlate with their adhesion ability.

### 5.4.3 Displacement of EAEC from adhesion

The displacement of EAEC from adhesion was to determine the abilities of the selected LAB to dislodge adhered EAEC cells from the enterocytes. Just like the CE assay, the LAB had a significant effect ($P < 0.05$) on the adhesion of EAEC to the enterocytes, but the results show strain-to-strain specificity between the bacteria (Figure 5.3). *L. plantarum* FS2 recorded the highest ($P < 0.05$) adhesion (19.6 %) to the enterocytes. This trend was followed by *L. acidophilus* ATCC 4356 (15.1 %), *P. pentosaceus* D39 (14.3 %), and *B. bifidum ATCC* 11863 (12.8 %). There was no difference between the adhesion competence of *L. acidophilus* ATCC 4356 and *P. pentosaceus* D39 and *B. bifidum ATCC* 11863 and *P. pentosaceus* D39 to the monolayers. Since *L. plantarum* FS2 had the highest AS to the

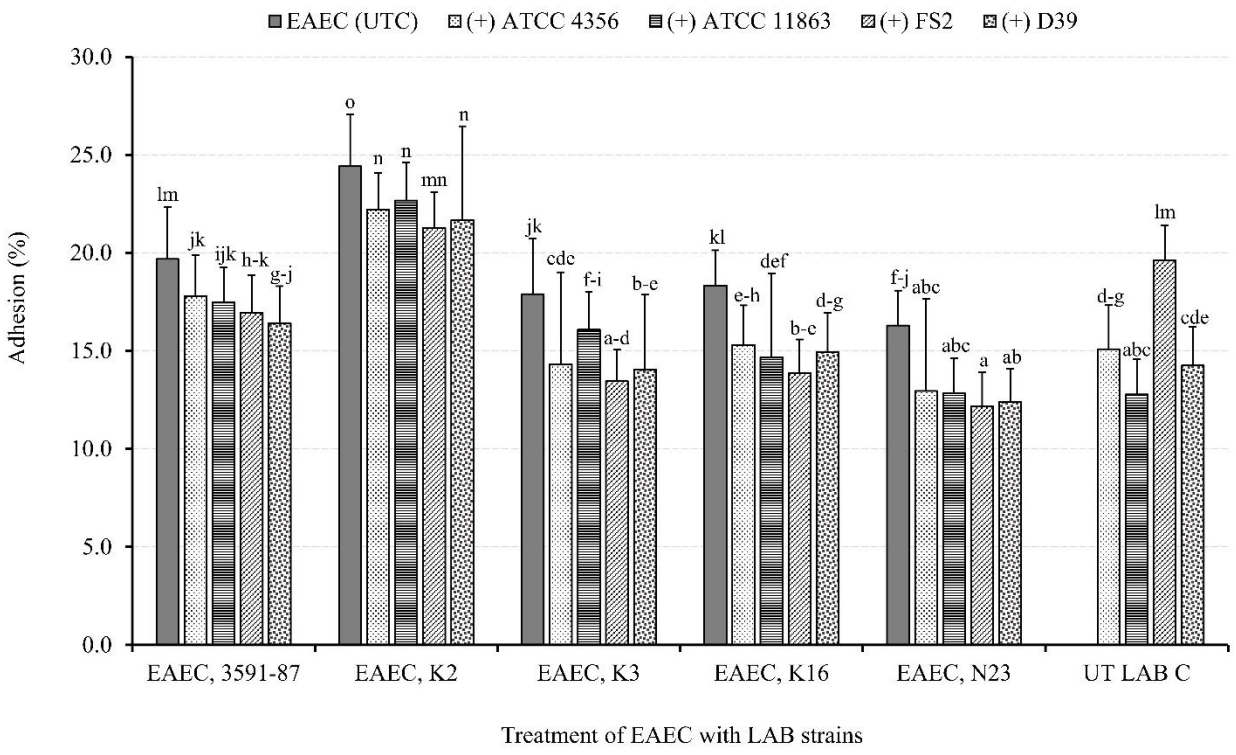


Figure 5.3: Displacement of enteroaggregative *E. coli* (EAEC) by lactic acid bacteria (LAB) from adhesion to Caco-2 monolayers

Results were expressed as per cent adhesion (mean ± standard deviation) for all treated and untreated EAEC controls [EAEC (UTC)] as well as untreated LAB controls [LAB (UTC)]. Bars with different letters significantly differ ($P < 0.05$) from one another (n=12).

EAEC strains: 3591-87; K2; K3; K16; and N23.

LAB strains: *L. acidophilus* ATCC 4356, *B. bifidum* ATCC 11863, *L. plantarum* FS2 and *P. pentosaceus* D39, respectively.

monolayers, it was expected to have the highest, whereas *B. bifidum ATCC* 11863 and *P. pentosaceus* D39 should have about the least displacement effects against the EAEC from adhesion. However, this expectation was not met across the selected EAEC strains in this study.

In the absence of LAB, D-EAEC K2 and ND-EAEC N23 recorded the highest (24.4 %) and the least (16.3 %) ASs, respectively, to the monolayers. Despite the highest AS of *L. plantarum* FS2 to the

monolayers, there was no difference in its displacement effect (2.8 %) and that of *P. pentosaceus* D39 (3.2 %) against the clinical diarrhoeagenic positive EAEC reference strain, 3591-87. Additionally, there was no difference among *L. acidophilus* (1.9 %) ATCC 4356, *B. bifidum ATCC* 11863 (2.2 %), and *L. plantarum* FS2 (2.8 %) against this D-EAEC. As expected, *L. plantarum* FS2 recorded the highest ($P < 0.05$) displacement (3.1 %) against D-EAEC K2 but its competence for the displacement of D-EAEC was not different from that of *L. acidophilus* ATCC 4356 (2.2 %) and *P. pentosaceus* D39 (2.8 %). Irrespective of the highest AS of untreated *L. plantarum* FS2 to the monolayers, its competence for the displacement of D-EAEC K3 (4.4 %) was not different from *L. acidophilus* ATCC 4356 (3.6 %). *L. plantarum* FS2 had the highest displacement effect (4.5 %) against the D-EAEC K16 and the ND-EAEC N23. There was no difference among the remaining LAB strains in their competence for the displacement of the D-EAEC K16 from adhesion.

### 5.4.4 EAEC Inhibition from adhesion

This experiment evaluated the LAB's competence to inhibit the EAEC from adhering to the Caco-2 monolayers. Like the two previous studies, the treatment of the monolayers with LAB had a significant effect ($P < 0.05$) on the exclusion of the selected EAEC strains from adhering to the enterocytes (Figure 5.4). Similarly, our results show strain specificity between the EAEC and the LAB. In the absence of LAB, the adhesion of EAEC K2 to the enterocytes was the highest (21.6 %). In contrast, the ND-EAEC N23 had the least (15.4 %). No difference was detected among the D-EAEC 3591-87, K3 and K16 to the enterocytes. As usual, among the LAB controls, *L. plantarum* FS2 had the highest ($P < 0.05$) AS (22.7 %), followed by *P. pentosaceus* D39 (18.0 %), *L. acidophilus* ATCC 4356 (16.9 %) and *B. bifidum ATCC* 11863 having the least (14.4 %). However, there was no difference between the adhesion of *L. acidophilus* ATCC 4356 and *P. pentosaceus* D39. Accordingly, we expected *L. plantarum* FS2 to exhibit the highest exclusion against the adhesion of the selected EAEC strains, followed by *P. pentosaceus* D39 and *L. acidophilus* ATCC 4356 and then finally by *B. bifidum ATCC* 11863. However, just like this study's two previous sections (5.4.2 and 5.4.3), there were a few

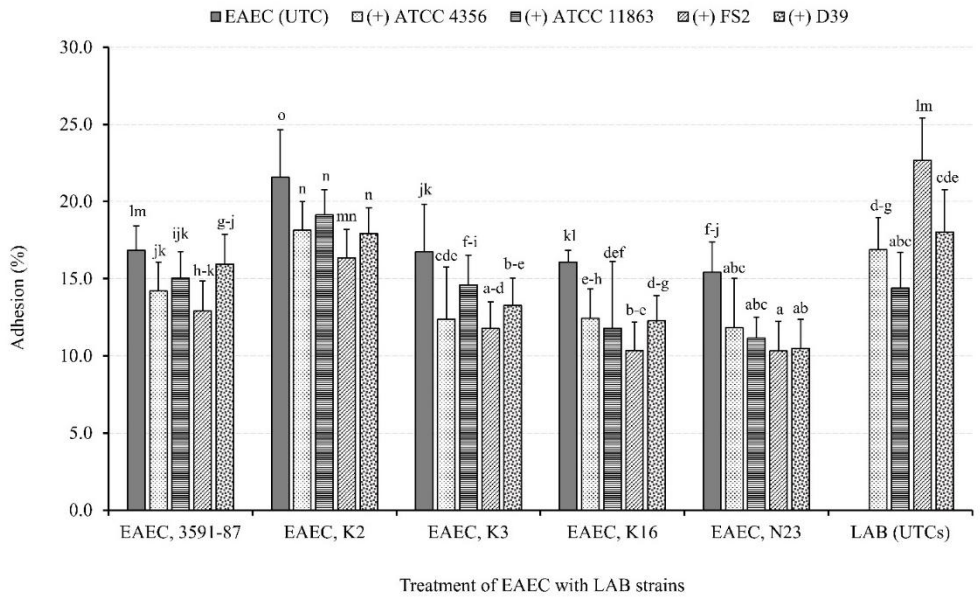


Figure 5.4: Exclusion of enteroaggregative *E. coli* (EAEC) by lactic acid bacteria (LAB) from adhesion to epithelial (Caco-2) monolayers

Results were expressed as per cent adhesion (mean ± standard deviation) for all treated and untreated EAEC controls [EAEC (UTC)] as well as untreated LAB controls [LAB (UTC)]. Bars with different letters significantly differ ($P < 0.05$) from one another (n=12).

EAEC strains: 3591-87; K2; K3; K16; and N23.

LAB strains: *L. acidophilus* ATCC 4356, *B. bifidum* ATCC 11863, *L. plantarum* FS2 and *P. pentosaceus* D39, respectively.

exceptions to this expectation. *L. plantarum* FS2 had the highest exclusion against the adhesion of D-EAEC 3591-87 (7.1 %). That was followed by *L. acidophilus* ATCC 4356 (5.8 %) and *B. bifidum ATCC* 11863 (5.0 %). *P. pentosaceus* D39 had the least exclusion effect against the D-EAEC 3591-87 (4.1 %). There was no difference between the exclusion competence of *B. bifidum ATCC* 11863 and *P. pentosaceus* D39 against the EAEC 3591-87. As expected, *L. plantarum* FS2 had the highest exclusion against the adhesion of EAEC K2, K3, K16 and N23 by 9.0, 7.3 and 6.4 and 6.9 %, respectively. No difference was found among the adhesion exclusion effects of *L. acidophilus* ATCC

4356 (7.2 %), *B. bifidum ATCC* 11863 (6.2 %) and *P. pentosaceus* D39 (7.4 %) against the EAEC K2. Similarly, there was no difference in the inhibitory effect of *L. acidophilus* ATCC 4356 (6.8 %) and *L. plantarum* FS2 (7.3 %) against the adhesion of EAEC K3. *B. bifidum ATCC* 11863 (4.5 %) and *P. pentosaceus* D39 (5.8 %) had the least inhibitory effects (2.2 %) against the adhesion of EAEC K3. Just like EAEC K2, there was no difference among the inhibitory effects of *L. acidophilus* ATCC 4356 (4.3 %), *B. bifidum ATCC* 11863 (5.0 %) and *P. pentosaceus* D39 (4.5 %) against the adhesion of EAEC K16. This trend was similar for ND-EAEC N23, where the inhibitory effects of *L. acidophilus* ATCC 4356, *B. bifidum ATCC* 11863, and *P. pentosaceus* D39 were 5.4, 6.1, and 6.7 %, respectively.

The adhesion of treated and untreated EAEC and control LAB strains were computed and presented as a heatmap (Figure 5.5). The adhesion of all EAEC strains in the presence of PBB was summed up. This summation was for all the different modes of infection. Thus, CEFA, DFA and IFA and each sum were expressed as a percentage of the total of the three. It was quite interesting to note that the percentage total ASs of the EAEC strains during the CEFA was the highest (36.3 %), followed by that of DFA (34.9 %) and then during the IFA assay (28 %). The original data was subjected to multiple-treatment comparison, and the results indicate that the different modes of infecting the Caco-2 monolayers significantly ($P < 0.05$) affected the adhesion of EAEC. Thus, CE and displacement of EAEC by LAB had mean values of 17.8 ± 5.2 % and 17.2 ± 3.8 %, respectively. Between these two modes of infection, there was no difference in the adhesion of EAEC. We were expecting the adhesion of EAEC during the displacement assay to be higher than that of the CE because there was no competing LAB for adhesion during the former phase.

On the other hand, the mean adhesion of EAEC during the exclusion assay was 14.5 ± 2.9 %, which was lower ($P < 0.05$) than the other two modes of infection. This result was not surprising because, during the exclusion assay, the monolayers were treated with EAEC for only one instead of 2 h for the other two assays. This is the first time this finding has been reported to the best of our knowledge.

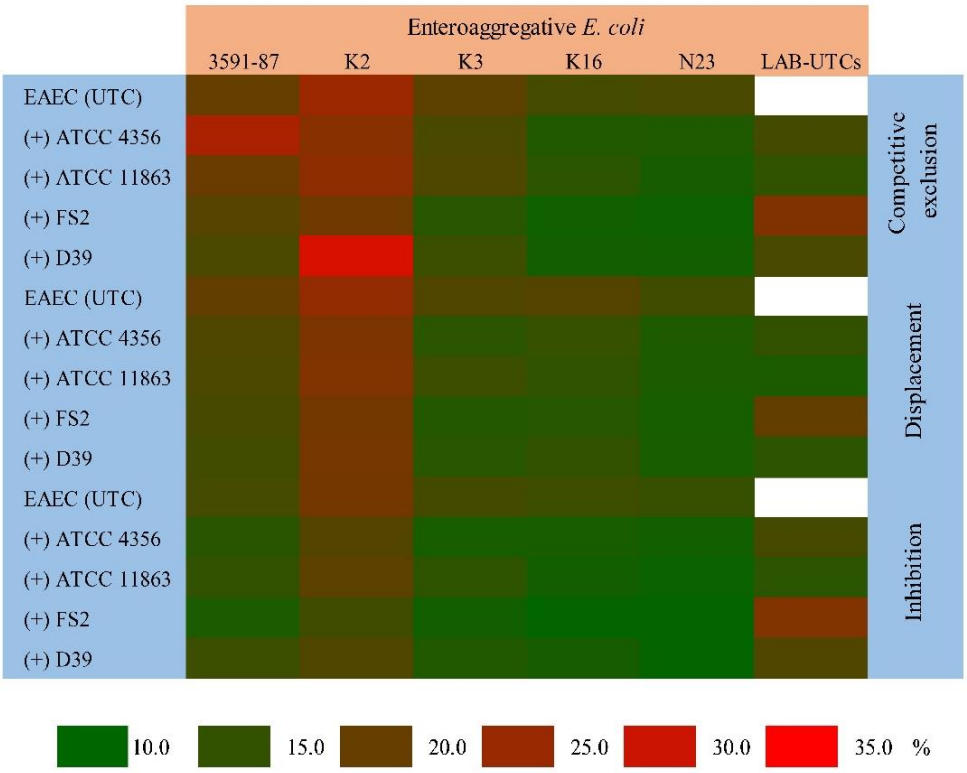


Figure 5.5: Heat map representation of competitive exclusion, displacement, and exclusion of Enteroaggregative *E. coli* (EAEC) of unpasteurized fresh milk origin from adhesion to the epithelial (Caco-2) monolayers by lactic acid bacteria from traditionally fermented *ogi*

Each cell represents the mean of three independent replicates (n=12) for the per cent bacterial adhesion during competitive exclusion; displacement and exclusion of EAEC 3591-87, K2, K3, K16 and N23 from adhesion to differentiated Caco-2 monolayers by lactic acid bacteria, including *L. acidophilus* ATCC 4356, *B. bifidum ATCC* 11863, *L. plantarum* FS2 and *P. pentosaceus* D39 respectively).

In a study to evaluate the potential of Zimbabwean commercial probiotic products and strains of *Lactobacillus plantarum* as prophylaxis and therapy against diarrhoea caused by *Escherichia coli* in children, it was confirmed that the LAB grossly inhibited diarrhoeagenic clinical *E. coli* strains compared to *L. rhamnosus* LGG strain and probiotic products, which were widely used for prophylaxis and therapeutic purposes (Chingwaru and Vidmar, 2017). Other independent studies further confirmed this finding (Karimi *et al.*, 2018b; Yang *et al.*, 2014).

Our results demonstrated that the profile of competitive exclusion, displacement, and exclusion of EAEC by the LAB differed. Differences in their surface characteristics could account for those differences. These results further suggest that the underlying mechanism for the antagonistic actions of the LAB against the selected EAEC could be a complex one involving several factors.

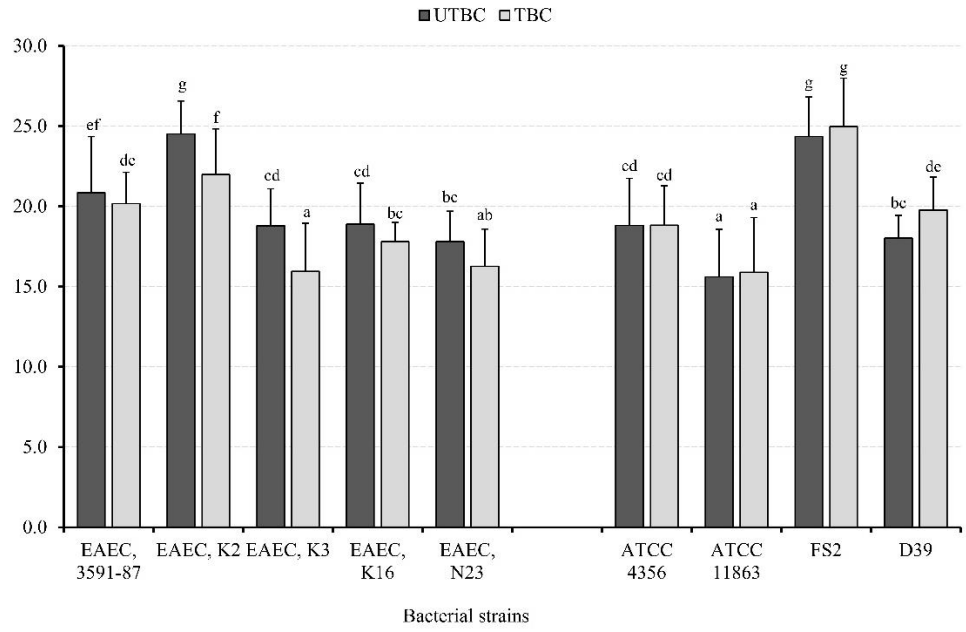


Figure 5.6: Effect of acidification (pH = 4.5) on the adhesion of EAEC and LAB to the monolayers

Results were expressed as per cent adhesion (mean ± standard deviation) for all treated bacterial cells (TBCs) and untreated bacterial cells (UTBCs). Bars with different letters were significantly different ($P < 0.05$) from one another (n=12).

EAEC strains: 3591-87; K2; K3; K16; and N23.

LAB strains: *L. acidophilus* ATCC 4356, *B. bifidum* ATCC 11863, *L. plantarum* FS2 and *P. pentosaceus* D39, respectively.

### 5.4.5 Effect of acidity (pH = 4.5) on adhesion of EAEC and LAB

The GIT, by its nature, has varying levels of acidity with their corresponding different pH values for the facilitation of its primary physiological functions, including digestion (Evans *et al.*, 1988). The ability of the LAB to tolerate bile salts and gastric acid was critical for their survival and growth within

the GIT (Argyri *et al.*, 2013; Guglielmetti *et al.*, 2008). Therefore, acid and bile salt tolerance constitute principal criteria for selecting LAB as probiotic candidates. Therefore, their ability to adhere to the GIT under these conditions is equally critical. However, the adherence of LAB and EAEC under these varying conditions have not been fully elucidated. The selected LAB from this study exhibited varying degrees of acid and bile tolerance at different pH values and, together with bile salts (Fayemi, 2015a).

Except for *Lactobacillus acidophilus ATCC* 4356, the acidification (pH = 2.5) lead to increments in the adhesion of all the LAB (Figure 5.6). However, these differences were not significant. The ASs of the selected EAEC strains, on the other hand, were significantly affected ($P < 0.05$) by acidification (pH = 4.5). EAEC K3 suffered the most severe reduction (2.9 %) in its adhesion followed by EAEC K2 (2.5 %), N23 (1.5 %), K16 (1.1 %) and 3591-87 (0.7 %). This result was not expected since ND-EAEC N23 had the least ASs in most cases throughout the study.

### 5.4.6 Effect of acidity (pH = 2.5) on adhesion of EAEC and LAB

Unlike the acidic treatment (pH = 4.5) of the Caco-2 monolayers, the acidification (pH = 2.5) accounted for significant reduction ($P < 0.05$) in the adhesion of all the EAEC strains (Figure 5.7). The adhesion of none of the LAB was affected by this treatment. Under this condition, *L. plantarum* FS2 adhered the most (24.9 and 25.0 %), whereas *B. bifidum ATCC* 11863 recorded the least AS (17.0 and 15.9 %). The monolayers' acidification (pH = 2.5) accounted for marginal increments in the adhesion of all the LAB. *L. plantarum* FS2 gained an increment (0.1 %) after the treatment. This trend was followed by *Lactobacillus acidophilus* ATCC 4356; *Bifidobacterium bifidum ATCC* 11863; and *Pediococcus pentosaceus* D39, which suffered 1.6, 1.1 and 1.1 %, respectively in their reductions to the monolayers. However, the differences due to the higher acidification were not significant. Contrarily, the selected EAEC strains suffered different degrees of significant reductions ($P < 0.05$) in their adhesion to the epithelial monolayers. Although EAEC K2 scored the highest adhesion to the monolayers under neutral and acidic conditions, it suffered the highest depreciation (7.1 %) in its adhesion to the monolayers. This trend was followed by D-EAEC K3 (4.9 %), D-EAEC 3591-87 (4.1

%) and then D-EAEC K16 (3.0 %). Interestingly, the ND-EAEC N23 recorded the least reduction (2.7 %) in its adhesion to the monolayers (Figure 5.7). The higher stability of the selected LAB to the higher acidic conditions as opposed to the EAEC is an indication that the LAB has the potential to protect the host against gastrointestinal inflammation due to EAEC strains. Additionally, our results

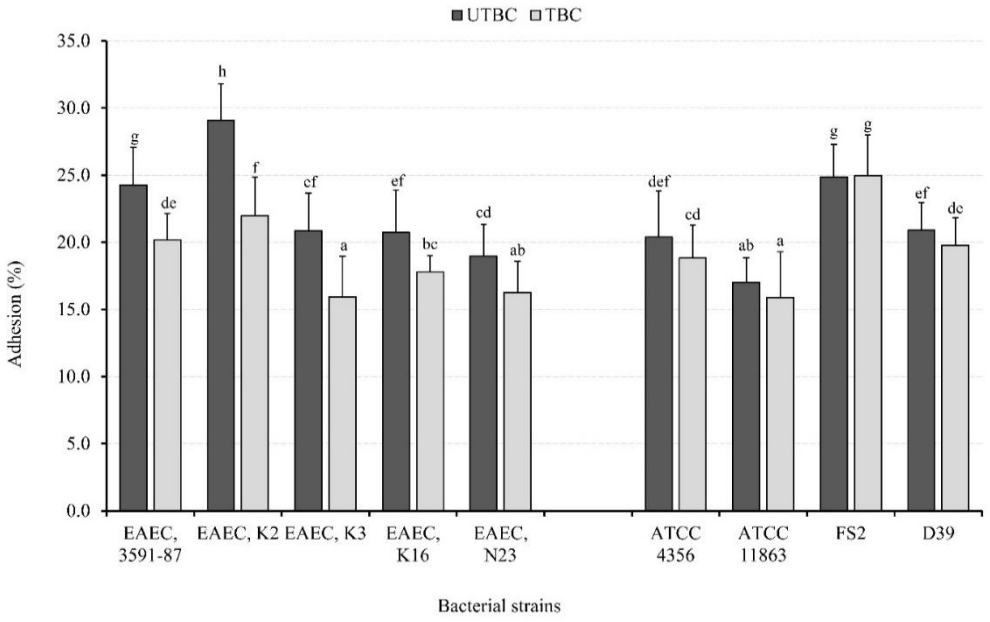


Figure 5.7: Effect of acidification (pH = 2.5) on the adhesion of EAEC and LAB to the monolayers

Results were expressed as per cent adhesion (mean ± standard deviation) for all treated bacterial cells (TBC) and untreated bacterial cells (UTBC). Bars with different letters were significantly different ($P < 0.05$) from one another (n=12).

EAEC strains: 3591-87; K2; K3; K16; and N23.

LAB strains: *L. acidophilus* ATCC 4356, *B. bifidum* ATCC 11863, *L. plantarum* FS2 and *P. pentosaceus* D39, respectively.

indicate that with prolonged exposure of the EAEC to these extreme acidic conditions might not be successful in eliciting their pathogenic and virulence effect on their host.

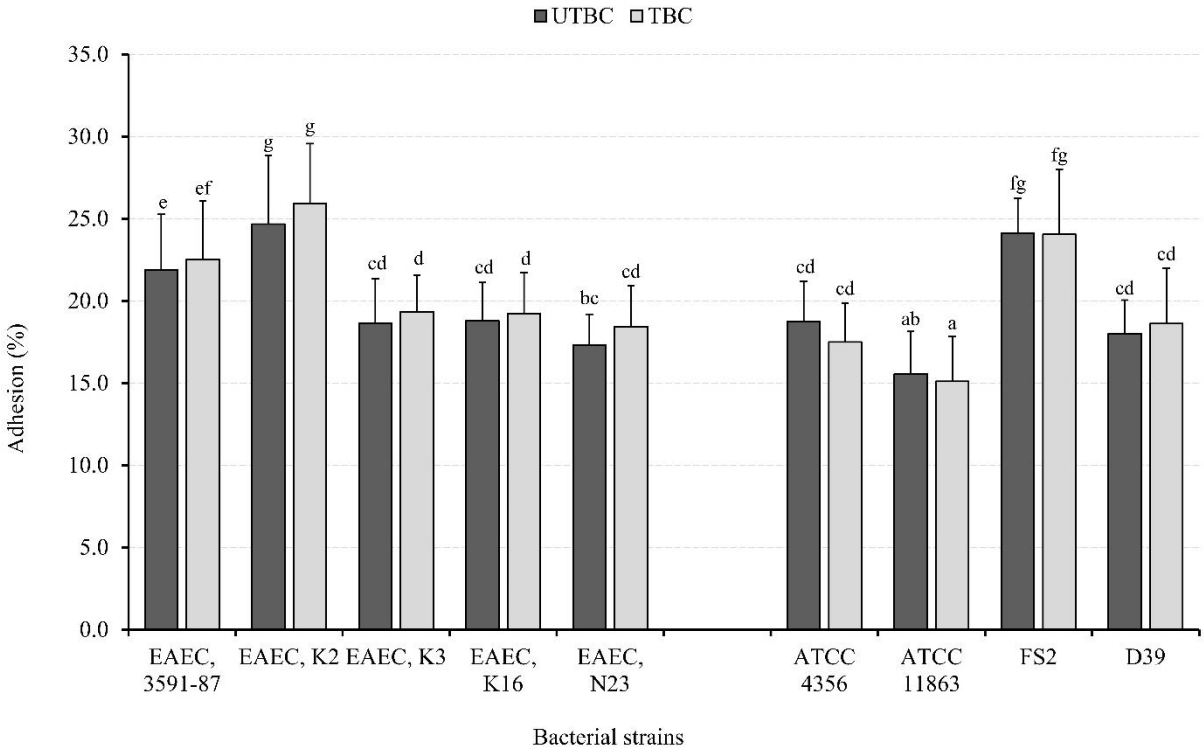


Figure 5.8: Effect of bile salts with acidification (pH = 6.5) on the adhesion of EAEC and LAB to the monolayers

Results were expressed as per cent adhesion (mean ± standard deviation) for all treated bacterial cells (TBC) and untreated bacterial cells (UTBC). Bars with different letters were significantly different ($P < 0.05$) from one another (n=12).

EAEC strains: 3591-87; K2; K3; K16; and N23.

LAB strains: *L. acidophilus* ATCC 4356, *B. bifidum* ATCC 11863, *L. plantarum* FS2 and *P. pentosaceus* D39, respectively.

### 5.4.7 Effect of bile salts (pH = 6.5) on adhesion of EAEC and LAB

Interestingly, unlike the adhesion of the EAEC strains under the two acidic conditions (pH of 4.5 and 2.5, the adhesion of all the EAEC were not affected by bile salts at a pH of 6.5 just like their LAB counterparts (Figure 5.8). Irrespective of both bacteria classes registering some increments and reductions to the monolayers, those differences were insignificant.

### 5.4.8 Scanning electron microscopy (SEM)

#### *5.4.8.1 Adherence of LAB to the enterocytes*

The scanning electron micrographs [Figure 5.9, 1(a – c] of uninfected monolayers, showed that the 15 days post-confluence Caco-2 cells differentiated into finger-like structures called enterocytes. These

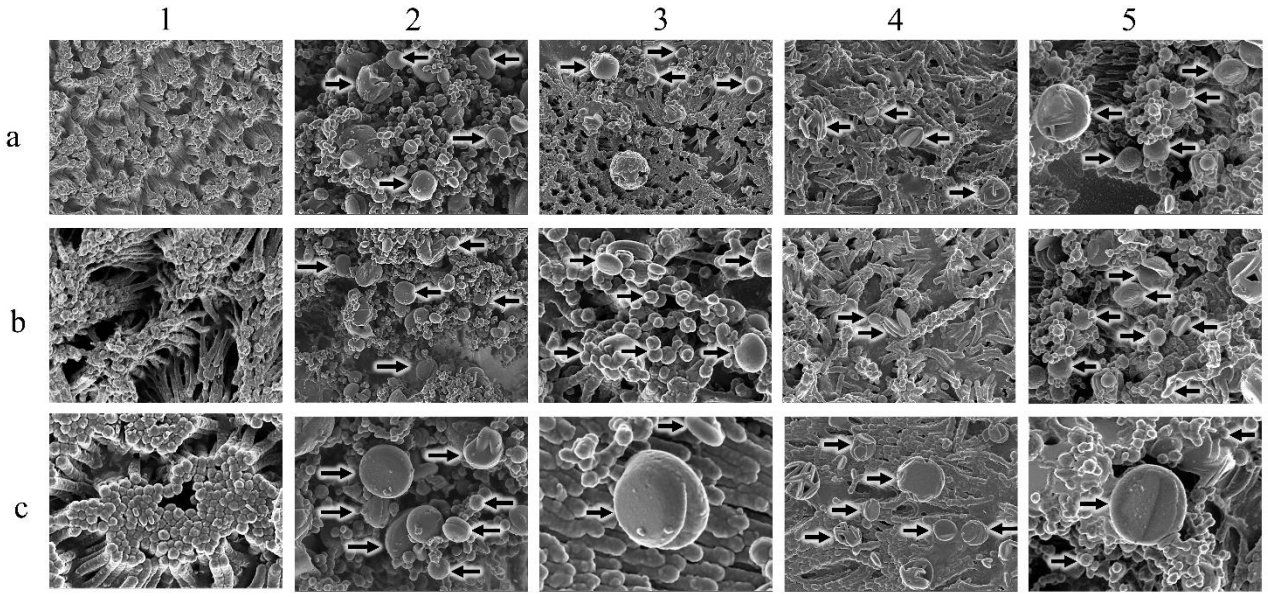


Figure 5.9: Scanning electron micrographs illustrating the adherence of lactic acid bacteria strains to the differentiated Caco-2 (epithelial) monolayers

Plates 1(a-c), 2(a-c), 3(a-c), 4(a-c) and 5(a-c) shows untreated differentiated Caco-2 monolayers infected with; no bacteria (uninfected/control); *L. acidophilus* ATCC 4356; *B. bifidum ATCC* 11863; *L. plantarum* FS2, and *P. pentosaceus* D39 respectively from lower to higher magnifications.

enterocytes possess numerous microvilli which cluster at their endings upon close examination. This observation implies that they can facilitate the adhesion of luminal substances, including bacteria cells. Their appearance and maturity state agreed with previous findings (Kimoto *et al.*, 1999; Medrano *et al.*, 2009). These results suggest that using them as cell culture models in experiments involving adhesion assays could produce results comparable to their in-vivo counterparts.

The results of our SEM micrographs indicate that the LAB cells not only attached to the brush border endings of the microvilli but were also trapped within two or more microvilli (Figure 5.9). Thus, in our study, both the reference PBB namely *L. acidophilus* ATCC 4356 and *B. bifidum ATCC* 11863

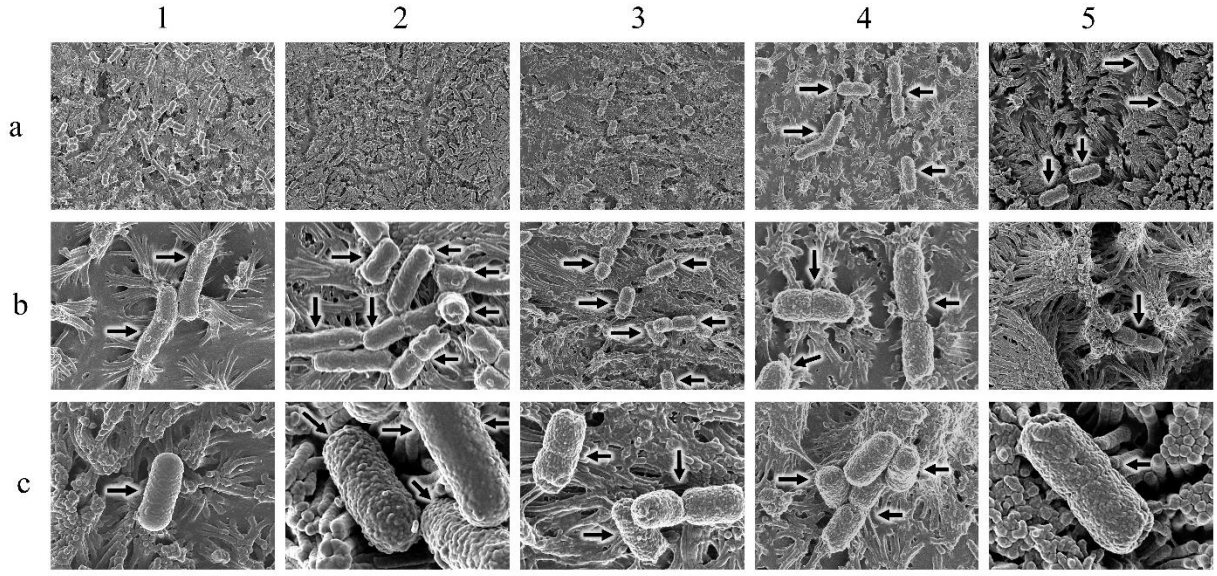


Figure 5.10: Scanning electron micrographs showing the adherence of enteroaggregative *E. coli* (EAEC) to the differentiated Caco-2 monolayers

Plates 1(a-c), 2(a-c), 3(a-c), 4(a-c) and 5(a-c) shows the adherence of EAEC 3591-87, K2, K3, K16 and N23 respectively to the differentiated Caco-2 monolayers from lower to higher magnifications.

and those from the fermented *ogi, L. plantarum* FS2 and *P. pentosaceus* D39 adhered to Caco-2 monolayers as illustrated by micrographs 1(a-c), 2(a-c), 3(a-c), 4(a-c) and 5(a-c) (Figure 5.9). The colonization of the intestinal epithelium by both bacteria seemed not to cause any damage to the enterocytes. These findings agreed with previous findings (Alp *et al.*, 2010; Kim *et al.*, 2003; Salas-Jara *et al.*, 2016a).

#### *5.4.8.2 Adherence of enteroaggregative E. coli to the enterocytes*

Unlike the LAB cells, the EAEC cells were mainly found to adhere to the brush border endings of the differentiated Caco-2 monolayers. Our results indicate that all the diarrhoeagenic and non-diarrhoeagenic EAEC adhered to the differentiated Caco-2 monolayers as shown in micrographs [Figure 5.10, 1(a-c), 2(a-c), 3(a-c), 4(a-c) and 5(a-c)]. These results indicate that the diarrhoeagenic strains may elicit inflammatory bowel disease (Aijuka *et al.*, 2018; Aijuka *et al.*, 2019) once they get ingested into the host's gut.

#### *5.4.8.3 Competitive exclusion, displacement, and exclusion of EAEC by LAB*

Micrographs [Figure 5.11, 1(a-c)] illustrated competition between EAEC K2 and *L. plantarum* FS2 for adhesion to the Caco-2 monolayers. During this process, the Caco-2 monolayers were simultaneously infected with the two microorganisms. Micrographs 2(a-c) (Figure 5.11) showed that *L. plantarum* FS2 directly adhered to EAEC K2. During this process, the monolayers were infected with EAEC K2 before *L. plantarum* FS2. The direct attack of the EAEC K2 by the *L. plantarum* FS2 could be explained by the fact that the adhesion of EAEC K2 to the monolayers rendered fewer adhesion sites available for the LAB cells. Similarly, pre-infecting the monolayers with *L. plantarum* FS2 before the EAEC K2, the former attached to the microvilli leaving fewer adhesion sites available for the EAEC K2 [Figure 5.11, 3(a-c)]. But in most cases, unlike the first instance where the monolayers were infected with the LAB, the EAEC simultaneously, the cells did not adhere directly to the LAB cells.

The ability of LAB to adhere to the intestinal epithelium, as observed in this study, is one of the critical considerations for selecting LAB as probiotic candidates (Conway, 1996; Dunne *et al.*, 2001a; Tuomola *et al.*, 2001). The process of bacterial adhesion to the gut epithelium is a critical stage for both LAB and enteric pathogens, signifying the possibility of interaction between the two. Adhesion of lactobacilli to the intestinal epithelium is associated with numerous beneficial health effects (Boirivant and Strober, 2007; Otutumi *et al.*, 2012). For example, they negate the colonization and

virulence of enteric pathogens within the gut of their host through several interventional mechanisms. In most situations where the EAEC adhered in significant numbers to the monolayers, they aggregated with their characteristic stacked brick arrangements. This result supports an earlier report (Andrade *et al.*, 2011; Nataro, 2005; Nataro *et al.*, 1992). We showed that enteric pathogens have great potential to invade and colonize the intestinal epithelium by setting up physical contacts between their cellular membranes or extracellular surface structures and the brush borders of the differentiated enterocytes. Similarly, adhesion of the LAB to the differentiated Caco-2 cells also suggests that they have

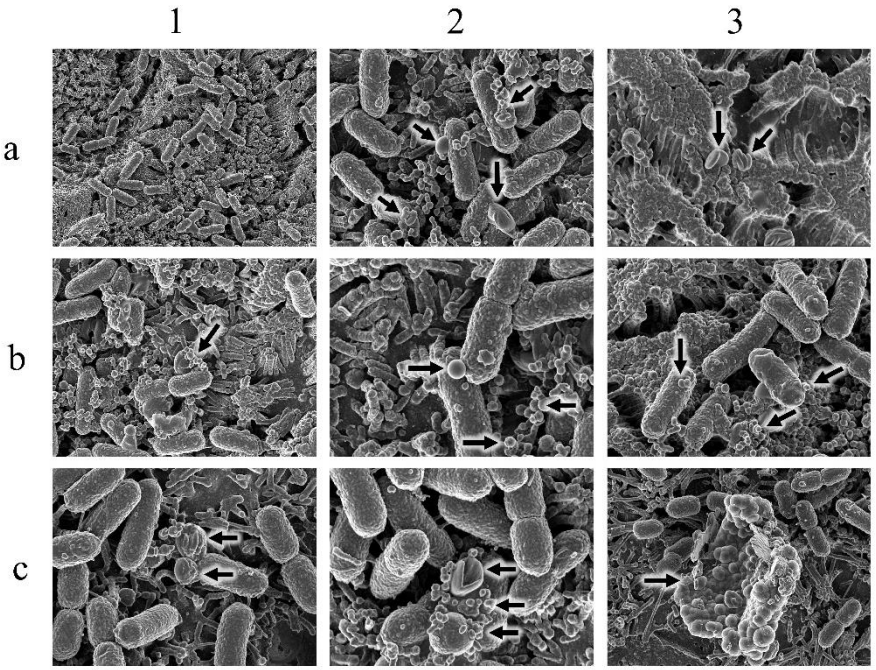


Figure 5.11: Scanning electron micrographs illustrating the adhesion of presumptive probiotic bacteria and their effect on Enteroaggregative *E. coli* to Caco-2 (epithelial) monolayers.

Competitive exclusion, displacement, and exclusion of EAEC K2 by *L. plantarum* FS2 are illustrated by plates 1(a-c), 2(a-c) and 3(a-c) respectively.

significant potential of colonizing and then occupying the adhesion sites of the epithelium, thereby preventing enteropathogens from adhering and invading the gut. Therefore, these LAB may avert the virulence of these enteric pathogens such as the D-EAEC against their host. We showed that enteric

pathogens have great potential to invade and colonise the intestinal epithelium by setting up physical contacts between their cellular membranes or extracellular surface structures and the brush borders of the differentiated enterocytes. Similarly, adhesion of the LAB to the differentiated Caco-2 cells also suggests that they have the potential to colonise and engage the adhesion sites of the epithelium and thereby preventing enteropathogens from adhering and invading the gut. Therefore, these LAB could avert the virulence of these enteric pathogens such as D-EAEC against their host.

Thus, our current studies revealed that under the given conditions, all the EAEC and the LAB adhered to the epithelial cells differently depending on the bacterial strain involved. Also, the selected LAB competitively excluded, displaced, and inhibited EAEC in varying degrees based on strain-strain specificity. Additionally, except for EAEC K2 and K3, the adhesion of both EAEC and LAB to the Caco-2 cells were generally not affected by simulated gastric fluid (pH of 4.5) conditioning. Furthermore, the adhesions of all the EAEC, unlike the LAB strains reduced in the presence of simulated gastric fluid (pH of 2.5). Finally, both EAEC and LAB exhibited some stability with their adhesion to the Caco-2 cells in the presence of SBS condition (pH 6.5).

We have identified a few strengths of our study. The current research effectively compared the EAEC and LAB adhesion potentials to Caco-2 cells in vitro. Secondly, our study shows the competence of LAB strains from the fermented cereal food, *ogi,* to competitively exclude, displace, and inhibit the various EAEC strains from adhesion. Thirdly, we explored the effects of the simulated gastric fluid and bile salts conditioning on the adhesion of the EAEC and the LAB. Most studies in this field lacked this component of the analysis. Fourthly, we illustrated the adhesion of both EAEC and LAB to the Caco-2 monolayers using SEM. Finally, we demonstrated the LAB's competitive exclusion, displacement, and exclusion abilities against the EAEC through the scanning electron micrographs, which most previous studies lacked.

Like many other studies, this research did not go without limitations. Due to logistical constraints, the bacterial adhesion to the Caco-2 monolayers was limited to 2 h, as reported by previous works. Meanwhile, bacterial adhesion to the intestinal epithelium was said to be a function of time right from the point of infection (Arico *et al.*, 1993; Danielsson *et al.*, 1977; Jankowska *et al.*, 2008). Thus, bacterial adhesion might go beyond two h from the point of infection in vivo. Secondly, specific physiological processes such as fermentation and producing organic acids and bacteriocins also occur in vivo. These processes are also time-dependent, mostly between 48 to 72 h (Avonts *et al.*, 2004; Boris *et al.*, 2001). These substances might have their own influence on bacterial survival and adhesion. These natural phenomena' influence on our results was not catered for (Danielsson *et al.*, 1977) single strains (Collado *et al.*, 2005). However, the experimental design of our current study failed to investigate the effect of combinations of the selected LAB on the adhesion profile of the pathogens.

The infection, trypsinisation, and the plating of the bacterial cells for enumeration might have suffered some timing discrepancies (bias) of ± 30 minutes. These inconsistencies are because the wells were treated one after the other and might not precisely fall within the various specified timings stated in the methods. The duration of vortexing test tubes and microcentrifuge tubes might not be exactly the specified time for all the treatments. These anomalies might have affected the homogeneity and the enumeration of the bacterial cells. The plating of the attached bacterial cells for counting mostly spanned across at least 6 h. Though the bacterial cells were kept on chiller ice to prevent cell multiplications, this might have affected the survival of bacterial cells plated later compared to those platted earlier. It was assumed that any adverse effect of this chiller ice and the different timings of the plating on the bacterial cells might have been negligible.

## 5.5 Conclusion

LAB that competitively exclude, displace, and inhibit (enteric) pathogens are excellent probiotic candidates for use against such tested pathogens under specific conditions. Our results demonstrate that the two LAB, *Lactobacillus fermentum* FS2 and *Pediococcus pentosaceus* D39 from *ogi*, just as the two reference PBB, *Lactobacillus acidophilus* ATCC 4356 and *Bifidobacterium bifidum ATCC* 11863 tested in this study exhibited probiotic characteristics by competitively excluding, displacing, and inhibiting at least three of the selected EAEC. However, the high specificities of these processes need to be deemed very important. Therefore, it is essential to characterise the properties of the specific PBB and pathogens strains, bearing in mind the target group. This will enable the selection of the best (combinations of) probiotic strains for specific interventions.

Additionally, this will allow the development of probiotics for managing specific disease conditions among specific target groups. Our results also report high specificities not only in the adhesion of the individual LAB and the EAEC to the enterocytes but also in the competitive exclusion, displacement, and exclusion of the selected EAEC strains by the LAB. Thus, the PBB need to be characterised on a case-by-case basis. This characterisation will enable the selection of LAB (PBB) strains with potential application for prophylactic or therapeutic management of specific gastrointestinal infections. However, we strongly recommend testing specific identified pathogens from a target population to select the best probiotic or probiotic combination ahead of interventions involving human volunteers.

# CHAPTER 6.

## RESEARCH CHAPTER 3

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## Immunomodulatory activities of potential probiotics, *Lactobacillus plantarum* and *Pediococcus pentosaceus* in enteroaggregative *Escherichia coli* (EAEC)-challenged Caco-2 cells

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This chapter (paper) was submitted to the journal, *Clinics and Research in Hepatology and Gastroenterology*.

**Immunomodulatory activities of potential probiotics, *Lactobacillus plantarum* and *Pediococcus pentosaceus* in enteroaggregative *Escherichia coli* (EAEC)-infected Caco-2 cells**

## 6.1 Abstract

The application of probiotics as an indispensable tool for prophylactic and therapeutic management of gastrointestinal infections caused by enteropathogens like Enteroaggregative *Escherichia coli* (EAEC) is evolving. Some Lactobacilli have been noted to inhibit enteropathogens' adhesion to protect epithelial barrier integrity and function. This study focused on the ability of lactic acid bacteria (LAB) with promising probiotic characteristics from West-African traditionally fermented food, *ogi*, to attenuate diarrhoeagenic EAEC-induced changes to the intestinal epithelial barrier. Our results demonstrate that challenging polarised Caco-2 cell monolayers with the selected EAEC strains reduced trans-epithelial electrical resistance (TEER) and increased inflammatory cytokine, interleukin 8 (IL-8) secretions. However, treating the monolayers with *Lactobacillus plantarum* FS2 and *Pediococcus pentosaceus* D39 from *ogi* restored the deviations in TEER and IL-8 from the diarrhoeagenic EAEC's damaging effects. Thus, the two LAB isolates conferred some protective effects on the intestinal epithelium and will protect and maintain its structure and function against the ravaging effects of the diarrhoeagenic EAEC, implying that they both have the potential to maintain and improve consumers' gut health.

## 6.2 Introduction

The World Health Organization (WHO) defined probiotics as "live microorganisms, which when administered in adequate amounts, confers a health benefit on the host" (Agostoni *et al.*, 2004; Guarner and Schaafsma, 1998). Probiotics mainly belong to the *Bifidobacterium* and *Lactobacillus* genera. They are primarily selected for their competence to survive gastrointestinal conditions, grow in dairy products, and attach to the epithelial cells (Delgado *et al.*, 2008; Dunne *et al.*, 2001b). However, their selection is most importantly based on their competence to address specific health needs (Gueimonde

and Salminen, 2006). Probiotics confer their beneficial effects through several demonstrated mechanisms: competitive exclusion, displacement and exclusion of enteropathogens (Agbemavor and Buys, 2021), improvement of epithelial and mucosal barrier integrity and function and regulation of the host's immune response (Bron *et al.*, 2017; Judkins *et al.*, 2020; Martens *et al.*, 2018). Several studies demonstrated that the effects of enteropathogens and probiotics to maintain intestinal epithelial barrier integrity and function could be assessed by measuring trans-epithelial electrical resistance (TEER) across the apical and basolateral sides of adjoining intestinal epithelial monolayer cells. For example, *Lactobacillus plantarum has* been reported to improve gut barrier integrity and function (Anderson *et al.*, 2018; Qiu *et al.*, 2017; Ren *et al.*, 2020). Although different studies have established several associations between gastrointestinal infection and various probiotics (Preidis *et al.*, 2020; Sebastian Domingo, 2017; Su *et al.*, 2020; Wilkins and Sequoia, 2017), studies involving applications of potential probiotics from other non-dairy sources such as fermented cereals for enhancing intestinal barrier integrity and function are limited.

This study aimed to evaluate the abilities of *L. plantarum* FS2 and *Pediococcus pentosaceus* D39 from a West-African fermented cereal, *ogi*, to maintain the epithelial barrier integrity and function from the destructive effects of selected diarrhoeagenic enteroaggregative *E. coli* (D-EAEC) strains from unpasteurized fresh milk. Enteroaggregative *E. coli* (EAEC) are emerging enteropathogens primarily involved in persistent and acute paediatric diarrhoea, leading to growth retardation mostly among pre-school children in developing and industrialized nations (Nataro and Kaper, 1998). It also largely accounts for acute diarrhoea in travellers to developing countries (Lääveri *et al.*, 2018) and persistent inflammatory bowel disease among HIV/AIDS patients (Durrer *et al.*, 2000). EAEC strains demonstrated substantial diversity in their genes encoding for several adhesins, toxins, and surface proteins concerning their virulence, strategies, and locations (jejunal, ileal, and colonic mucosa) of infection due to the presence of heavy molecular weight plasmids carrying several genes associated with pathogenesis (Estrada-Garcia and Navarro-Garcia, 2012; Guerrieri *et al.*, 2020). Such plasmids

account for the acquisition or deletion of virulent genes leading to heterogenicity within the pathotype (Hebbelstrup Jensen *et al.*, 2017; Hosseini Nave *et al.*, 2016).

The selected LAB strains were previously evaluated for their abilities to auto-aggregate and co-aggregate with different D-EAEC, tolerate gastrointestinal conditions and adhere to intestinal epithelial cells (IECs) (Agbemavor and Buys, 2021). The selected EAEC strains were also studied for their ability to cause alterations and induce the secretion of different inflammatory cytokines. However, the studied EAEC were not challenged with any PBB. This would have revealed LAB effect on the EAEC to cause intercellular tight junction disruption. Like the EAEC, the probiotic strains also demonstrated extensive heterogeneity in different characteristics, such as their antimicrobial properties due to differences in antimicrobial compounds like the bacteriocins they produce through their metabolic activities (Alvarez-Sieiro *et al.*, 2016). These characteristics account for their varying competencies for ameliorating different diseases (Alvarez-Sieiro *et al.*, 2016). Thus, their efficacy is strain dependent with several proposed mechanisms of action (Clarke *et al.*, 2012).

## 6.3 Materials and methods

### 6.3.1 Bacterial strains and culture conditions

Two selected diarrhoeagenic (3591-87 and K2), and a non-diarrhoeagenic *E. coli* (ND-EAEC) N23 strains (Aijuka *et al.*, 2018; Ntuli *et al.*, 2017) (Table 6.1); isolated from unpasteurized fresh milk (except for 3591-87 being a positive clinical reference control) were used in this study. Two LAB, *L. plantarum* FS2 and *P. pentosaceus* D39, with some good probiotic characteristics, were obtained out of the several isolates from a previous study involving traditional non-alcoholic fermentation of maize for the production of a West African gruel (*ogi*) from an earlier study (Fayemi and Buys, 2017; Fayemi *et al.*, 2017). The other LAB (probiotics) used in this study and their culturing conditions were described (Table 6.1).

### 6.3.2 Cell culturing and maintenance conditions

Human epithelial intestinal cell from colorectal adenocarcinoma, Caco-2 [American Type Culture Collection (ATCC) catalogue number (CN), HTB-37, Maryland, USA] was obtained and sustained in Dulbecco's modified Eagle medium (DMEM; Gibco, ThermoFisher, USA) containing 4,500 mg/L D-glucose, non-essential amino acids, 110 mg/L of sodium pyruvate as described earlier (Agbemavor and Buys, 2021). Briefly, the media was supplemented with 10% (v/v) gamma-irradiated, heat-inactivated foetal bovine serum (FBS, Gibco) with 1% penicillin-streptomycin. The cells were mostly cultivated in T75 (75 cm2) cell culture flasks [CN, "658940" (Greiner Bio-One GmbH, Frickhausen, Germany)] and sub-cultured (60 to 70 % confluence) into a ratio of 1:3 and incubated (37 ℃, 5% CO2) in a $CO_2$ humidified (95 % air) incubator (Healforce, HF 212UV, China). The cells were subcultured every 3-5 days after trypsinization [(0.5 % trypsin- ethylenediaminetetraacetic acid (trypsin-EDTA), ThermoFisher, USA]. Monolayers were considered polarized when their TEER value was at least 1000 $\Omega.cm^2$. Therefore, based on previous studies, polarized Caco-2 cell monolayers (PCC-2CMLs) having TEER values from ~1000 to ~ 2000 Ω cm2 were used for the analysis (Aijuka *et al.*, 2019; Karimi *et al.*, 2018a). Monolayers having TEER values below 1000 Ω $cm^2$ were excluded due to the possibility of having high permeabilities. Caco-2 cells within passages 30-39 were used in all experiments. The cell cultures were routinely examined and confirmed to be void of bacterial and mycoplasma contaminations before their usage for all experiments. At least two hours before the onset of the various experiments, the cells were nourished with serum- and antibiotic-free medium.

### 6.3.3 Preparation of epithelial cells for inflammation assays

Caco-2 cells were seeded (5.0 x $10^5$ cells/1.12 $cm^2$) using Corning 12-well plates with sterile Coaster Snapwell collagen-coated polytetrafluoroethylene semipermeable filter inserts (Transwell®-COL, 12 mm diameter, 1.12 $cm^2$ cell growth area, 0.4 µm pore with CN, 3493, Corning B.V., Arizona, United States). The cells were allowed to differentiate for 21 days post-confluence. The PCC-2CMLs were washed thrice with phosphate-buffered saline (PBS) to remove FBS and the antibiotics at least 2 h

before bacterial infection. Appropriate volumes of freshly prepared serum- and antibiotic-free DMEM (containing 4,500 mg/L D-glucose, 1 % (v/v) non-essential amino acids, and 110 mg/L of sodium pyruvate) were then added to the apical and basolateral compartments of the wells with and without epithelial cells.

### 6.3.4 The effects of EAEC and LAB on epithelial barrier integrity

This study evaluated the competence of the selected LAB to protect and maintain the epithelial-like PCC-2CMLs from the ravaging effects of diarrhoeagenic EAEC. Briefly, cultured (18 h old) bacterial strains were standardized (EAEC, 6.0 x $10^8$ and LAB, 6.0 x $10^9$ CFU/mL, Table 6.1) with PBS using the McFarland densitometer (DEN-1 Model, Grant-bio, Sia Biosan, Riga, Latvia). These strains were

Table 6.1: Sources and characteristics of selected enteroaggregative *E. coli* (EAEC) and lactic acid bacteria (LAB) strains used in this study States).

| Bacteria strain | Characteristic | Source |
|---|---|---|
| EAEC 3591-87 | Clinical and diarrhoeagenic (positive reference strain) | [a]NICD of NHLS |
| EAEC K2 | Diarrhoeagenic | [b]Unpasteurised fresh milk |
| EAEC N23 | Non-Diarrhoeagenic | [b]Unpasteurised fresh milk |
| *Bifidobacterium bifidum ATCC* 11863 | Reference probiotic bacteria | [c]ATCC Collections |
| *Lactobacillus plantarum* FS2 | Promising probiotic characteristics | [d]Traditional fermented food (*ogi*) |
| *Pediococcus pentosaceus* D39 | Promising probiotic characteristics | [d]Traditional fermented food (*ogi*) |

[a]National Institute for Communicable Diseases (NICD), a division of the National Health Laboratory Service (NHLS), Johannesburg, the Republic of South Africa.

[b]Previously isolated by Aijuka *et al.* (2018) and Ntuli *et al.* (2017).

[c]American Type Culture Collection (ATCC, USA).

[d]Previously isolated by Fayemi and Buys (2017) Fayemi and Buys (2017).

further homogenized in serum- and antibiotic-free DMEM to form final bacterial densities (EAEC, 6.0 x $10^7$ and LAB, 6.0 x $10^8$ CFU/mL). Selected wells of PCC-2CMLs were monoinfected by replacing the cell culture medium from the apical chambers with 25 µL each of DMEM-bacterial suspension and PBS. In contrast, others were infected with different combinations of 25 µL each of an EAEC (3591-87, K2 and N23) and LAB (*B. bifidum* ATCC 11863; *L. plantarum* FS2 and *P. pentosaceus* D39) (Table 6.2). The challenged PCC-2CMLs were incubated (37 ℃, 5% CO2, 6 h). The initial and final

Table 6.2: Enteroaggregative *E. coli* (EAEC) and lactic acid bacteria (LAB) treatment template for Caco-2 monolayers infection

| LAB Bacteria Strains | EAEC Strains | | |
|---|---|---|---|
| | 3591-87 | K2 | N23 |
| 11863 | 3591-87 + 11863 | K2 + 11863 | N23 + 11863 |
| FS2 | 3591-87 + FS2 | K2 + FS2 | N23 + FS2 |
| D39 | 3591-87 + D39 | K2 + D39 | N23 + D39 |

Note: The EAEC strains include 3591-87, K2, K3, K16, and N23 and the LAB include *L. acidophilus* ATCC 4356 and *Bifidobacterium bifidum ATCC* 11863, *L. plantarum* FS2, and *P. pentosaceus* D39, respectively.

resistance measurements were then taken across the PCC-2CMLs for the determination of TEER, whilst supernatants (25 µL) were harvested from the apical chamber and stored (-20 ℃) for IL-8 assay (Aijuka *et al.*, 2019).

### 6.3.5 Effect of bacterial infection dose (BID) on epithelial barrier integrity

In this study, the effect of D-EAEC K2 infection dose on TEER and IL-8 induction was monitored as previously reported (Lodemann *et al.*, 2015) with a few modifications. Briefly, EAEC K2 cultures (18 h old) were standardized (1.5 x $10^9$ CFU/mL) as previously described (section 6.3.4). The bacterial cells were homogenized with serum- and antibiotic-free DMEM to a final concentration of (1.5 x $10^8$

CFU/mL). This bacterial cell-culture medium suspension was taken through ten-fold serial dilutions with further homogenizations to obtain various bacterial concentrations up to 1.5 x $10^2$ CFU/mL. These bacterial cells were then used to challenge PCC-2CMLs by replacing the serum- and antibiotic-free DMEM with the bacterial suspension, followed by incubation (section 6.3.4). The initial and final resistance measurements were taken across the PCC-2CMLs to estimate TEER, whilst supernatants were harvested and kept (-20 ℃) for IL-8 assay as previously described (Aijuka *et al.*, 2019).

### 6.3.6 Bacterial infection mode and treatment time (TT) effects on epithelial barrier integrity

This assay was carried out to determine the effect of TT and bacterial infection mode; 1. simultaneously with EAEC and LAB; 2. with EAEC an hour before LAB or 3. LAB an hour before EAEC on TEER and IL-8 of the PCC-2CMLs termed bacterial (EAEC) competitive exclusion from adhesion (BCEFA) assay, bacterial (EAEC) displacement from adhesion (BDFA) assay, and bacterial (EAEC) inhibition from adhesion (BIFA) assay, respectively. This study was restricted to EAEC K2 and *L. plantarum* FS2. Bacterial cultures (18 h old) were standardized (1.5 x $10^8$ and 1.5 x $10^9$ CFU/mL) (section 6.3.4) and used to infect PCC-2CMLs in different modes (Agbemavor and Buys, 2021) resulting in final BIDs of 7.5 x $10^7$ and 7.5 x $10^8$ CFU/well for EAEC and LAB, respectively. The challenged PCC-2CMLs were incubated (37 ℃, 5 % $CO_2$) and assessed for their initial and final trans-epithelial resistance (4, 8, 12, 16, 20, 24 and 28 h) for the determination of TEER, whilst their corresponding supernatants were collected from the apical chambers and isolated (-20 ℃) for IL-8 assay as previously described (Aijuka *et al.*, 2019).

### 6.3.7 Bacterial infection mode and TT effects on adhesion

This study aimed to determine the effects of TT and different modes of infection on the competence of bacterial adhesion to PCC-2CMLs. The selected bacterial cultures (EAEC K2 and *L. plantarum* FS2, 18 h old) were standardized as described earlier (section 6.3.4). The PCC-2CMLs were mono- and coinfected in different modes with the selected bacteria (Agbemavor and Buys, 2021), resulting

final bacterial infection densities (section 6.3.4). The experimental setups were then incubated (section 6.3.4). The bacterial cells (EAEC and LAB) were evaluated for their competence for adhesion to the monolayers at different times (2, 4, 8, 12, 16, 20, 24 and 28 h).

### 6.3.8 Interleukin 8 (IL-8) assay

A commercially available sandwich Enzyme Linked-Immunosorbent Assay (ELISA) kit (Elabscience Biotechnology Inc., Texas, United States) (CN, E-EL-H0048) was purchased and used to evaluate IL-8 strictly according to the manufacturer's instructions. Briefly, anti-human IL-8 pre-coated 96 well strip plates were individually treated with serially diluted reference standards and then incubated [room temperature (RT), 1 h]. The plates were washed (3x) with PBS, followed by treatment of each well with biotinylated antibody reagent and incubation (RT, 1 h). The plates were rewashed (3x) followed by treatment with 100 µL of streptavidin-Horseradish Peroxidase (HRP) solution, covered with petri film and then incubated (25 ℃, 30 mins). This procedure was followed by washing (3x) and then treating each well with 100 µL of TMB (3,3',5,5'-tetramethylbenzidine). The plates were further incubated (RT, 30 mins, dark room). Each well was finally treated with 100 µL of the stop solution to terminate the reactions. The plates' optical density readings (500 nm) were measured using a filter-based multi-mode microplate reader (FLUOstar Omega, BMG LabTech, Ortenberg Germany). The experiment was repeated by replacing the serially diluted reference standards with the thawed and preincubated (RT, 15 minutes) harvested supernatants. The IL-8 concentrations of the samples were calculated regarding the linear equation generated from the optical densities of the reference standards.

### 6.3.9 Trans-epithelial Electrical Resistance (TEER) assay

This assay was carried out by following previously laid down protocols (Aijuka *et al.*, 2019) with a few modifications. The electrical resistance was measured across the monolayers (from the apical to the basolateral sides) using a Millicell ERS-2 electrode (MERSSTX01) volts/ohmmeter resistance system (Millipore Corporation, Bedford, MA, USA). The background resistance for the cell culture

membrane inserts with (the serum- and antibiotic-free) medium was subtracted from the initial and final resistance readings to obtain the actual resistance values. The TEER value was calculated as a product of the resistance value and the membrane area (in $cm^2$) of the cell culture insert.

### 6.3.10 Data analysis

Analysis of variance (ANOVA) with the Fisher's least significant difference (LSD) test for multiple comparisons at 95 % confidence level was used to compare the different response variables for the various treatments. The analysis was performed using Statgraphics Centurion XVI (Statpoint Technologies Inc, 2020). The data were presented generally in bar charts whereas explorations of statistical relations between dependent variables (correlations) were presented in linear graphs as scatter plots. All experiments were independently carried out in triplicates with at least four internal replicates to cater for intra-assay variation. All experiments were conducted independently in triplicates and each experiment was repeated at least three times to cater for likely intra-assay variations.

## 6.4 Results and discussion

This section involves the interpretation and description of the significance of the major findings regarding the stated research problem(s) (questions) subjected to investigation within the context of this research. It further explains a few new understandings and insights that emerged from this study.

### 6.4.1 Cytokine secretion from Caco-2 monolayers in the presence or absence of EAEC and LAB

Our results indicate that both EAEC and LAB induced the secretion of the proinflammatory cytokine, interleukin 8 (IL-8), from PCC-2CMLs. However, the amount of IL-8 induced by LAB strains was much lower ($P < 0.05$) than with EAEC strains (Figure 6.1). Mono-infection with EAEC strains demonstrated that D-EAEC K2 induced IL-8 secretion to the highest order, followed by the positive

clinical reference D-EAEC 3591-87 and ND-EAEC N23 by 14.5, 13.3 and 4.2 folds, respectively, over that of the control setups. These results are not surprising because the adhesion of D-EAEC K2 was previously reported to be the highest, whereas that of ND-EAEC N23 was the least (Agbemavor and Buys, 2021). The selected LAB mitigated against the three EAEC in their abilities to induce IL-8

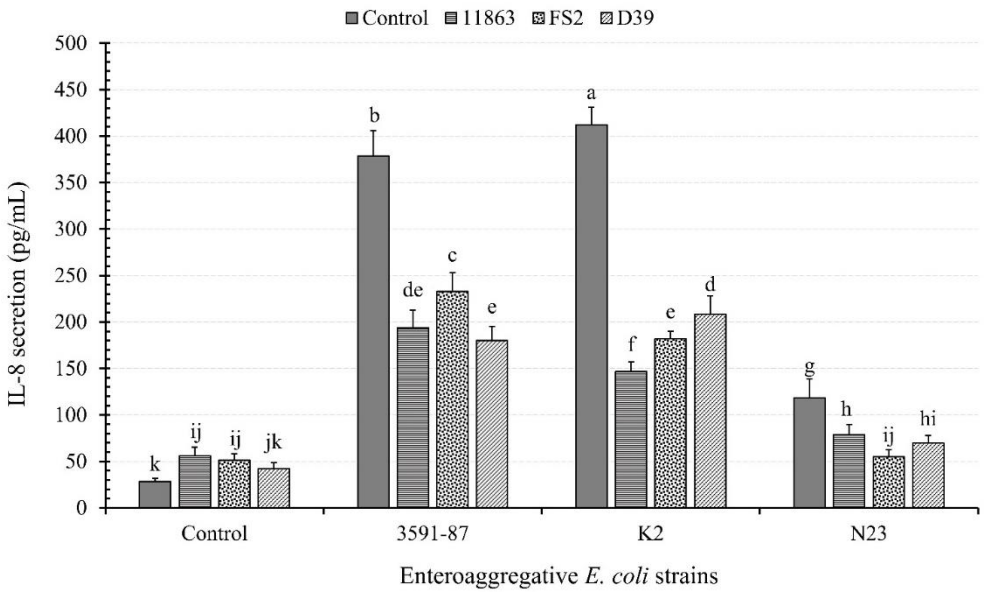


Figure 6.1: The effect of enteroaggregative *E. coli* (EAEC) and lactic acid bacteria (LAB) on the induction of interleukin 8 (IL-8) from polarized Caco-2 cell monolayers

The EAEC include diarrhoeagenic 3591-87 and K2 and a non-diarrhoeagenic (N23) strains. *B. bifidum;* ATCC, 11863 *L. plantarum* FS2 and *P. pentosaceus* D39 constitute the lactic acid bacteria (LAB) strains. Each bar is a mean of two independent replicates (n=4) with its corresponding standard deviation. Bars with different letters (a-k) indicate significant differences ($P \leq 0.05$) according to Fisher's least significant difference (LSD) test.

secretion in different capacities, which heavily depended on strain-strain specificity. Against the clinical D-EAEC reference strain 3591-87, *P. pentosaceus* D39 reduced its IL-8 induction ability from the differentiated Caco-2 cell monolayers (DCC-2CMLs) the most, followed by *B. bifidum* ATCC

11863 and then *L. plantarum* FS2 by 198.53 pg/mL (52.4 %), 184.9 pg/mL (48.8 %), and 145.8 pg/mL (38.5 %), respectively. *B. bifidum* ATCC 11863 mitigated the IL-8 secretion ability of the D-EAEC K2 the most, followed by *L. plantarum* FS2 and then *P. pentosaceus* D39 by 265.6, 230.2, and 203.7 pg/mL representing 64.4, 55.6 and 49.4 %, respectively. On the other hand, the IL-8 secretion induction ability of the ND-EAEC N23 was mitigated by *L. plantarum* FS2, followed by *P. pentosaceus* D39, and then *B. bifidum* ATCC 11863 by 63.3, 48.6, and 39.5 pg/mL signifying 53.5, 41.3, and 33.4 %, respectively.

These results were contrary to our expectations because we expected the mitigatory effect of *L. plantarum* FS2 against the selected EAEC to be the highest. This expectation was because the *L. plantarum* FS2 recorded the highest adhesion ability to the DCC-2CMLs whilst demonstrating excellent competitive exclusion, displacement, and inhibitory abilities against most of the EAEC strains (Agbemavor and Buys, 2021). Several cytokines were shown to regulate the intercellular tight junctions, cytoskeletal structure, and function (Cai *et al.*, 2018; Sluysmans *et al.*, 2017). IL-8 is a popular inducer of proinflammatory cytokine which recruits neutrophils antigen, phagocytes, and other neutrophils to the sites of injured tissues or infection and has been associated with pathogen-induced modifications of intercellular tight junctions (Domínguez-Díaz *et al.*, 2021; Dubreuil, 2017). Our current study showed that except for the ND-EAEC N23, the other two D-EAEC strains were associated with substantial increases in the secretion of IL-8. However, this was not the case with the selected LAB as they were mainly associated with low levels of IL-8 expression suggesting possibilities for the prevention of intestinal epithelial inflammation whilst maintaining epithelial barrier integrity and function.

Our results show that mono-infection, particularly with the pathogens, EAEC 3591-87 and K2 caused higher severity of inflammation (IL-8 secretions) than coinfections with the selected LAB agreeing with previous findings (Garcia-Gonzalez *et al.*, 2018; Jeffrey *et al.*, 2018; Soo *et al.*, 2019). Contrarily, inflammations due to mono-infection with LAB were much lower ($P < 0.05$) than mono-infection with

the pathogens corresponding to previously reported findings (Soo *et al.*, 2019; Soo *et al.*, 2016). Previous reports indicate that induced levels of IL-8 moderately correlated with

endothelial and epithelial permeability, suggesting that IL-8 may be reliably used as an in-vitro biomarker to measure the severity of inflammatory-related illnesses. In some cases, it was reported that IL-8 might not immediately reflect the actual state of endothelial/epithelial permeability but rather takes some time, even up to two days, to cause a significant rise in the permeability of the model (Soo *et al.*, 2019). This observation was attributed to low levels of secreted IL-8, usually occurring during the first post-infection day.

### 6.4.2 BID effect on IL-8 induction

The results for the effect of BID demonstrated that the ability of EAEC K2 to induce IL-8 from the DCC-2CMLs was bacterial dose-dependent (Figure 6.2). IL-8 secretion increased from the control setups to those treated with a final bacterial concentration of 3.7 $\log_{10}$ (CFU/well) by 208.4 pg/mL (4.1 folds). The IL-8 induction ability of this D-EAEC strain was further increased by 262.4, 309.4, 315.4, 325.5, 345.8 and 360.3 pg/mL, signifying 4.9, 5.6, 5.7, 5.9, 6.2 and 6.4 folds regarding the controls for the DCC-2CMLs infected with 4.7, 5.7, 6.7, 7.7, 8.7 and 9.7 $\log_{10}$ (CFU/well), respectively.

Adhesion and colonization of the gut by enteropathogens stimulate the indigenous inflammatory response systems leading to the secretion of IL-8 and other pro-inflammatory substances. Subsequently, this leads to subsequent engagement of neutrophils together with other inflammatory cells. Occasionally, prolonged, and massive intrusion of neutrophils can cause perpetual inflammation, eventually resulting in cell damage, deterioration of epithelial barrier and function with the onset of diarrhoea. Our results demonstrate that the selected LAB did not trigger IL-8 secretion from the PCC-2CMLs. Additionally, about $10^8$ CFU/mL LAB dose has proved effective in preventing the IL-8 secretion by the PCC-2CMLs, agreeing with previous findings (Lodemann *et al.*, 2015; Yu *et al.*, 2015). These results demonstrate that the selected LAB may prevent enteropathogens from inducing

IL-8 secretion. Reports indicate that enteropathogen-triggered gut inflammation changes the microbiome's composition and stability and interrupts colonization resistance whilst promoting the

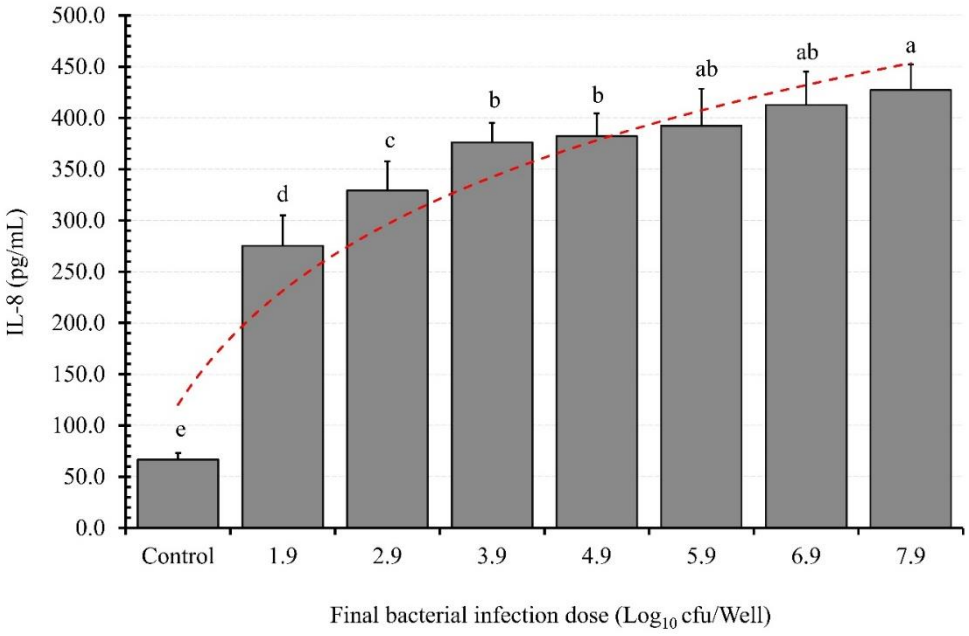


Figure 6.2: The effect of bacterial (enteroaggregative *E. coli*, EAEC K2) infection dose on IL-8 induction from polarized Caco-2 cell monolayers

Each bar is a mean of two independent replicates (n=4) with its corresponding standard deviation. Bars with different letters (a-e) indicate significant differences ($P < 0.05$) according to Fisher's LSD test.

proliferation of pathogens within the gut (Brosschot and Reynolds, 2018; Stecher and Hardt, 2008). The fact that *L. plantarum* FS2 and *P. pentosaceus* D39 are competitively excluded, displaced, and inhibited D-EAEC from the intestinal epithelium suggests they can serve as promising candidates for developing functional foods. According to our expectations, but contrary to data reported from previous studies (Sharma *et al.*, 2006), our results demonstrated a proportional relationship between the BID and IL-8 response of intestinal epithelial cells (IECs). Our results illustrate that EAEC induced the IL-8 secretion from the PLCC-2CMLs. Thus, pathogen adhesion to the epithelial cells increased with BID resulting in elevated secretion of proinflammatory cytokines agreeing with previous findings by Lodemann *et al.* (2015).

### 6.4.3 Bacterial infection mode and TT effects on IL-8 secretion

Our results (Figure 6.3) indicate that, unlike the untreated DCC-2CMLs and those treated with *L. plantarum* FS2, the ability of EAEC K2 to induce the secretion of IL-8 was significantly affected ($P < 0.05$) by TT (incubation time). Contrary to the LAB, the IL-8 induction ability of EAEC K2 demonstrated a progressive increment after the 8$^{th}$, 12$^{th}$, 16$^{th}$, 20$^{th}$, 24$^{th}$, and 28$^{th}$ treatment hours by 0.5, 1.2, 2.1, 2.4, 3.0, and 3.3 folds, respectively. The BCEFA results demonstrated continuous increments from the 4$^{th}$ to the 8$^{th}$ and 12$^{th}$ hours by 0.4 and 0.9 folds, after which the rate of increment declined from the 16$^{th}$, 20$^{th}$, 24$^{th}$, and 28$^{th}$ hour by 0.7, 0.6, 0.4 and 0.3 folds concerning the 4$^{th}$ hour, respectively. The BDFA results also demonstrated increments with IL-8 secretion from the 4$^{th}$ to the 12$^{th}$ hour by additional 0.7 folds. The IL-8 gradually reduced up to the 28$^{th}$ h by additional 0.3 folds regarding the 4$^{th}$ h. Similarly, BIFA results showed a gradual increase in IL-8 secretion up to the 12$^{th}$ h by 0.7 folds which gradually reduced up to the 28$^{th}$ h by 0.4-fold regarding the 4$^{th}$ h. However, from the 4$^{th}$ to the 12$^{th}$ h, BCEFA and BDFA did not reduce but increased IL-8 secretion due to pathogen virulence. This observation could be explained by the fact that the IL-8 induction reduction effect needed a relatively longer time than these. These two modes of infection rather reduced the IL-8 secretion from the 16$^{th}$ to the 28$^{th}$ hour.

IL-8 induction during the BIFA was quite different from the two previous modes of bacterial infection. No difference was detected between EAEC K2 and *Lactobacillus plantarum* FS2, followed by EAEC K2 to induce IL-8 secretion from PCC-2CMLs after the 8$^{th}$ infection hour. The earlier infection of the DCC-2CMLs with the LAB before the EAEC K2 can explain this result. This infection mode enabled the LAB to confer protection from the pathogen virulence and keep IL-8 secretion in check. From the 12$^{th}$ to the 28$^{th}$ h, this infection mode consistently reduced the IL-8 secretion effect of EAEC K2. Our results confirmed previous findings that reductions in epithelial barrier function caused by enteropathogens virulence followed by IL-8 secretion are dependent on BID and TT (Lodemann *et al.*, 2015; Yang *et al.*, 2018b; Yu *et al.*, 2013). A study involving coinfection of *Anaplasma*

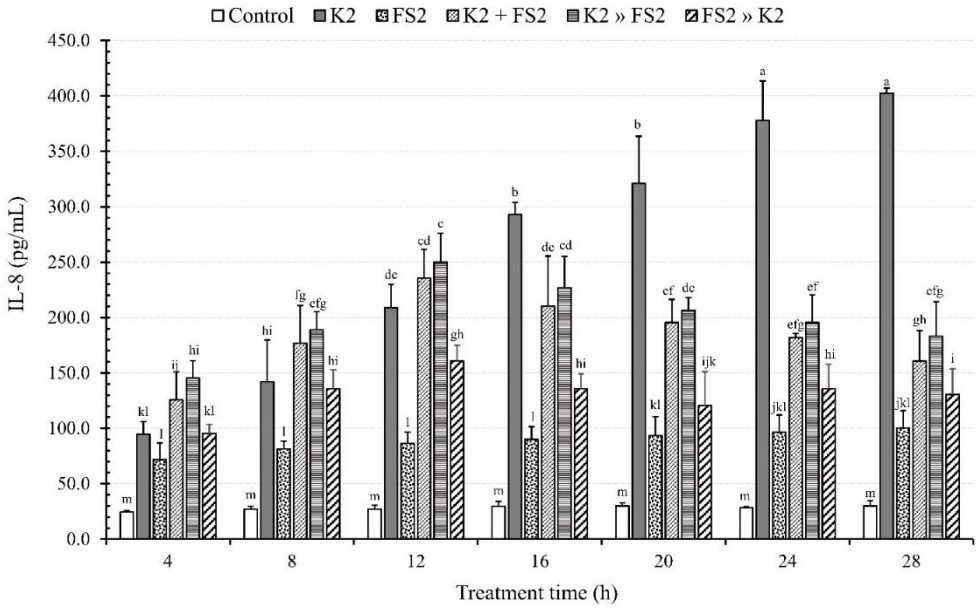


Figure 6.3: The effect of bacterial (enteroaggregative *E. coli*, EAEC K2 and *L. plantarum* FS2) infection mode and treatment time on IL-8 induction from polarised Caco-2 cell monolayers

Each bar is a mean of two independent replicates (n=4) with its corresponding standard deviation. Bars with different letters (a-m) indicate significant differences ($P < 0.05$) according to Fisher's LSD test.

K2 + FS2: Bacterial competitive exclusion from adhesion between K2 and FS2,

K2 » FS2: Bacterial (EAEC) displacement from adhesion by FS2, and

FS2 » K2: Bacterial (EAEC) inhibition from adhesion by FS2

*phagocytophilum* and *B. burgdorferi* showed that secreted IL-8 levels corresponded to TEER formation, strongly indicating the function of IL-8 as a biomarker for severity measurements (Grab *et al.*, 2007). Intercellular tight junction proteins disruption coupled with increased endothelial permeability was associated with increased IL-8 secretion (Kelley *et al.*, 2012; Yu *et al.*, 2013). This finding implies that higher severity, as shown by increased endothelial permeability, could be triggered

by higher IL-8 levels, as demonstrated by most mono-infection with EAEC, than coinfection with the selected LAB. In a separate study on HIV infection, more increased IL-8 was associated with the stimulation of viral replication (Grønborg *et al.*, 2017; Lehtoranta *et al.*, 2020; Yu *et al.*, 2019). Accordingly, increased endothelial permeability was associated with higher viral titre (Dewi *et al.*, 2004), bolstering the current findings of this study.

### 6.4.4 Effects of bacterial mono-infection on TEER

Results from monoinfected DCC-2CMLs demonstrated that *B. bifidum* ATCC 11863 caused a reduction (6.2 %) in TEER for the control setups (non-infected DCC-2CMLs) from 104.6 % to 98.5 %. Contrarily, DCC-2CMLs monoinfected with *L. plantarum* FS2 or *P. pentosaceus* D39 deteriorated TEER by 9.0 and 7.3 % to 95.6 and 97.3 %, respectively (Figure 6.4). Among the EAEC strains, D-EAEC K2 declined ($P < 0.05$) TEER score to 58.6 % (by 46.0 %). This trend was followed by the clinical positive reference D-EAEC 3591-87 (-39.1, 65.5 %) and ND-EAEC N23 (-18.7, 86.0 %) being the least. Thus, our results for both EAEC and LAB strains demonstrated strain-strain dependent effects, which agrees with previous reports (Anderson *et al.*, 2010).

The state of structural and functional maintenance or variations which is directly related to the epithelial barrier integrity and permeability is instrumentally measured as TEER (Henry *et al.*, 2017; Lodemann *et al.*, 2015; Nicolas *et al.*, 2021). TEER measurement is dependent on the cellular and shunt resistances which operate in parallel. None of the three LAB showed any destructive effect but somewhat improved and maintained the epithelial barrier integrity, which agrees with previous findings (Hansen *et al.*, 2021; Lodemann *et al.*, 2015; Yang *et al.*, 2018c). In a separate study, *Lactobacillus rhamnosus* HN001, *Lactobacillus rhamnosus* L34, *Lactobacillus acidophilus*, and *L. plantarum* were reported to demonstrate a considerable reduction in pathogen virulence (Cosme-Silva *et al.*, 2019; Liu *et al.*, 2019; Panpetch *et al.*, 2018). Contrarily, *L. plantarum*, and *L. rhamnosus* were characterized to stimulate the host immune response (Han *et al.*, 2021; Kazun *et al.*, 2020). Our current study indicates that the selected LAB can improve the intercellular tight junctions.

### 6.4.5 Effects of LAB and EAEC coinfection on TEER

Our coinfection results demonstrated that the three selected LAB show varying abilities to ease the TEER level reduction caused by the EAEC deteriorative effects on the PCC-2CMLs. After the 6 h of

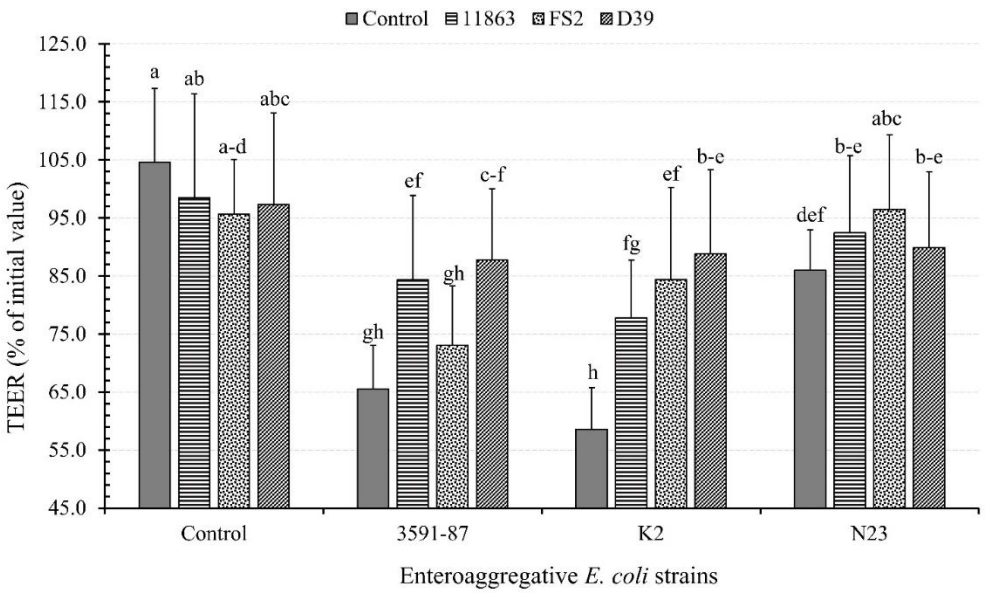


Figure 6.4: The effect of enteroaggregative *E. coli* (EAEC) and lactic acid bacteria (LAB) on transepithelial electrical resistance (TEER) of polarised Caco-2 cell monolayers

The EAEC include diarrhoeagenic 3591-87 and K2 and a non-diarrhoeagenic (N23) strains. *B. bifidum* ATCC 11863 *L. plantarum* FS2 and *P. pentosaceus* D39 constitute the lactic acid bacteria (LAB) strains. Each bar is a mean of two independent replicates (n=4) with its corresponding standard deviation. Bars with different letters (a-h) indicate significant differences ($P < 0.05$) according to Fisher's LSD test.

treatment, the PCC-2CMLs previously monoinfected with D-EAEC 3591-87 had their TEER (65.5 %) restored the most by *P. pentosaceus* D39 by a margin of 22.2 to 87.7 % (Figure 6.4). This competence was followed by *B. bifidum* ATCC 11863 (18.8, 84.4 %) and *L. plantarum* FS2 (7.5, 73.1 %). Similarly, coinfection with *P. pentosaceus* D39 restored the disrupted TEER value (58.6 %) due to EAEC K2 the most (30.2, 88.8 %), followed by *L. plantarum* FS2 (25.8, 84.4 %) and then *B. bifidum* ATCC 11863 (19.2, 77.8 %). The ND-EAEC N23, which slightly reduced TEER (86.0 %) compared to its

diarrhoeagenic counterpart, had its TEER restored the most by 10.6 % (96.5 %) when it was coincubated with *L. plantarum* FS2. This ability was followed by *B. bifidum* ATCC 11863 (6.5, 92.4 %) and *P. pentosaceus* D39 (3.9, 89.9 %). The LAB were indifferent to restoring the TEER values due to infection of the DCC-2CMLs with the ND-EAEC N23. These results were contrary to our expectations because, in our previous studies (Agbemavor and Buys, 2021), *L. plantarum* FS2 was reported to be the most adherent LAB. Therefore, it was expected to be the most competent LAB in restoring the TEER caused by disruptions by the virulent D-EAEC.

In this study, PCC-2CMLs were challenged by EAEC in the presence or absence of different adhering LAB strains. At the end of the treatment, unchallenged monolayers had their TEER values remaining virtually the same (maintained their intestinal barrier integrity). This finding agrees with previous reports (Lodemann *et al.*, 2015). Challenging the PCC-2CMLs with the two EAEC resulted in reductions in the TEER values as previously reported (Bhat *et al.*, 2019b; Lodemann *et al.*, 2015; Yuan *et al.*, 2020). The reductions in TEER signify deteriorations in intestinal barrier integrity demosntrated some strain dependent effects and to a larger extent agreeing with some previously reported findings (Lodemann *et al.*, 2015).

Reductions in TEER leading to cellular structural damage and cytokine induction varied with bacterial species and strains irrespective of pathogenicity. Scanning electron micrographs revealed that co-culturing of PCC-2CMLs with EAEC, unlike LAB for 18 h, mostly deteriorated the integral structure of Caco-2 cells (data not shown). D-EAEC 3591-87 and K2 decreased TEER of PCC-2CMLs more than their non-diarrhoeagenic counterpart, EAEC N23 and the LAB strains (Figure 6.4). The three LAB co-cultured independently with PCC-2MCLs seemed not to influence their respective TEERs over the 6 h incubation. Although cytotoxic pore formation in cells contributes to reductions in TEER, it also depends on the physiological regulation of intercellular tight junctions. The tight junction structure is maintained by claudins, occludins, and zonal occludins (ZO), ZO-1, ZO-2 and ZO-3 proteins (Gvoic *et al.*, 2021).

### 6.4.6 BID effect on TEER

BID effect on TEER was determined using EAEC K2. The results show an exponential decrease with increasing BID (Figure 6.5). Uninfected PCC-2MCLs demonstrated the highest TEER (97.3 %), indicating that the intercellular tight junctions (monolayers) were intact (epithelial barrier integrity). However, with BID (0.36 $\log_{10}$/well), the epithelial barrier integrity (TEER) dropped significantly ($P < 0.05$) by 20.8 % to 76.4 %, which further deteriorated (38.7, 58.5 %) with BID (1.36 $\log_{10}$/well). This drop continued until the epithelial barrier integrity was reduced by 95.2 % to a final TEER value

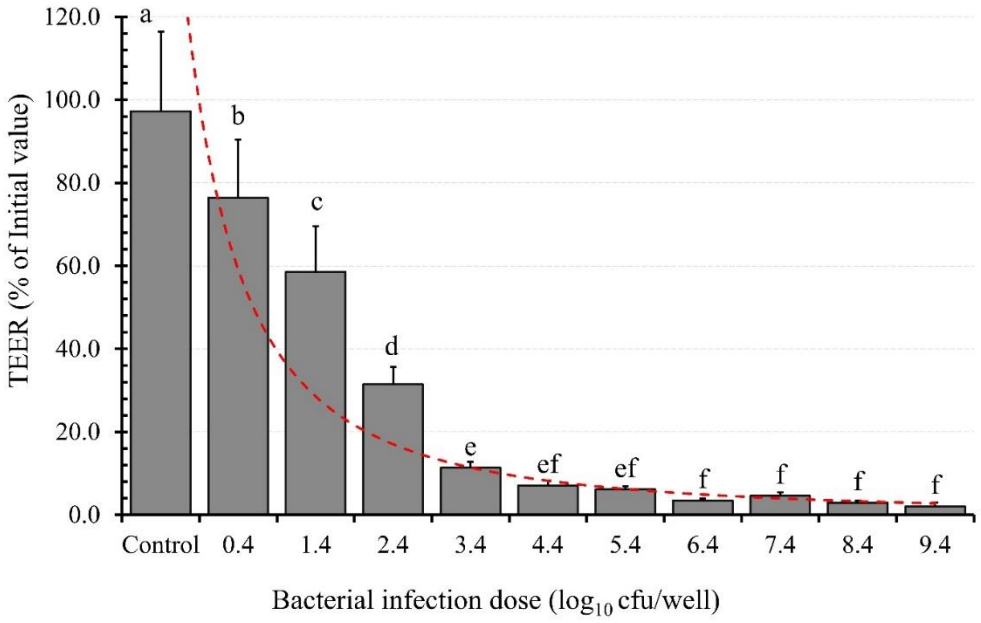


Figure 6.5: The effect of bacterial [enteroaggregative *E. coli* (EAEC) K2] infection dose on transepithelial electrical resistance (TEER) of polarized Caco-2 cell monolayers

Each bar is a mean of two independent replicates (n=4) with its corresponding standard deviation. Bars with different letters (a-f) indicate significant differences ($P < 0.05$) according to Fisher's LSD test.

(2.0 %). This study showed that epithelial barrier integrity (TEER) depends on BID. The higher the BID, the lower the TEER and the intercellular tight junctions (epithelial barrier integrity). As previously reported, bacterial adhesion is expected to increase with infection dose (Medrano *et al.*,

2009). Adherent bacteria such as the EAEC used in the current study mostly adhere to the epithelium's apical sides (Shigetomi and Ikenouchi, 2019). Therefore, as expected, TEER induction must vary with BID, as demonstrated by the results of our current study (Lodemann *et al.*, 2015).

### 6.4.7 Bacterial infection mode and TT effects on TEER

Our results show that the intercellular tight junctions of D-EAEC K2 monoinfected PCC-2CMLs deteriorated significantly ($P < 0.05$) from the 4$^{th}$ (61.4 %) to the 28$^{th}$ h (4.6 %) of treatment, signifying 40.0 to 104.5 % deviations from their controls, respectively (Figure 6.6). The different modes of coinfecting the intestinal epithelium revealed that infecting the PCC-2CMLs with *L. plantarum* FS2 an hour before the D-EAEC K2 (Figure 6.6) was mostly effective than the other two in the recovery of the intercellular tight junctions. This result was followed by the simultaneous coinfection of the two bacteria. Pre-infecting the monolayers with the D-EAEC K2 an hour before the *L. plantarum* FS2 was generally the least effective in alleviating the epithelial barrier. These results were not surprising because, with simultaneous coinfection, the LAB was introduced to commence the battle against the EAEC right from scratch. More so, by infecting the monolayers with the LAB an hour before the pathogen, the LAB would have started some prophylactic processes before the introduction of EAEC. Therefore, when the pathogen was first introduced, the therapeutic ability of the LAB might delay compared to the other two modes of infection.

The current study has further confirmed the way probiotics exert their beneficial effects whilst highlighting the competence of the selected LAB to safeguard the polarized epithelial cells from the harmful impacts of diarrhoeagenic *E. coli* at various levels. Furthermore, our results illustrated that treatment with the chosen LAB ameliorated the ravaging effects of D-EAEC on epithelial barrier integrity and functions. We also demonstrated that the selected D-EAEC caused reductions in TEER and might lead to increased epithelial permeability. The results further implied that the selected LAB might be valuable for the protection and maintenance of intercellular tight junctions and epithelial barrier integrity, as previously reported (Choi *et al.*, 2017; Lepine *et al.*, 2018).

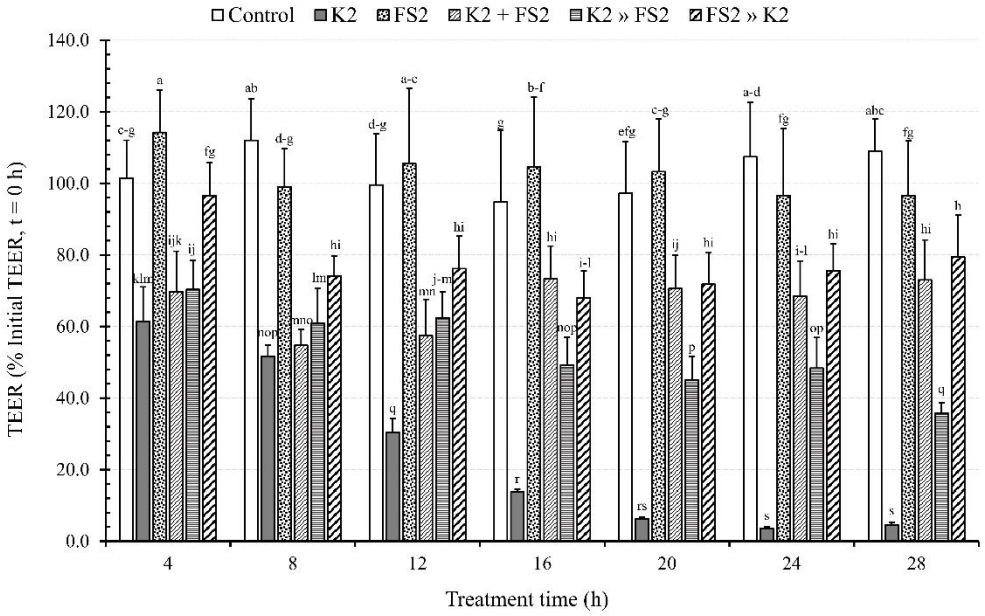


Figure 6.6: The effect of bacterial (enteroaggregative *E. coli*, EAEC K2 and *L. plantarum* FS2) infection mode and treatment time on transepithelial electrical resistance (TEER) of polarised Caco-2 cell monolayers

Each bar is a mean of two independent replicates (n=4) with its corresponding standard deviation. Bars with different letters (a-s) indicate significant differences ($P < 0.05$) according to Fisher's LSD test.

K2 + FS2: Bacterial competitive exclusion from adhesion between K2 and FS2,

K2 » FS2: Bacterial (EAEC) displacement from adhesion by FS2, and

FS2 » K2: Bacterial (EAEC) inhibition from adhesion by FS2

While several *Lactobacillus* strains were reported through clinical studies to confer beneficial health effects on their host by producing bactericidal (bacteriostatic) agents (Mao *et al.*, 2020; Yi *et al.*, 2016), regulation of immunomodulatory effects (Ferreira Dos Santos *et al.*, 2016; Vareille-Delarbre *et al.*, 2019), or competitive exclusion of pathogens (Agbemavor and Buys, 2021; Siedler *et al.*, 2020; Zuo *et al.*, 2019), their direct mechanisms of action remain unclear. The current findings indicate that *L. plantarum* FS2 and *P. pentosaceus* D39, as well as the *B. bifidum* ATCC 11863, can maintain the

intestinal barrier functions in different capacities by preventing disruptions caused by enteropathogens. This maintenance function will be achieved by upregulating PCC-2CMLs TEER whilst downregulating its permeability by limiting the secretion of inflammatory cytokines, amongst others, as previously reported (Han *et al.*, 2019; Wang *et al.*, 2018b).

### 6.4.8 Bacterial infection mode and TT effects on adhesion

Though the adhesion of the D-EAEC K2 to the DCC-2CMLs was more ($P < 0.05$) than that of *L. plantarum* FS2 in a few instances (12 and 24 h), they both demonstrated significant progressive increases with TT ranging from 25.6 – 73.2 and 24.3 – 70.9 %, respectively (Figure 6.7). This observation confirms that bacterial adhesion depends on exposure duration (time). Primarily, gastrointestinal infections result from compositional and functional disorders of the human gut microbiome (Feng *et al.*, 2018; Lobionda *et al.*, 2019; Quigley, 2020; Zhu *et al.*, 2020). Probiotics form an integral part of the gut microbiome and thereby affecting its composition and function whilst mainly contributing to the maintenance of human health. The application of novel alternative treatments and preventive techniques like probiotics have become indispensable, especially with the advent of antibiotic-resistant pathogens (Davoodabadi *et al.*, 2015a; Pamer, 2016). Our results indicate that the three probiotics have different competitive exclusion, displacement, and inhibitory competencies against the selected EAEC, confirming earlier reports (Agbemavor and Buys, 2021; Pazhoohan *et al.*, 2020b; Tran *et al.*, 2018).

The DCC-2CMLs were treated with the two bacteria (D-EAEC K2 and *L. plantarum* FS2) during the three infection modes; BCEFA, BDFA, and BIFA. Throughout the different instances, *L. plantarum* FS2 competitively excluded EAEC K2 from adhesion ($P < 0.05$) with progressively increasing margins of 6.1, 11.4, 12.1, 19.6, 21.6, 27.8, 31.7 and 44.0 % for the 2, 4, 8, 12, 16, 20 24 and 28 h, respectively (Figure 6.7). Our results further showed that except for 8 h, LAB increasingly displaced EAEC K2 with TT. Thus, after 2, 4, 8, 12, 16, 20, 24, and 28 h, LAB expelled EAEC K2 by 5.5, 20.7, 16.9, 24.8, 24.8, 34.2, 40.0 and 54.0 %, respectively. The LAB's ability to displace was generally

higher than that for competitive exclusion of EAEC. These results contradicted our expectations and are quite difficult to explain. However, without the competing LAB, the EAEC cells relaxed their rate of adhesion to the epithelial cells than in the presence of the LAB, as confirmed previously (Agbemavor and Buys, 2021). Infecting the DCC-2CMLs with *L. plantarum* FS2 an hour before the EAEC K2 resulted in the progressive exclusion of the pathogen with TT. The LAB inhibited

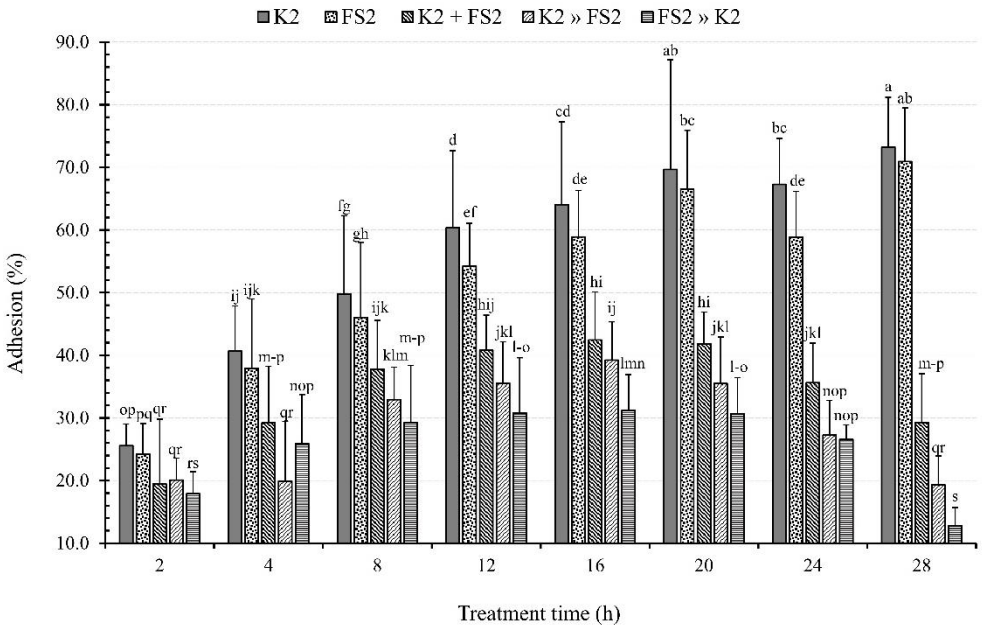


Figure 6.7: The effect of infection mode and treatment time on the adhesion of enteroaggregative *E. coli*, (EAEC), K2 and *L. plantarum* FS2 to polarised Caco-2 cell monolayers

Each bar is a mean of two independent replicates (n=4) with its corresponding standard deviation. Bars with different letters (a-s) indicate significant differences ($P < 0.05$) according to Fisher's LSD test.

K2 + FS2: Bacterial competitive exclusion from adhesion between K2 and FS2,

K2 » FS2: Bacterial (EAEC) displacement from adhesion by FS2, and

FS2 » K2: Bacterial (EAEC) inhibition from adhesion by FS2

the EAEC K2 by 7.7, 14.7, 20.6, 29.6, 32.9, 39.0, 40.7 and 60.4 % after 2, 4, 8, 12, 16, 20, 24, and 28 h, respectively. The magnitude of EAEC K2 inhibited from adhesion was in all instances more than

those competitively excluded and displaced from adhesion which agrees with previous reports (Gueimonde *et al.*, 2006). Thus, our results demonstrate that the competence of the selected LAB to competitively exclude, displace, and inhibit the tested pathogens depend on the pathogen strains tested, which tallies with previous reports (Agbemavor and Buys, 2021; Davoren *et al.*, 2019; Gharbi *et al.*, 2019).

### 6.4.9 Exploration of possible linear relations between different variables

Even though bacterial adhesion generally decreases with TEER, our results indicate that, unlike the D-EAEC K2, exhibiting a powerful negative correlation [correlation coefficient (R) = 0.9746; $P < 0.05$],

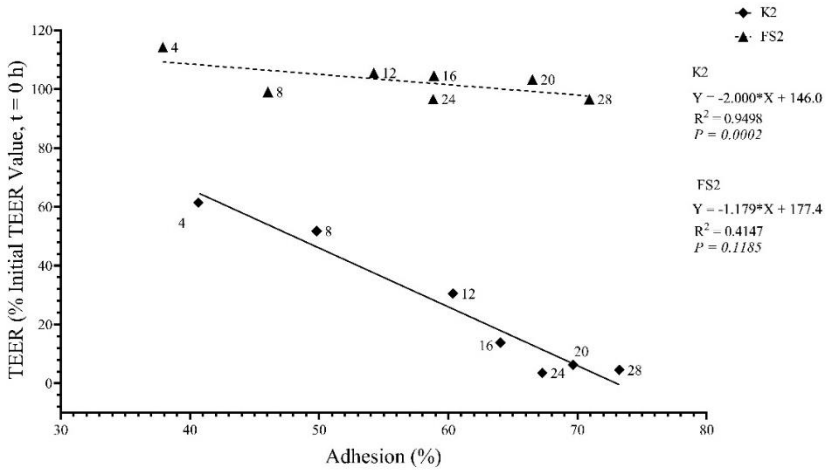


Figure 6.8: Exploration of relationship between bacterial (enteroaggregative *E. coli*, EAEC K2 and *L. plantarum* FS2) adhesion and transepithelial electrical resistance (TEER) of Caco-2 monolayers

Each data point is a mean of two independent replicates (n=4)

*L. plantarum*, whereas FS2 showed no correlation [R = 0.6440; $P > 0.05$], between adhesion and TEER variables (Figure 6.8). This finding partly agrees with previous studies (Pilkington *et al.*, 2014). Interestingly, both bacteria showed some linearity between adhesion and IL-8 secretion. This finding confirmed the evidence of powerful positive correlations between adhesion abilities and IL-8 secretion (R = 0.9552; $P < 0.05$) and (R = 0.9546; $P < 0.05$) as demonstrated by EAEC K2 and *L. plantarum*,

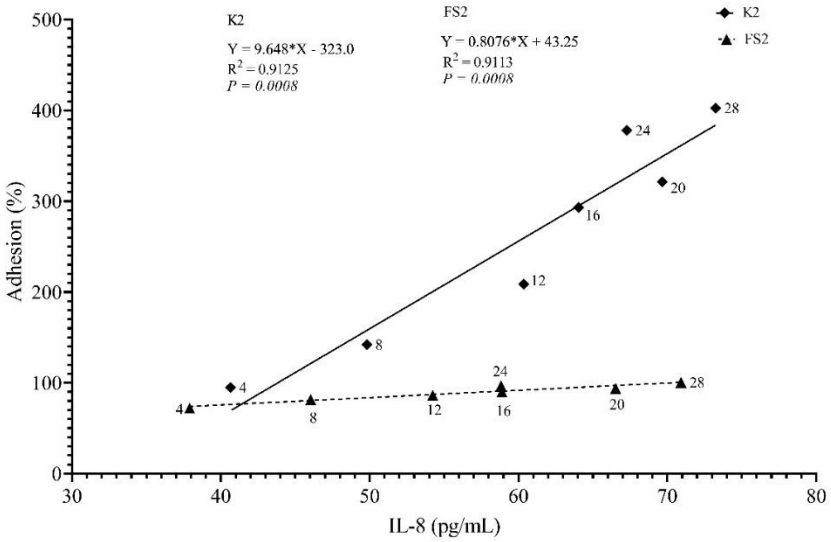


Figure 6.9: Exploration of relationship between bacterial (enteroaggregative *E. coli*, EAEC K2 and *L. plantarum* FS2) adhesion and interleukin 8 (IL-8) induction of Caco-2 monolayers

Each data point is a mean of two independent replicates (n=4)

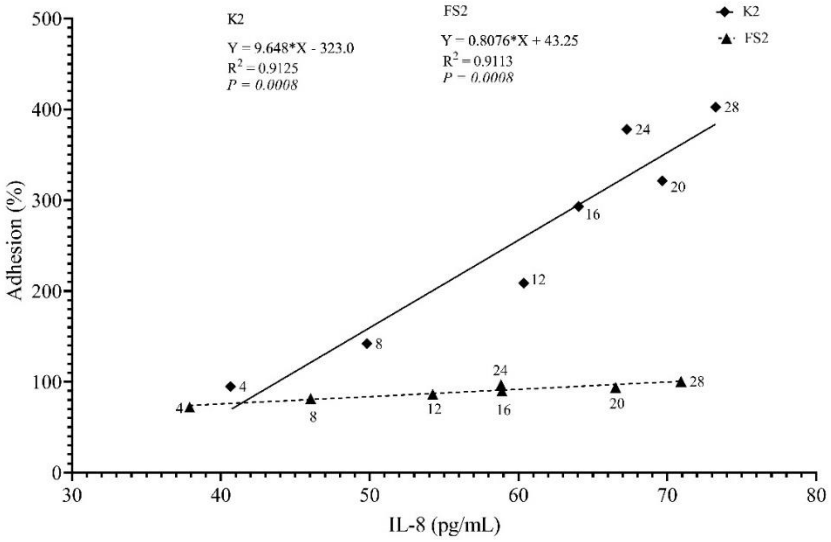


Figure 6.10: Exploration of relationship between transepithelial electrical resistance (TEER) and interleukin 8 (IL-8) induction of Caco-2 monolayers

Each data point is a mean of two independent replicates (n=4).

respectively (Figure 6.9). However, our findings disagreed with previous reports (Morita *et al.*, 2002). Furthermore, both EAEC K2 and *L. plantarum* FS2 demonstrated the induction for copious secretion of IL-8 for instances of low TEER values and vice versa, as witnessed by a strong negative (R = 0.9740;

*P* < *0.05*) and a strong negative (R = 0.7906; *P* < *0.05*) correlations by EAEC K2, and *L. plantarum*, respectively (Figure 6.10). To the best of our knowledge, no study reported the possible correlation between IL-8 induction and TEER.

## 6.5 Conclusion

*L. plantarum* FS2 and *P. pentosaceus* D39 can offer valuable biotechniques for preventing and treating gastrointestinal infections. Microbial communities from fermented (cereal) foods like animals may serve as an essential pool for specific microbes such as LAB with vital characteristics qualifying them as probiotic candidates for prophylactic and therapeutic management of a wide range of diseases for both humans and livestock. Beneficial microbes like these may possess and exert different valuable health impacts, such as the exclusion of pathogen proliferation and function within their host. They may also stimulate the host's immune response and function by improving their intestinal barrier integrity. By diverse mechanisms of probiosis, the probiotics may produce varying positive effects at different stages. When administered, probiotics can counteract the incidence of various gastrointestinal diseases. They may also suppress the duration and severity of disorders during treatment. The advancement of knowledge and skillset in the human microbiome, coupled with the logical selection of probiotics based on their established mechanisms of action, can optimize, and strategize their therapeutic management. Eventually, probiotics are expected to boost microbial communities' stability and diversity to prevent diseases and improve human health.

# CHAPTER 7.

## RESEARCH CHAPTER 4

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**Potential probiotics, *Lactobacillus plantarum* (FS2) and *Pediococcus pentosaceus* (D39) inhibit enteroaggregative *Escherichia coli* impaired Caco-2 cells viability and permeability**

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# Potential probiotics, *Lactobacillus plantarum* (FS2) and *Pediococcus pentosaceus* (D39) inhibit enteroaggregative *E. coli* impaired Caco-2 cells viability and permeability

## 7.1 Abstract

The application of probiotics as preventive medicine is emerging as an indispensable tool in managing foodborne infections and inflammatory bowel syndromes. The current study aimed to evaluate the *in vitro* prophylactic and therapeutic abilities of two lactic acid bacteria (LAB), (*Lactobacillus plantarum* FS2 and *Pediococcus pentosaceus* D39) against diarrhoeagenic enteroaggregative *Escherichia coli* (EAEC) induced damage to intestinal epithelial barrier function using Caco-2 cells. Intestinal cells exposed to EAEC demonstrated very low trans-epithelial electrical resistance (TEER) levels (2.50 ± 0.05 $\Omega.cm^2$) coupled with significantly higher ($P < 0.01$) phenol red flux levels contrary to controls and LAB ($10^9$ CFU/mL) treated cells. Nevertheless, the EAEC-induced hyperpermeability was significantly restored when the EAEC were competitively excluded, displaced, and inhibited by the two potential probiotic LAB. Meanwhile, significantly high numbers of EAEC cells (0.8 – 3.8 $\log_{10}$ CFU/ml) were recorded translocating across the differentiated Caco-cells when challenged with the former. In conclusion, *L. plantarum* FS2 and *P. pentosaceus* D39 restrained and restored EAEC-impaired intestinal barrier function by improving the expression and distribution of important tight junction proteins. Thus, they can be applied as indispensable food supplements and additives to address different diseases, particularly gut-related ones.

## 7.2 Introduction

Inflammatory bowel syndromes constitute significant public health concerns since they constitute the underlying factors of morbidity and mortality. Diarrheal diseases accounted for over 1.6 million deaths globally in 2016, out of which 27 % involved children below five years of age (Troeger *et al.*, 2018a). The principal aetiological agents of gastroenteritis include Calicivirus, Rotavirus and diarrhoeagenic *Escherichia coli*. The diarrhoeagenic *E. coli* were characterised into different pathotypes based on their

spectrum of pathogenicity, clinical symptoms, and combinations of various virulence factors and pathogenic mechanisms (Croxen *et al.*, 2013). Among these diarrhoeagenic *E. coli* pathotypes, enteroaggregative and enteropathogenic were reported to be mostly associated with diseases and account for 30 to 40 % diarrheal incidents among low and middle income countries (Raghavan *et al.*, 2017).

Enteroaggregative *Escherichia coli* (EAEC) is associated with inflammatory bowel syndrome as reported in numerous clinical symptoms (Harrington *et al.*, 2006; Harrington *et al.*, 2005). Several epidemiologic studies indicate that EAEC was the most frequently occurring bacterial pathogen among diarrheal patients across all ages (Nataro *et al.*, 2006). EAEC pathogenesis commences with adherence to the intestinal epithelium, followed by secretion of cytotoxins and enterotoxins. The adhesion of EAEC to human intestinal cells is mostly mediated by aggregative adhesive fimbriae (AAF) which is similar to adhesins of diarrhoeagenic and uropathogenic *E. coli* (Nataro, 2005). EAEC just like several enteric pathogens induce IL-8 production and secretion of interleukin 8 (IL-8) leading to decrease in transepithelial electrical resistance (TEER) in polarized epithelial cells. Epithelial barrier (function) disruption may result in direct secretion of electrolytes and fluids which is popularly known as the leak-flux model (Ma *et al.*, 2006; Schulzke *et al.*, 2009; Simonovic *et al.*, 2000) contributing to malabsorption and mucosal protein loss. This can enable enteric pathogens that desire this path of entry to access the basolateral compartment. One of our previous chapters reported the persistent loss of epithelial barrier integrity complemented by delocalisation of tight junction proteins as a results EAEC pathogenesis.

The gastrointestinal tract of human adults is customarily inhabited by *Lactobacillus* and *Bifidobacterium* species. The microbial strains from these genera are commonly found in dairy products out of which some are characterised as probiotics because of their abilities to exert certain physiological functions within the gut of their host (Dunne *et al.*, 1999; Isolauri *et al.*, 2004). Regardless of their wide range of applications, their underlying mechanisms of action have not been

fully understood. The gut epithelium was, however, firmly believed to play a significant role in the organisation of the induced effects (Ma *et al.*, 2004). Multiplex interactions transpire between the probiotics and gut ecosystem (prevailing microflora and immune and epithelial cells) (Umesaki and Setoyama, 2000). The development and maintenance of gut activities heavily depend on these interactions.

Like functional foods, probiotics are generally regarded as safe. Despite several studies involving LAB strains, none reported any likelihood of toxicity towards epithelial cells, unlike their enteric pathogen counterparts (Er *et al.*, 2015; Messaoudi *et al.*, 2012). Most of these probiotics were noted for maintaining the intercellular tight junctions and, for that matter, epithelial integrity, and functions (Chaiyasut *et al.*, 2022; Wang *et al.*, 2018b). Safety concerns involving probiotic bacteria for the use of mankind has been subjected to numerous reviews by authorities in food safety (Adams, 1999; Bernardeau *et al.*, 2006; Salminen *et al.*, 1998). Based on their long history of safe use as dietary and food supplements, some of these reviews embrace the suitability and safety some LAB species for consumption as oral probiotics.

This study aimed to evaluate the abilities of *L. plantarum* FS2 and *Pediococcus pentosaceus* D39 isolated from a West-African fermented cereal, *ogi*, to mitigate cytotoxicity and epithelial barrier permeability due to the pathogenetic effects of selected diarrhoeagenic enteroaggregative *E. coli* (D-EAEC) strains from unpasteurized fresh milk.

## 7.3 Materials and Methods

### 7.3.1 Bacterial strains and culture conditions

Two selected diarrhoeagenic (3591-87 and K2) and a non-diarrhoeagenic *E. coli* (ND-EAEC), N23 strains (Table 7.1); were isolated from unpasteurised fresh milk (except for 3591-87 being a positive clinical reference control) were used in this study (Aijuka *et al.*, 2018; Ntuli *et al.*, 2017). Two LAB, *L. plantarum* FS2 and *P. pentosaceus* D39, with some potential probiotic characteristics, were obtained

out of the several isolates from a previous study involving traditional non-alcoholic fermentation of maise for the production of a West African gruel (*ogi*) from an earlier study (Fayemi and Buys, 2017; Fayemi *et al.*, 2017). The other LAB (probiotics) used in this study and their culturing conditions were described (Table 7.1).

Table 7.1: Sources and characteristics of selected enteroaggregative *E. coli* (EAEC) and lactic acid bacteria (LAB) strains used in this study

| Bacteria strain | Characteristic | Source |
|---|---|---|
| EAEC 3591-87 | Clinical and diarrhoeagenic (positive reference strain) | [a]NICD of NHLS |
| EAEC K2 | Diarrhoeagenic | [b]Unpasteurised fresh milk |
| EAEC N23 | Non-Diarrhoeagenic | [b]Unpasteurised fresh milk |
| *Bifidobacterium bifidum* ATCC 11863 | Reference probiotic bacteria | [c]ATCC Collections |
| *Lactobacillus plantarum* FS2 | Promising probiotic characteristics | [d]Traditional fermented food (*ogi*) |
| *Pediococcus pentosaceus* D39 | Promising probiotic characteristics | [d]Traditional fermented food (*ogi*) |

[a]National Institute for Communicable Diseases (NICD), a division of the National Health Laboratory Service (NHLS), Johannesburg, the Republic of South Africa.

[b]Previously isolated by Aijuka *et al.* (2018) and Ntuli *et al.* (2017).

[c]American Type Culture Collection (ATCC, USA).

[d]Previously isolated by Fayemi and Buys (2017) Fayemi and Buys (2017).

### 7.3.2 Cell culturing and maintenance conditions

Human epithelial intestinal cells from colorectal adenocarcinoma, Caco-2 (ATCC catalogue number HTB-37, Maryland, USA) was obtained and sustained in Dulbecco's modified Eagle medium (DMEM; Gibco, ThermoFisher, USA) containing 4 500 mg/L D-glucose, non-essential amino acids, 110 mg/L of sodium pyruvate as described earlier (Agbemavor and Buys, 2021). Briefly, the media was supplemented with 10% (v/v) gamma-irradiated, heat-inactivated foetal bovine serum (FBS, Gibco)

with 1% penicillin-streptomycin. The cells were primarily cultivated in T75 (75 cm2) cell culture flasks [with catalogue number (CN), "658940" (Greiner Bio-One GmbH, Frickhausen, Germany)] and sub-cultured (60 to 70 % confluence) into a ratio of 1:3 followed by incubation (37 ℃, 5% CO2) in a $CO_2$ humidified (95 % air) incubator (Healforce, HF 212UV, China). The cells

Table 7.2: Treatment template of enteroaggregative *E. coli* (EAEC) with lactic acid bacteria (LAB)

| LAB Bacteria Strains | EAEC Strains | | |
|---|---|---|---|
| | 3591-87 | K2 | N23 |
| 11863 | 3591-87 + 11863 | K2 + 11863 | N23 + 11863 |
| FS2 | 3591-87 + FS2 | K2 + FS2 | N23 + FS2 |
| D39 | 3591-87 + D39 | K2 + D39 | N23 + D39 |

Note: The EAEC strains include 3591-87, K2, K3, K16, and N23 and the LAB include *L. acidophilus* ATCC 4356 and *Bifidobacterium bifidum ATCC* 11863, *L. plantarum* FS2, and *P. pentosaceus* D39, respectively.

were subcultured every 3-5 days after trypsinisation [0.5 % trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA), ThermoFisher, USA]. The resulting monolayers were considered polarised when their TEER value was at least 1000 $\Omega.cm^2$. Therefore, this study used polarised Caco-2 cell monolayers (PCC-2CMLs) with TEER values from ~1000 to ~2000 $\Omega$ $cm^2$ were used for this study based on previous studies (Aijuka *et al.*, 2019; Karimi *et al.*, 2018a).

### 7.3.3 Trans-Epithelial Electrical Resistance (TEER) assay

TEER of Caco-2 monolayers was assessed by following procedures previously described (Aijuka *et al.*, 2019) with a few modifications. Electrical resistance across the monolayers was measured using a Millicell ERS-2 electrode (MERSSTX01) volts/ohmmeter resistance system (Millipore Corporation, Bedford, MA, USA). Background (blank) resistance ($R_b$) was measured for wells with cell culture inserts containing medium (serum- and antibiotic-free). This resistance value ($R_a$) was subtracted from the initial and final resistance readings to obtain the net resistance. A product of the net resistance and the cell culture inserts membrane's effective surface area (cm2) constitutes the TEER (Equation 7.1).

PCC-2CMLs with TEER values under 1000 Ω cm2 were excluded because they might have high permeabilities. Caco-2 cells within passages 39-40 were used in all experiments. The cell cultures were routinely examined and confirmed to be void of bacterial and mycoplasma contaminations before their usage for all experiments. At least two hours before the onset of the various experiments, the cells were nourished with serum- and antibiotic-free medium.

Equation 7.1: Trans-epithelial electrical resistance (TEER)

$$TEER = (R_a - R_b) \times A_{CCIM}$$

### 7.3.4 Cell viability assay

Metabolic activity of cells was evaluated by adopting in vitro colourimetric 3-(4,5-dimethylthiazole 2-yl)-2,5-phenyl tetrazolium bromide (MTT) assay (Garcia-Gonzalez *et al.*, 2018). This protocol was modified slightly to assess the potential cytotoxic effects of selected EAEC strains on human intestinal cells. Briefly, human adenoma carcinoma (Caco-2) cells were seeded (4.0 x $10^2$ cells / well, 96-well plates) and incubated (37 ℃, 5% CO2, 48 h) to confluent. The cell culture medium was removed and replaced with sterilized phosphate buffer saline (PBS). The Caco-2 cells were treated with standardized EAEC or LAB (1 x $10^8$ CFU/ml) individually or in combination (Table 7.2) in different challenge modes including (i) bacterial competitive exclusion from adhesion (BCEFA) during which the Caco-2 cells were infected with both EAEC and LAB simultaneously and incubated (37 ℃, 5% CO2, 8 h), (ii) bacterial displacement from adhesion (BDFA): where the Caco-2 cells were first infected with EAEC and incubated (37 ℃, 5% CO2, 4 h) followed by infection with LAB and incubation (37 ℃, 5% CO2, 4 h), and (iii) bacterial inhibition from adhesion (BIFA): where the Caco-2 cells were initially infected with LAB, incubated (37 ℃, 5% CO2, 4 h) followed by EAEC and incubation (37 ℃, 5% CO2, 4 h). The bacterial cells were lysed at the end of the incubation period by substituting the PBS with sterile distilled water (100 µL) to eliminate living bacterial cells which might interfere with the interpretation of experimental results. This procedure was followed by adding MTT (5 mg/mL, 10 µL)

with 100 µL of PBS to each well and incubated (37 °C, 5% CO2, 3 h). The supernatants were removed, completely dissolving the formazan crystals by adding acidified isopropanol (100 µL). The 96-well plate was shaken (10 min), after which the Optical Densities (ODs) of the wells were taken at 570 and 630 nm using a filter-based multi-mode microplate reader (FLUOstar Omega, BMG LabTech, Ortenberg Germany). Provisions were made for blank wells (without Caco-2 cells) and control wells (without bacterial cells) to eliminate background noise for absorbance readings and provide blank measurements. The data obtained was expressed as a percentage of cellular metabolic activity according to Equation 7.2.

Equation 7.2: Percentage cell viability based on cellular metabolic activity

$$Cell\ viability = \left[\frac{OD_{TS}}{OD_{NC}} \times 100\right]$$

Where:

$OD_{TS}$ is the optical density of tested sample (epithelial cells with bacteria) and

$OD_{NC}$ is the optical density of negative control (epithelial cells without bacteria).

### 7.3.5 Intestinal epithelial barrier permeability and translocation assays

#### *7.3.5.1 Intestinal epithelial barrier permeability assay*

The effects of EAEC and LAB on intestinal barrier permeability were assessed by phenol red (dye) flux and trans-epithelial electrical resistance (TEER) assays using Caco-2 cells. These assays were carried out as previously outlined (Bhat *et al.*, 2019c). Briefly, Caco-2 cells were seeded [1 x $10^5$ cells/well (24mm, 0.4 µm pores; Coaster Corning, NY, USA)] and were allowed to grow and differentiate (2.5 x $10^6$ cells/well) for 18 days, post-confluence. Prior to challenging the differentiated monolayers with EAEC or LAB, the cell culture medium was replaced with one without FBS and antibiotics, after which the Caco-2 cell monolayers (CC-2CMLs) were checked for their integrity by

phenol red diffusion (Bhat *et al.*, 2019c) and TEER (Aijuka *et al.*, 2019; Barnett *et al.*, 2016) assays. The experiment was carried out in three infection modes as described earlier (Section 7.3.4).

#### *7.3.5.2 Phenol red flux assay*

This assay was carried out with differentiated Caco-2 cell monolayers cultured on collagen-treated Transwell cell culture inserts. The apical and basolateral chambers of the cell culture insert with the polarised Caco-2 cell monolayers were rinsed with phosphate-buffered saline (PBS). The rinsing was followed by adding the 1.5 mL of phenol red with DMEM (0.16 g/L, 1.5 mL, Sigma Aldrich) to the upper chamber, whilst the same volume of phenol red-free DMEM was added to the lower chamber. The bicameral system was incubated (37 °C, 5 % $CO_2$, 1 h) in a humidified incubator. The medium from the basolateral compartment was mixed with NaOH (1 N, 20 µL), and its final absorbance (100 µL) was measured (558 nm) as a measure of the integrity of the Caco-2 cell monolayers. The amount of phenol red diffused across the PCC-2CMLs was expressed as a percentage diffusion, and wells with less than 1 % phenol red flux were selected for the experiments. PCC-2CMLs were challenged with standardised EAEC ($1x10^8$ CFU/well) (Table 7.2) as inflammatory agents to determine LAB ($1x10^9$ CFU/well)]) effects on intestinal cell monolayer integrity, as described earlier for the different bacterial infection modes (Section 7.3.4) and reported (Bhat *et al.*, 2019a; Bhat *et al.*, 2019b; Saliganti *et al.*, 2015). The set-up was incubated (37 °C, 5 % $CO_2$, 6 h). The optical densities (absorbance readings, 558 nm) of the media (containing the phenol red) from the apical chamber of the cell culture inserts before and after the treatments were measured as described earlier. These data were used to deduce the per cent change in the phenol red flux (permeability) across the Caco-2 cell monolayers.

#### *7.3.5.3 Bacterial translocation assay*

This assay was carried out as previously outlined (Clark *et al.*, 2005). Briefly, this experiment was carried out simultaneously as an extension to the intestinal barrier permeability assay as previously described (section 7.3.5.1). The bacteria (EAEC) cells translocated from the apical chamber into the

basolateral chamber were enumerated by serial dilution and plating on appropriate agar and then expressed as CFU/ml in % to the initial microbial concentration.

### 7.3.6 Data analysis

Analysis of variance (ANOVA) with the Fisher's least significant difference (LSD) test for multiple comparisons at 95 % confidence level was used to compare the different response variables for the various treatments. The analysis was performed using Statgraphics Centurion XVI (Statpoint Technologies Inc, 2020). The data were presented in bar charts. All experiments were independently carried out in triplicates with at least four internal replicates to cater for intra-assay variations. All experiments were conducted independently in triplicates and each experiment was repeated at least three times to cater for likely intra-assay variation.

## 7.4 Results and discussion

### 7.4.1 Viability of Caco-2 cells

Epithelial cell viability is of utmost importance to infection studies. Therefore, Caco-2 cells were incubated with *Bifidobacterium bifidum* ATCC 11863, *L. plantarum* FS2, and *P. pentosaceus* D39 to determine their apoptotic or proliferative cellular response to selected enteroaggregative *Escherichia coli* (EAEC) strains. The effects of LAB and EAEC on the viability of the epithelial cells were conducted in three different modes as described earlier (Section 7.3.4). Regardless of the infection mode adopted and the EAEC diarrhoeagenic status, all the EAEC strains reduced Caco-2 cell viability (Figure 7.1, Figure 7.2, and Figure 7.3).

During the BCEFA, the D-EAEC K2 had the severest apoptotic effect (57.9 %) on Caco-2 cells, followed by the positive clinical reference, D-EAEC 3591-87 (52.5 %) (Figure 7.1). Among the EAEC strains, the ND-EAEC and N23 caused the least reduction (35.4 %) in epithelial cell proliferation. To determine the effects of our selected potential probiotic LAB, they were coincubated with the EAEC-challenged Caco-2 cells. Per our results, there were no differences ($P > 0.05$) among the three possible

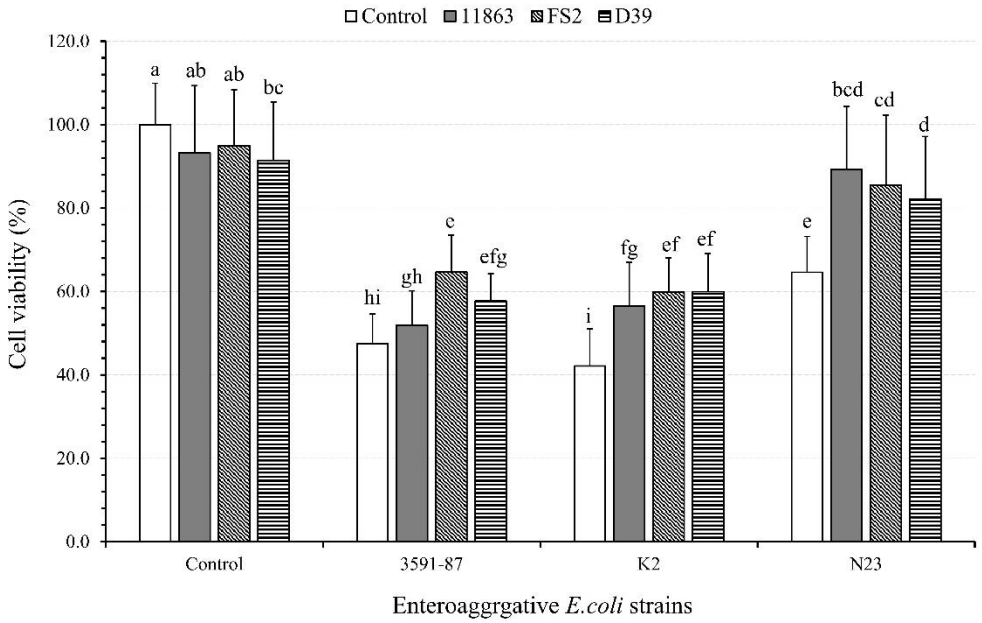


Figure 7.1: The effects of enteroaggregative *E. coli* (EAEC) and lactic acid bacteria (LAB) on Caco-2 cell viability after bacterial competition from adhesion assay

The EAEC include diarrhoeagenic (3591-87, and K2) and a non-diarrhoeagenic (N23) strains. *B. bifidum ATCC* 11863; *L. plantarum* FS2 and *P. pentosaceus* D39 constitute LAB strains. Each bar is a mean of three independent replicates (n=18) with its corresponding standard deviation. Bars with different letters (a-i) indicate significant differences ($P \leq 0.05$) according to Fisher's least significance difference (LSD) test.

LAB in their competence against the selected EAEC in reducing Caco-2 cell death (apoptosis). *L. plantarum* FS2 reduced Caco-2 cell death following treatment with D-EAEC 3591 to the highest order (17.1 %), followed by *P. pentosaceus* D39 (10.2 %) and *B. bifidum* ATCC 11863 (4.4 %). Both *L. plantarum* FS2 and *P. pentosaceus* D39 reduced epithelial cell death following treatment with D-EAEC K2 to the highest order (17.8 %), followed by *B. bifidum* ATCC 11863 (14.3 %). Caco-2 cells treated with ND-EAEC N23 also had their death margin decline by 24.7, 20.9 and 17 % when coincubated with *B. bifidum* ATCC 11863, *L. plantarum* FS2 and *P. pentosaceus* D39, respectively. With the BDFA however, the selected EAEC 3591-87, K2 and N23 caused cytotoxicity of Caco-2 cells by 53.4, 52.9 and 27.3 %, respectively. Thus, ND-EAEC N23 caused the least apoptosis of Caco-

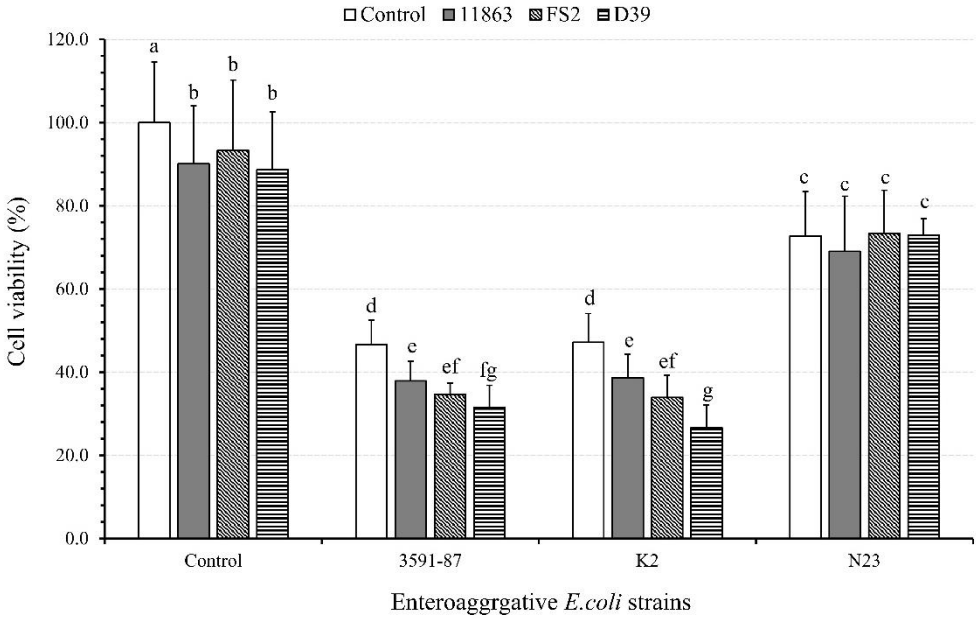


Figure 7.2: The effects of enteroaggregative *E. coli* (EAEC) and lactic acid bacteria (LAB) on Caco-2 cell viability after bacterial displacement from adhesion assay

The EAEC include diarrhoeagenic (3591-87, and K2) and a non-diarrhoeagenic (N23) strains. *B. bifidum ATCC* 11863; *L. plantarum* FS2 and *P. pentosaceus* D39 constitute LAB strains. Each bar is a mean of three independent replicates (n=18) with its corresponding standard deviation. Bars with different letters (a-g) indicate significant differences ($P \leq 0.05$) according to Fisher's LSD test.

2 cells, whereas there was no difference between D-EAEC 3591-87 and K2 to induce cell apoptosis (Figure 7.2). The competence of *P. pentosaceus* D39 to decline Caco-2 cell death due to treatment with EAEC 3591-87 and K2 was significantly higher ($P < 0.05$) than by *B. bifidum* ATCC 11863 (15.1 and 20.5 %, respectively). However, this competence for *L. plantarum* FS2 (12.0 and 13.3 %) was not different from those of *B. bifidum* ATCC 11863 and *P. pentosaceus* D39. The three potential probiotic bacteria decreased cell death following treatment with ND-EAEC N23 almost to the same level ($P > 0.05$) as their non-treated counterpart.

The results for cell viability during BIFA also indicate that EAEC 3591-87, K2, and N23 caused Caco-2 cell death by 53.4, 52.9 and 27.3 %, respectively (Figure 7.3). The selected LAB were not different from one another in their competence to reduce Caco-2 cell death due to EAEC treatment. *B. bifidum* ATCC 11863, *L. plantarum* FS2, and *P. pentosaceus* D39 demoted cell death due to treatment with

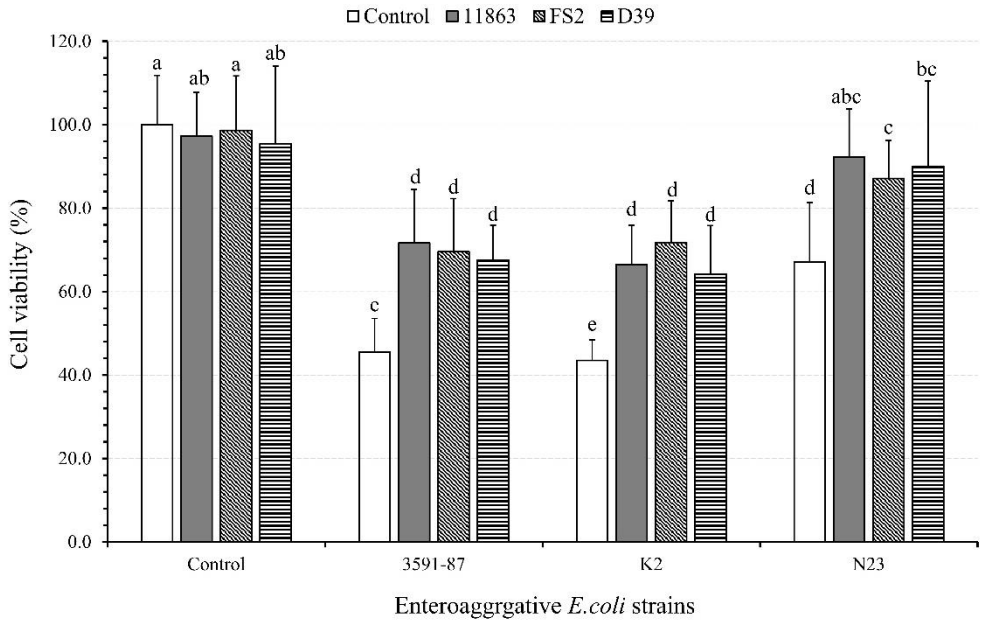


Figure 7.3: The effects of enteroaggregative *E. coli* (EAEC) and lactic acid bacteria (LAB) on Caco-2 cell viability after bacterial inhibition from adhesion assay

The EAEC include diarrhoeagenic (3591-87, and K2) and a non-diarrhoeagenic (N23) strains. *B. bifidum ATCC* 11863; *L. plantarum* FS2 and *P. pentosaceus* D39 constitute LAB strains. Each bar is a mean of three independent replicates (n=18) with its corresponding standard deviation. Bars with different letters (a-e) indicate significant differences ($P \leq 0.05$) according to Fisher's LSD test.

EAEC 3591-87 by 26.1, 24.0, and 22.0 %, respectively. These LAB also reduced Caco-2 cell death owing to treatment with EAEC K2 by 23.0, 28.3 and 20.7 %, respectively. Upon treatment with ND-EAEC N23, the selected potential LAB moderated cell death by 25.1, 20.0 and 22.9 %, respectively.

Notably, the LAB strains did not show any difference ($P > 0.05$) in their competence to reduce epithelial cell death across the different EAEC strains.

The results presented here from our *in vitro* experiments indicate that our *L. plantarum* and *P. pentosaceus* strains isolated from *ogi* appear to be promising probiotic candidates showing that cereal foods can equally be good sources of probiotic bacteria. The epithelial cell viability results were found to be dependent on the LAB and pathogen strains as well as on the bacteria infection mode as reported by previous studies (Bailey *et al.*, 2011). Prior to their use as feed additives, it is essential to critically assess the safety status of potential probiotics strains. This assessment must comprise adhesion and cytotoxicity to intestinal epithelial cells (IECs), susceptibility to other microbes and antibiotics of human and veterinary significance, evaluation for the existence of virulence and transmissible antimicrobial resistance genes. This study was aimed to determine the abilities of two LAB, *L. plantarum* FS2 and *P. pentosaceus* D39 with promising probiotic characteristics on the viabilities of EAEC-impaired IECs in different bacterial modes of infection.

One key requirement of probiotics is their abilities to adhere to the intestinal epithelium without inflicting cytotoxic effects against them to guarantee extended stability within the gastrointestinal tract (García-Hernández *et al.*, 2016; Piatek *et al.*, 2012). Probiotics' ability to adhere to the intestinal epithelium enhances their antagonistic combats, further empowering them to outcompete pathogens from epithelial cell adhesion sites (Corr *et al.*, 2009). All the selected LAB strains from the current study adhered to Caco-2 cells with different capacities, varying with the strain (Agbemavor and Buys, 2021). In previous research, the adhesion of LAB to Caco-2 cells was reported to be a strain, matrix and dose-dependent (Jensen *et al.*, 2012). The EAEC-impaired cytotoxicity levels against Caco-2 cells from the current study were generally higher, ranging from 27.3 to 54.4 %, than what was reported from previous findings (Ayala *et al.*, 2019; Baylor *et al.*, 2003; Olivas-Quintero *et al.*, 2022; Poormontaseri *et al.*, 2017). Interestingly, however, the current cytotoxicity scores were much lower than those previously reported (Bailey *et al.*, 2011; Bhat *et al.*, 2019a) whilst partially agreeing with

the findings of (Poormontaseri *et al.*, 2017; Yoon and Choi, 2012). Just like our positive reference probiotic, *B. bifidum* ATCC 11863, the two fermented cereal-based LAB, *L. plantarum* FS2 and *P. pentosaceus* D39 can be relied upon as feed additives without compromising the viability of epithelial cells, though subject to further studies. We have illustrated that EAEC-imposed cell death was mitigated to various degrees by the selected LAB, *L. plantarum* and *P. pentosaceus* strains based on the EAEC strain and the bacterial infection mode involved, as previously reported elsewhere (Bailey *et al.*, 2011; Mohsin *et al.*, 2015). Our results so far demonstrated that the two probiotics might be

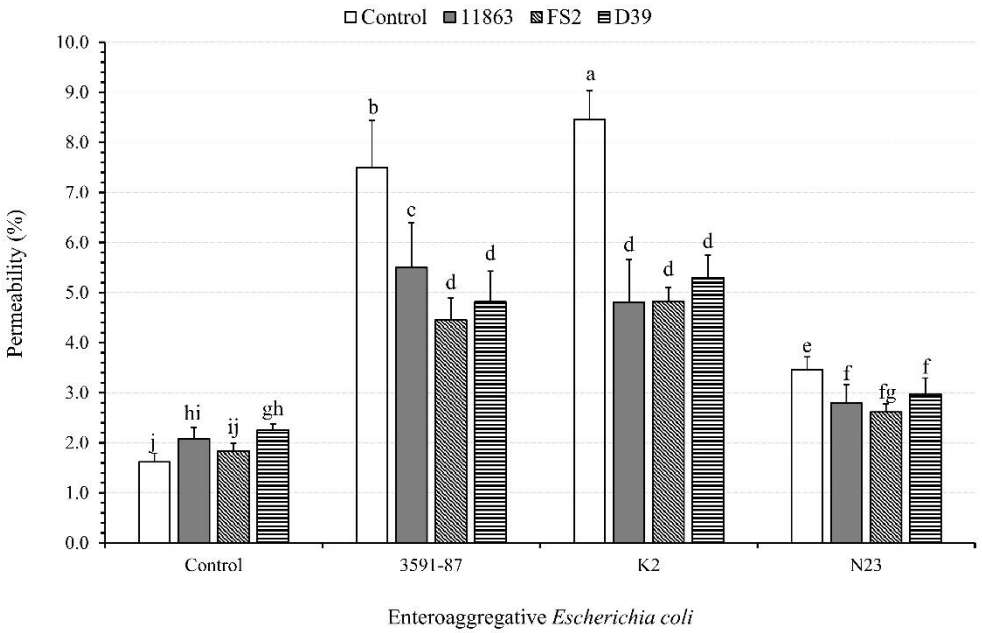


Figure 7.4: The effects of enteroaggregative *E. coli* (EAEC) and lactic acid bacteria (LAB) on Caco-2 cell permeability after bacterial competitive exclusion from adhesion assay

The EAEC include diarrhoeagenic (3591-87, and K2) and a non-diarrhoeagenic (N23) strains. *B. bifidum ATCC* 11863; *L. plantarum* FS2 and *P. pentosaceus* D39 constitute LAB strains. Each bar is a mean of three independent replicates (n=12) with its corresponding standard deviation. Bars with different letters (a-j) indicate significant differences ($P \leq 0.05$) according to Fisher's LSD test.

effective in the prophylactic and therapeutic management of inflammatory bowel syndrome, as reported by previous studies (Anukam *et al.*, 2008; Bibiloni *et al.*, 2005). In a recent study (Agbemavor and Buys, 2021), even though more EAEC cells were excluded during bacterial (EAEC) inhibition from adhesion assay than the others, LAB's ability to mitigate IEC cytotoxicity during this bacterial infection mode was not up to the expected levels. The reason could be that during this mode of bacterial

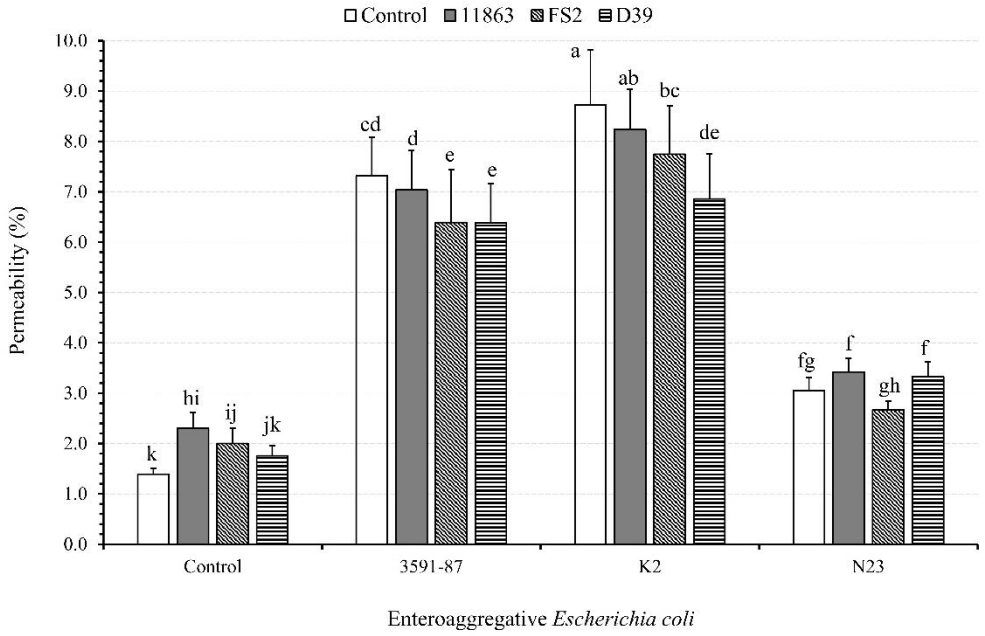


Figure 7.5: The effects of enteroaggregative *E. coli* (EAEC) and lactic acid bacteria (LAB) on Caco-2 cell permeability after bacterial displacement from adhesion assay

The EAEC include diarrhoeagenic (3591-87, and K2) and a non-diarrhoeagenic (N23) strains. *B. bifidum ATCC* 11863; *L. plantarum* FS2 and *P. pentosaceus* D39 constitute LAB strains. Each bar is a mean of three independent replicates (n=12) with its corresponding standard deviation. Bars with different letters (a-j) indicate significant differences ($P \leq 0.05$) according to Fisher's LSD test.

infection, the LAB cells pre-occupied the intestinal epithelium receptors before their opponents (EAEC strains) were introduced. This process should offer some level of protection to the Caco-2 cells before the arrival of the EAEC. Thus, even though the Caco-2 cell viabilities were comparatively higher in

some cases, these levels should have been higher than recorded. To the best of our knowledge, reports of this nature were minimal. Our current study illustrates that the two LAB can considerably mitigate epithelial cell death.

### 7.4.2 Permeability of Caco-2 cells

Regardless of the bacterial infection mode, the 6 h exposure of Caco-2 cell monolayers to the selected EAEC caused a significant elevation in phenol red flux (permeability) compared to their control counterparts (Figure 7.4, Figure 7.5, and Figure 7.6). However, the competence of the potential LAB to reduce the phenol red influx varied from one EAEC strain to the other and with bacterial infection mode. During the BCEFA, treatment with EAEC 3591-87, K2 and N23 for 6 h significantly ($P < 0.05$) increased phenol red flux (permeability) by 7.5, 8.5, and 3.5 %, respectively, across the Caco-2 cell monolayers as compared to untreated control cells (1.6 %) (Figure 7.4). Co-incubating the EAEC-challenged Caco-2 cells with the potential probiotics demonstrated varying competencies to reduce the EAEC-induced permeability increments. *B. bifidum* ATCC 11863, *L. plantarum* FS2, and *P. pentosaceus* D39 reduced permeability by 2.0, 3.0 and 2.7 %, respectively. When challenged with EAEC K2, the selected LAB downregulated Caco-2 cell permeability by 3.7, 3.6, and 3.2 %, respectively, as against 0.7, 0.8, and 0.5 % when challenged with EAEC N23. Interestingly, except for the treatment with EAEC 3591-87, there was no difference ($P > 0.05$) among the LAB in their competence to reduce elevation in permeability following infection with EAEC K2 and N23.

During BDFA, Caco-2 cell permeability was significantly ($P < 0.05$) elevated by 7.3, 8.7, and 3.1 % following infection with EAEC 3591-87 regarding the control cells (Figure 7.5). However, these permeability increments were reduced by 0.3, 0.9 and 0.9 % when the challenged Caco-2 cells were co-infected with *B. bifidum* ATCC 11863, *L. plantarum* FS2, and *P. pentosaceus* D39, respectively. Infection with EAEC K2 indicates that the elevated permeabilities of Caco-2 cell monolayers were slashed by 0.5, 1.0, and 1.9 %, respectively. Challenging the Caco-2 monolayers with EAEC N23 followed by LAB indicates that only *L. plantarum* FS2 lowered the permeability (0.4 %). In contrast,

marginal increments (0.4 and 0.3 %) were recorded by *B. bifidum* ATCC 11863 and *P. pentosaceus* D39. Despite these results, no difference was detected in the competence of these LAB to reduce the phenol red influx across the Caco-2 cell monolayers.

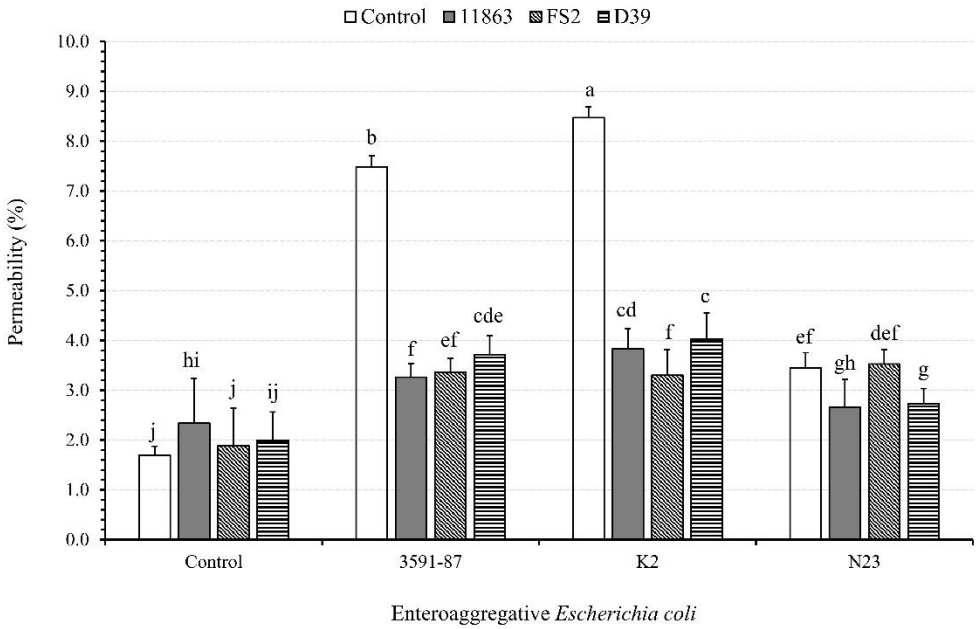


Figure 7.6: The effects of enteroaggregative *E. coli* (EAEC) and lactic acid bacteria (LAB) on Caco-2 cell permeability bacterial inhibition from adhesion assay

The EAEC include diarrhoeagenic (3591-87, and K2) and a non-diarrhoeagenic (N23) strains. *B. bifidum ATCC* 11863; *L. plantarum* FS2 and *P. pentosaceus* D39 constitute LAB strains. Each bar is a mean of three independent replicates (n=12) with its corresponding standard deviation. Bars with different letters (a-j) indicate significant differences ($P \leq 0.05$) according to Fisher's LSD test.

The results obtained during the BIFA indicate that EAEC 3591-87, K2, and N23 increased the phenol red flux across the Caco-2 cell monolayers by 5.8, 6.8 and 1.8 %, respectively, compared to the non-treated cells (Figure 7.6). These increased permeabilities were suppressed to different degrees by the selected LAB. *B. bifidum* ATCC 11863, *L. plantarum* FS2, and *P. pentosaceus* D39 increased

permeabilities by 4.2, 4.1, and 3.8 % when the epithelial cell was infected with EAEC 3591-87. Alternatively, when treated with EAEC K2, Caco-2 cell permeability was restored by 4.6, 5.2, and 4.4 %, respectively contrary to 0.8, 0.1, and 0.7 %, respectively, when the cells were treated with EAEC N23. The intestinal barrier provides an active physical barrier that promotes continuous communication with the gut microbiota and the immediate immune system, essential for maintaining gut homeostasis and functionality.

Reductions in microflora diversity and stability coupled with elevations in pro-inflammatory bacteria are characteristic of alterations in the gut microbiome composition. These reductions subsequently affect the gut barrier functions leading to the incidence of metabolic syndromes, allergies, and inflammatory diseases (West *et al.*, 2015). Certain probiotic bacteria in diverse formulations have been demonstrated to improve the gut microbiome stability with various health-promoting characteristics, including immune system modulation, anti-hypercholesterolemic, anti-obesity, anti-anxiety/anti-depressive, and several others (Markowiak and Slizewska, 2017). Nevertheless, regardless of solid scientific proofs underscoring the numerous benefits of probiotics, the mechanistic pathways undertaken by specific probiotic strains are yet to be fully comprehended, hence demanding more studies in this direction.

The current study was conducted to understand potential probiotic bacteria' effects on intestinal barrier function and permeability using a diarrhoeagenic EAEC-inflamed *in vitro* Caco-2 cell model. *E. coli* resides in the intestines and is associated with more than 90 % of humans. Even though *E. coli* constitutes below 1 % of the intestinal microbiome; it predominates the aerobic microorganisms within the gut. It is known to be the microbe to colonise the human neonatal intestine and, therefore, was considered to help prepare the gut to be inhabited by other commensals that later become more important in life. Initially, the scientific community assumed that only the intestinal pathogens, unlike commensal bacteria, elicited an intestinal pro-inflammatory response. The occurrence of this event with commensal bacteria was thought to hinder the mutualistic relationships between the bacteria in

question and their human host. However, non-pathogenic enteric *E. coli* K-12, its lipopolysaccharides and other metabolites were demonstrated to induce varying levels of increased intestinal epithelial permeability (Markowiak and Slizewska, 2017; Nakata *et al.*, 2017) and pro-inflammatory responses (Bhat *et al.*, 2019b; Zargar *et al.*, 2015) by secretions of small diffusible protein molecules like the flagellin. Hence, we evaluated *L. plantarum* FS2 and *P. pentosaceus* D39 as probiotic bacteria regarding *B. bifidum* ATCC 11863 for prophylactic and therapeutic procedures with EAEC from fresh unpasteurised milk as test pathogens.

The intestinal epithelial monolayer limits the free passage of nutrients and toxins, owing to its primary role as a perfect physical barrier between the systemic circulation and the external environment. Therefore, a compromised (leaky) intestinal epithelium is mainly associated with the continuous development of several syndromes, which are not just restricted to the gut but involve other organs. Thus, appropriate gut physiology and health require an integrated intestinal barrier. Its integrity level is determined chiefly as TEER (Trans Epithelial Electrical Resistance) as well as with phenol red and FITC-dextran (fluorescein isothiocyanate-labelled dextran) flux assays as well as TEER (Trans Epithelial Electrical Resistance) measurements (Ismail, 1999; Tan *et al.*, 2018). Following the phenol red diffusion and TEER assays, our EAEC strains resulted in leaky monolayer development illustrated by a considerable rise in the diffusion of phenol red tracer dye coupled with a significant reduction in TEER across the Caco-2 monolayer cells. These indices were restored to significant levels when intestinal cells were treated with the selected probiotics *B. bifidum* ATCC 11863, *L. plantarum* FS2, and *P. pentosaceus* D39 indicating their ability to repair the intestinal barrier. Like our current interpretations, previous studies similarly reported the shielding effects of different Bifidobacteria and lactobacilli strains against other enteropathogenic bacteria during various *in vitro* and *in vivo* experimental models (Han *et al.*, 2016; Ziegler *et al.*, 2018, 2019). *L. plantarum* WCFS1 (during a pre-treatment) was recently demonstrated to protect Caco-2 cell monolayers against phorbol ester-induced leaky intestinal epithelium whilst augmenting the intestinal barrier integrity among healthy

human subjects (Karczewski *et al.*, 2010). Reductions in TEER and increases in mannitol flux rates reported for porcine IPEC-J2 and human Caco-2 cells were significantly inhibited when the epithelial cells were pre-treated with probiotic *E. faecium* followed by exposure to Enterotoxigenic *E. coli* ETEC (Lodemann *et al.*, 2015). Probiotics have severally demonstrated their abilities to improve the intestinal barrier integrity by interactions and inductions of intercellular tight junctions signalling pathways that eventually induce tight junction proteins (genes) expressions (Alvarez *et al.*, 2016; Llewellyn and Foey, 2017).

### 7.4.3 Bacterial translocation across Caco-2 cells

The results for bacterial translocation (passage) across the Caco-2 cell monolayers indicated that no viable count was recorded for any of the LAB strains and the non-infected Caco-2 monolayers (Figure 7.7). These findings were contrary to that of the selected EAEC strains. EAEC K2 recorded the highest (3.8 $\log_{10}$ CFU/ml) passage ($P < 0.05$) across the differentiated Caco-2 monolayers (Figure 7.7). This was followed by EAEC 3591-87 (3.5 $\log_{10}$ CFU/ml) and N23 (0.8 $\log_{10}$ CFU/ml) recording the least passage. Our results agree with previous findings (Beeckmans *et al.*, 2020; Clark *et al.*, 2005).

Sufficient evidence has been established that the pathogenesis of several disorders, including inflammatory bowel syndrome, sepsis and even multiple organ failures, is preceded by loss of intestinal barrier function, which is mediated mainly by cellular factors and cytokines (Clayburgh *et al.*, 2004; Schlegel *et al.*, 2021; Schoultz and Keita, 2019). We hypothesised that paracellular permeability mediated by intercellular tight junction disruption increases with mucosal penetration of luminal bacteria and bacterial antigens, triggering or prolonging an inflammatory response. The current *in vitro* study investigated the possibility of bacterial translocation from the apical chamber into epithelial monolayers. This observation suggests that the epithelial monolayers might have been rendered porous, permeating bacteria passage. The key finding is that extended exposure of Caco-2 monolayers to EAEC cells stimulates the apical to basolateral translocation of bacterial cells, which might induce

cytokine secretion and changes whilst promoting bacterial invasion (internalisation) (Bevivino and Monteleone, 2018; Goethel *et al.*, 2018).

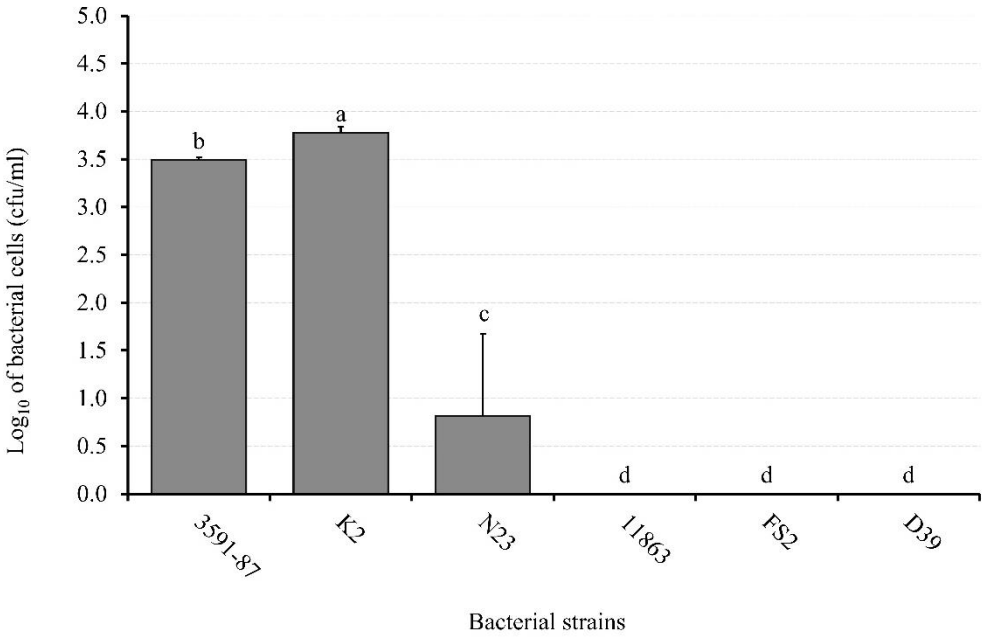


Figure 7.7: The translocation of enteroaggregative *E. coli* (EAEC) across polarized Caco-2 cell monolayers

The EAEC include diarrhoeagenic (3591-87, and K2) and a non-diarrhoeagenic (N23) strains. Each bar is a mean of three independent replicates (n=18) with its corresponding standard deviation. Bars with different letters (a-c) indicate significant differences ($P \leq 0.05$) according to Fisher's LSD test.

This phenomenon might, in turn, promote molecular changes within intercellular tight junctions and even paracellular permeability (Bevivino and Monteleone, 2018; Munoz *et al.*, 2019). Probiotics and other agents that interfere with lipid rafts were demonstrated to prevent bacterial translocation across epithelial monolayers (Nagpal and Yadav, 2017; Sato *et al.*, 2017). These findings dispute the perception that tight junction disruption is essential for cytokine-induced passage of non-pathogenic bacteria into the epithelial tissue (Catalioto *et al.*, 2011; Clark *et al.*, 2005).

According to the “leaky gut” hypothesis, intercellular tight junctions are believed to break down. This breakdown allows bacteria with other toxins to move across the epithelial barrier upon infestation with enteropathogens. It also leads to an inflammatory response that triggers a sequence of diseases during which more cytokines and other cellular substances are secreted, further impairing the intercellular tight junctions. However, up to date, there has not been decisive proof that an increase in bacterial translocation leads to a corresponding increase in gut permeability (Chen *et al.*, 2020; Ohlsson *et al.*, 2019). These parameters increased significantly with hemorrhagic shock and lipopolysaccharide administration in mice. However, gut permeability appeared comparatively normal to the extent of bacterial translocation in both studies (Maes *et al.*, 2012). It was recently concluded from an *in vivo* study that bacterial translocation occurs even without a considerable rise in gut permeability (Crapser *et al.*, 2016).

## 7.5 Conclusion

The current study revealed that the potential probiotic bacteria, *L. plantarum* FS2 and *P. pentosaceus* D39 intervention inhibited EAEC-impaired intestinal barrier function as witnessed in the improvement in viabilities and reductions in Caco-2 cell monolayer permeabilities. The inhibition could be due to the moderation of genes’ expression, which attenuates or enhance EAEC-impaired intestinal barrier functions. Therefore, the presumptive probiotic bacteria, *L. plantarum* FS2 and *P. pentosaceus* D39, have proved to be potential candidates to be used as a food additive for the prevention of inflammatory bowel syndromes based on their effectiveness during competition and exclusion assays. However, these species need to be evaluated through clinical trials to substantiate their safety and suitability before incorporating them into food systems for human consumption.

# CHAPTER 8.

# GENERAL DISCUSSIONS

This general discussion section will firstly focus on reviewing selected laboratory analytical techniques used in this study. Secondly, it will focus on discussing some pertinent research findings from the different research chapters to consolidate and align them with the principal objective of the current study.

## 8.1 Methodological reviews

Like most scientific research, this study faced some procedural limitations that might influence its findings. These deficiencies were investigated across the four research chapters (sections) with some recommendations for consideration for future investigations.

### 8.1.1 Bacterial strains and culturing conditions

Out of the five enteroaggregative *Escherichia coli* (EAEC) strains, four were obtained from the microbial bank of the Food Microbiology laboratory of the Department of Consumer and Food Sciences, University of Pretoria, Pretoria, South Africa. These strains (K2, K3, K16, and N23) were previously isolated from unpasteurised fresh milk samples being sold to rural folks for consumption (Mpumalanga Province, South Africa) in a separate study (Aijuka *et al.*, 2018; Aijuka *et al.*, 2019; Ntuli *et al.*, 2017). The four comprised of three diarrhoeagenic enteroaggregative *Escherichia coli* [D-EAEC (K2, K3, and K16)] and one non-diarrhoeagenic enteroaggregative *Escherichia coli* [ND-EAEC (N23)]. The last EAEC strain, 3591-87, was obtained from the microbial culture bank of the National Institute for Communicable Diseases (NICD), a division of the National Health Laboratory Service (NHLS), Sandringham, Johannesburg of the Republic of South Africa; under the independent approvals from the South African Council for Non-Proliferation of Weapons of Mass Destruction and the National Department of Health. This strain was isolated as clinical patients and characterised to be

EAEC which was used as a positive clinical reference strain in the current study. Two (*L. plantarum* FS2 and *P. pentosaceus* D39) of the four lactic acid bacteria (LAB) used in this study were isolated from a West African fermented cereal, *ogi* (Fayemi and Buys, 2017; Fayemi *et al.*, 2017), preserved and stored in the microbial culture bank of the same Food Microbiology laboratory. The other two (*B. bifidum* ATCC 11863 and *L. acidophilus* ATCC 4356) were obtained from the American Type Culture Collection (ATCC) and used as positive probiotic controls. The choice for the four EAEC was because out of the several *E. coli* isolates were characterised as diarrhoeagenic and a few non-diarrhoeagenic (Aijuka *et al.*, 2018; Aijuka *et al.*, 2019; Ntuli *et al.*, 2017). The two LAB isolates, *L. plantarum* FS2 and *P. pentosaceus* D39, stood very tall in their characteristics as probiotics amongst several LAB isolates obtained (Fayemi and Buys, 2017; Fayemi *et al.*, 2017).

The study initially involved five EAEC and four LAB strains totalling nine. Since the target was to harvest these bacterial cells within 18 hours, completing the harvest process within a reasonable time was challenging due to the large number of bacterial strains involved. In most cases, the procedure could take up to 2 h. This work overload could be avoided by working with a fewer number of bacterial strains from both EAEC and LAB.

### 8.1.2 Caco-2 cell culturing

The Caco-2 cells used for the experimental procedures in this study ranged from passage 30 to 42. Even though previous reports indicate that older-passaged cells attained their stationary phase sooner than younger ones, no morphological difference was detected between different passages when subjected to scanning electron microscopy (Briske-Anderson *et al.*, 1997; Lea, 2015; Mohammadi Farsani *et al.*, 2018). However, some of the cells grown on membranes were not monolayers but rather a several cells thick with different morphologies. Critical examination of these multi-layered areas indicates that the behaviour was inherent in the cell based on the conditions under which they were grown. The research team concluded that their results buttress the inherent irregularity among the

Caco-2 cell models. They strongly recommend closely monitoring the cultural attributes during growth and differentiation under specified conditions. Unfortunately, this monitoring was not carried out throughout our study. Therefore, to bolster findings from studies, there will be the need to observe this closely. Running these tests in parallel with other cell lines such as HT29 and T84 cells will go a long way toward overcoming these challenges.

### 8.1.3 Bacterial enumeration

Several experimental procedures during this study involved enumeration of bacterial colonies, including the determination of the antimicrobial effect of LAB against EAEC, EAEC and LAB adhesion to the host epithelial (Caco-2 cell), the impacts of the two potential probiotics on the exclusion, displacement, and inhibition of the selected EAEC strains, and infection mode and treatment time effects on adhesion, as well as gastric acid and bile salt stress response of EAEC and LAB for adhesion to the Caco-2 monolayers. The enumeration of bacterial cells throughout this study involved traditional plating technique (Collado *et al.*, 2007a; Collado *et al.*, 2005; Pazhoohan *et al.*, 2020a; Rajan *et al.*, 2018). This technique was very cumbersome, laborious, and expensive considering the number of samples to be analysed per given time (Rohde *et al.*, 2017; Sloan *et al.*, 2017; Wu *et al.*, 2019a). The technique can also be saddled with issues relating to the reliability and accuracy of its findings compared to other modern methods. Per the experimental procedures involving bacterial adhesions, the entire process of plating out the attached LAB and EAEC took not less than 6 hours due to the volume of work involved, as indicated by the EAEC-LAB pairing treatment plan (Table 4.2, Table 5.2 and Table 6.2). Even though the trypsinized bacterial cells were kept on ice (0 – 4 °C) to avoid the likelihood of multiplication, the long hours were not fair to samples plated later. Additionally, this procedure only allowed us to enumerate EAEC cells from samples treated with either EAEC alone or EAEC with the LAB. The LAB cells could not be enumerated from samples treated with both EAEC and LAB. Besides, this method fails to count dead or injured bacteria cells if there are any. However, these shortcomings can be overcome by using the flow cytometer technique, which provides accurate

enumeration and even absolute bacterial cell concentration, depending on the equipment's sophistication level, as reported by previous studies (Kivens and Shimizu, 2019; Ou *et al.*, 2017; Van Asten *et al.*, 2018). The attached bacterial cells can equally be conducted using the quantitative real-time polymerase chain reaction (qRT-PCR) technique with the thermal cycler as previously reported (Boguslawska *et al.*, 2016; Bujko *et al.*, 2015; Polisetti *et al.*, 2016). Like the flow cytometric method, this method is accurate, sensitive, and rapid for detecting and quantifying several bacterial genera and species adhering concurrently to epithelial cell monolayers.

### 8.1.4 Electron microscopy of challenged Caco-2 monolayer cells

The current study examined the formation of microvilli which results from the differentiation of Caco-2 cells, in addition to the morphological (topographical) imaging of challenged (bacterial infected) Caco-2 monolayer cells by using scanning electron microscopy (SEM) as previously reported (Behbahani *et al.*, 2019; Salas-Jara *et al.*, 2016b; Schimpel *et al.*, 2015). Even though over 85 % of microscopic fields show fully matured microvilli representing real-life (*in vivo*) situations, a few areas did not show such maturity levels. In contrast, a few other fields indicated either non-differentiated Caco-2 cells or a few open spaces (Goyer *et al.*, 2016; Kucki *et al.*, 2017). Situations like these will affect the accuracy of the bacterial adhesion findings throughout the various experiments since it was assumed that the maturity levels of the differentiated microvilli were similar throughout all the experiments. Even though this situation was not within the control of the current study, the results can be compared to another parallel experiment using a different mammalian epithelial cell line like HeLa, HT29, and T84. Besides these limitations, the current study failed to evaluate the effects of both diarrhoeagenic and non-diarrhoeagenic EAEC as well as the selected LAB on the internal cellular structure and organelles using transmission electron microscopy (TEM) as indicated by previous reports (Da Silva Santos *et al.*, 2015; Dos Santos *et al.*, 2015; Sanchez-Villamil *et al.*, 2019c; Stalb *et al.*, 2018). The scanning electron micrographs revealed highlighted homogeneously distributed darker lines between neighbouring enterocytes for control samples (uninfected Caco-2 cell monolayers) and

those treated with the LAB. Contrarily, monolayers treated with EAEC were showing wider (disrupted) intercellular tight junctions (ITJ) as illustrated by the darker highlighted lines (Finamore *et al.*, 2018; Goyer *et al.*, 2016; Puschhof *et al.*, 2021), this study failed to further emphasise these findings by immunofluorescent staining of junctional proteins (E-cadherin and ZO-1) to reveal adherens junctions and tight junctions of the intestinal epithelial cells (IECs), correspondingly using confocal laser scanning microscopy (CFLSM) (Gill and Hecht, 2018; Sarkar *et al.*, 2018). The results from this assay would have better revealed the degree of impairment inflicted by the D-EAEC on the differentiated enterocytes resulting in the disorganisation (delocalisation) of the intracellular spaces at both adherens and tight junctions levels (Chang *et al.*, 2020b; Goyer *et al.*, 2016; Salih *et al.*, 2020).

### 8.1.5 Evaluation of intestinal epithelial inflammation

In most epidemiological studies involving the outbreak of pathogens among human patients, the measurement of a single cytokine using only one analytical platform as conducted in this study is not representative enough. Instead, a spectrum (cocktail) of inflammatory, pro-inflammatory, and anti-inflammatory cytokines using at least two analytical platforms, for example, qRT-PCR with enzyme-linked immunosorbent assay (ELISA), have become increasingly important (Amsen *et al.*, 2009; Monastero and Pentyala, 2017). This because all analytical techniques have some advantages and limitations (Amsen *et al.*, 2009; Hosseini *et al.*, 2018; Laserna-Mendieta *et al.*, 2019). Due to logistical constraints, the current study evaluated IL-8 as a pro-inflammatory cytokine (Table 2.1) using only the ELISA analytical platform. The ELISA technique allows secreted cytokines to be detected at the protein level. However, one major limitation associated with this method is that it is mainly difficult to obtain sufficient tissue fluids due to the consumption of cytokines by the cells, and as a result, there might be an underestimation of the actual cytokine levels (Amsen *et al.*, 2009; Hosseini *et al.*, 2018). It is therefore recommended to assay a selection of these cytokines using at least two analytical platforms in future studies.

### 8.1.6 Cell viability assay

This study assayed cell viability using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or tetrazolium salt (MTT) analytical technique. Even though this technique performs very well in terms of accuracy and reproducibility of results, it is destructive, quite cumbersome, and time-consuming compared to other methods like lactate dehydrogenase (LDH) alamarBlue® using resazurin and 5-Carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) based on 5-carboxyfluorescein diacetate acetoxymethyl ester. These techniques are not destructive but quick and easy to run (Abel and Baird, 2018; Bahadar *et al.*, 2016; Bopp and Lettieri, 2008). Therefore, future studies, especially those with the same cells to be investigated for other endpoints, might consider other non-destructive alternative analytical techniques.

## 8.2 Research Findings

This study evaluated the influence of the four potential probiotic bacteria on the selected EAEC. These analyses include auto-aggregation, co-aggregation, bacterial surface hydrophobicity, adhesion, epithelial barrier integrity and inflammation, and IEC viability. The findings for these various sections are discussed in the following.

### 8.2.1 Characterisation of bacterial surface and antimicrobial properties

This study (Chapter 4) found varying levels of responses for both EAEC and LAB strains in terms of bacterial surface properties, including auto-aggregation, their co-aggregation and bacterial surface hydrophobicity, and the antimicrobial effect of the LAB against the selected EAEC strains. The variations among the different species and strains can be attributed to their genetic diversity being the driving force contributing adaptive evolution of the organisms (Schrader and Schmitz, 2019; Young *et al.*, 2017; Zhao *et al.*, 2019). Many other studies have indicated strain variability as the leading contributor accounting for the differences in bacterial characteristics in terms of auto-aggregation, co-aggregation, and their adhesion to IECs (Khojah *et al.*, 2022; Mallappa *et al.*, 2019). Another principal

observation for both EAEC and LAB is that the auto-aggregation for each bacterial strain increased with time (Table 4.3), agreeing with other studies (Jalowiecki *et al.*, 2018; Trunk *et al.*, 2018a). The rate of bacterial aggregation has been reported to be dependent on the presence or absence of some extracellular features like agglutinin, adhesins, pili, specialised surface proteins and the bacteria itself, extending from a few minutes to several hours or even overnight (Jalowiecki *et al.*, 2018; Trunk *et al.*, 2018a). Interestingly, except for the ND-EAEC N23, the auto-aggregation of all the D-EAEC were comparatively lower than their LAB counterparts. These differences could be attributed to the reasons that were explained earlier.

Auto-aggregation is vital for bacterial colonisation, kin and kind recognition and survival (Nwoko and Okeke, 2021; Trunk *et al.*, 2018a; Wall, 2016). It is directly facilitated by explicit communications between cell surface structures (organelles) or proteins of interacting cells or indirectly by the existence of secreted macromolecules like exopolysaccharides and environmental deoxyribonucleic acid (eDNA) (Bamford and Howell, 2016; Hobley *et al.*, 2015; Laventie and Jenal, 2020). Selected auto-aggregation effecters are self-association and offer fascinating concepts for protein interactions. Auto-aggregation can be helpful or harmful at different times and places. Therefore, it is regulated chiefly epigenetically by phase variation or transcriptional or post-transcriptional mechanisms (Nwoko and Okeke, 2021; Trzilova and Tamayo, 2021). auto-aggregation can contribute to bacterial adhesion to host epithelial cells, biofilm development or other high-ranking functions (Ageorges *et al.*, 2020; Sorroche *et al.*, 2018). Nevertheless, only a few bacteria require auto-aggregation for these phenotypes. Probiotic auto-aggregation seems to be vital for adhesion to IECs and their competence to aggregate with other bacteria like enteropathogens to form a barrier to prevent colonisation by pathogenic bacteria (Campana *et al.*, 2017; Gharbi *et al.*, 2019; Mojgani *et al.*, 2015; Simoes *et al.*, 2021). Therefore, the need to study, detect and measure auto-aggregation for bacteria using qualitative and quantitative *in vitro* and *in vivo* techniques. A comprehensive understanding of bacteria phenotype like

this holds the potential for discovering new therapeutic objectives that can be exploited cost-effectively.

Our findings further revealed that the levels of co-aggregation and growth inhibition of LAB against EAEC strains (antimicrobial) were principally dependent on the specific EAEC-LAB pair involved, as previously reported (Figure 4.7) (Campana *et al.*, 2017; Reuben *et al.*, 2019). Thus, co-aggregation between the selected EAEC and LAB were strain specific. This observation for co-aggregation further supports evidence for the interaction between cell surface molecules from the two participating bacteria. The co-aggregation scores for both bacteria after 2 h were higher than after an hour (Figure 4.7), further emphasising the strain and time dependency of auto- and co-aggregation (Aarti *et al.*, 2018; Collado *et al.*, 2007c; Tomičić *et al.*, 2022; Vijayalakshmi *et al.*, 2020). The same trend was usually observed for the growth inhibition of EAEC by LAB (Bajpai *et al.*, 2016; Hati *et al.*, 2018; Kumar *et al.*, 2017; Vijayalakshmi *et al.*, 2020). In some cases, however, a few EAEC strains were not susceptible to the antimicrobial tendency of a few LAB such that the colony counts of surviving EAEC after the 5$^{th}$ HOT was either indifferent or lower than that after the first HOT as reported by some previous reports (De Almeida Júnior *et al.*, 2015). Thus, the LAB from this study aggregated with the selected EAEC strains. Their ability to aggregate allows the LAB to directly inhibit EAEC growth by producing organic acids and other proteinaceous substances in a time-dependent LAB strain (Aarti *et al.*, 2018; Ohshima *et al.*, 2016). Co-aggregation is a considerable microbial phenomenon which is directly related to the ability of a microorganism like LAB to interact with the other such as pathogens closely (Cozzolino *et al.*, 2020; Gómez *et al.*, 2016). The tendency of the LAB to co-aggregate with the selected EAEC could contribute to their probiotic potential and prevent gut colonisation of the EAEC. The co-aggregation of lactobacilli with other pathogenic bacteria was strongly endorsed by Collado *et al.* (2007c), Grześkowiak *et al.* (2012), and Jena *et al.* (2013).

Cell surface hydrophobicity has been extensively measured by bacterial adhesion to hydrocarbons in LAB and other bacteria. Cell surface hydrophobicity was analysed in this study using an apolar solvent

and xylene extraction on live cells. The results from this study show that both EAEC and LAB demonstrated varying affinities to the three hydrocarbons (chloroform, ethyl ester, and xylene) (Figure 4.3). Whist their attraction to xylene (an apolar solvent) truly represents their cell SHs, their affinities to chloroform and ethyl ester (polar solvents) represent the abilities to donate and accept electrons, respectively, from these hydrocarbons (Duan *et al.*, 2022; Khojah *et al.*, 2022; Pieniz *et al.*, 2015). The affinities of EAEC and LAB to hydrocarbon (xylene) indicate that except for EAEC K3, N23, and the two reference LAB, *L. acidophilus* ATCC 4356 and *B. bifidum* ATCC 11863, all the other EAEC and LAB strains had their hydrophobicity abilities (HAs) above the critical threshold (40 %) (Abbasnezhad *et al.*, 2011; García-Cayuela *et al.*, 2014). The current study suggests that bacterial surface HAs were strain dependent, as previously reported in other studies (Arkoun *et al.*, 2017; García-Cayuela *et al.*, 2014; Tokatli *et al.*, 2015). The HA findings from this study suggest that bacterial cell surface hydrophobicity (BCSH) might not be the main predicting factor for determining bacterial aggregation abilities. Specific cell wall proteins and lipo(teichoic) acids render the bacterial surface hydrophobic, while polysaccharides make the same hydrophilic. Several microbial activities are known to be controlled by physicochemical characteristics of the cell wall. These play significant roles in hydrophobic interactions during the bacterial adhesion to host IEC (Dufrene, 2015; Monteagudo-Mera *et al.*, 2019; Stones and Krachler, 2016), which might even promote biofilm formation. Generally, most bacteria cells exhibit hydrophobic tendencies due to the presence of their resultant negative surface charge and this phenotype is mainly associated with bacterial adhesiveness, which varies with different microorganisms and strains depending on the bacterial age, surface structures, and the growth medium (Carniello *et al.*, 2018; Liao *et al.*, 2015; Zhao *et al.*, 2015). Thus, bacterial phenotypes including auto-aggregation, co-aggregation, and cell surface hydrophobicity have some tendency to influence bacterial adherence to host IECs.

### 8.2.2 Preventing the adhesion of D-EAEC to IECs: the role of the potential probiotics from *ogi*

In this study, EAEC and LAB adhesion to the epithelial cells was studied using the Caco-2 cell model as carried out by other researchers (Ferreira *et al.*, 2021; Noohi *et al.*, 2016; Rohani *et al.*, 2015; Vieira *et al.*, 2020). Results from comparative adhesion of EAEC and LAB to the host IEC indicate that their adhesion scores were microbial and strain dependent. The adhesion scores of EAEC were generally higher than LAB counterparts. D-EAEC K2 recorded the highest adhesion score (27.9 %) to the Caco-2 cells, whereas ND-EAEC N23 had the least (18.9 %) (Figure 5.1 and Figure 5.10). *L. plantarum* FS2 scored the highest adhesion (25.5 %) as against *B. bifidum* ATCC 11863, scoring the least (16.9 %) (Figure 5.1 and Figure 5.9). Bacterial adhesion to host IEC is a complex process requiring physical contact between the microbial plasmalemma and the interacting surfaces. Investigations on the structure, composition and forces of interaction associated with bacterial adhesion to IECs (Benhamed *et al.*, 2014; Guerin *et al.*, 2018; Polak-Berecka *et al.*, 2014) and mucus (Corfield, 2018; Dufrêne and Persat, 2020; Mays *et al.*, 2020; Sotres *et al.*, 2017) have extensively been studied.

The adhesion of pathogens to the intestinal epithelium is one of the essential prerequisites for colonisation of the gut to cause their pathogenesis through physical disruption and toxin elaboration. Results from this study show that the EAEC (both diarrhoeagenic and non-diarrhoeagenic) strains adhered to the Caco-2 cells significantly to trigger inflammatory bowel syndromes, as reported (Da Silva Santos *et al.*, 2015; Ferreira *et al.*, 2021; Rajan *et al.*, 2018; Vieira *et al.*, 2020). The adhesion levels of the selected D-EAEC strains from this study suggests that they can cause intestinal cell inflammation leading to inflammatory bowel disease.

The competence to attach to mucosal surfaces and epithelial cells has been recommended to be an essential characteristic for screening probiotic bacteria strains (Carasi *et al.*, 2014; Kumar and Kumar, 2015; Ochangco *et al.*, 2016; Pringsulaka *et al.*, 2015).

Like the EAEC strains, the LAB in this study also demonstrated satisfactory adherence to the IECs, suggesting that they can impede (prevent) the adhesion of enteropathogens like EAEC to the IECs

(Figure 5.1 and Figure 5.9). Consequently, they can also control the selected EAEC from causing inflammation of the IECs to prevent inflammatory bowel syndromes. Since the adhesion of *L. plantarum* FS2 was higher than that of *P. pentosaceus* D39, this suggests that the former should have the upper hand in limiting EAEC's binding to the Caco-2 cell monolayers. *L. plantarum* is a commonly distributed and adaptable lactic acid bacterium representing the microbiota of several foods and feeds, comprising dairy, fermented products (e.g., pickled vegetables, sauerkraut, and sourdoughs), fish, meat, hay, and vegetables. It naturally inhabits animal and human mucosa (gastrointestinal tract, oral cavity, vagina, etc.). *L. plantarum* is primarily valuable for controlled fermentation due to its associations with desirable properties in many fermented foods (Adesulu-Dahunsi *et al.*, 2020; Campaniello *et al.*, 2020; Şanlier *et al.*, 2019). To impact these properties, it is added to enhance the health benefits and their qualities of foods (Behera *et al.*, 2018; Ge *et al.*, 2019; Mirkovic *et al.*, 2018).

This study further evaluated the abilities of *L. plantarum* FS2 and *P. pentosaceus* D39 together with the positive reference probiotic bacteria (*L. acidophilus* ATCC 4356 and *B. bifidum* ATCC 11863) to competitively exclude, displace and inhibit the selected EAEC strains (EAEC 3591-87, K2, K3, K16, and N23) from securing adhesion to the IECs in a procedure as described earlier (sections 5.3.4, 5.3.5, and 5.3.6). Our results demonstrate that the selected LAB could competitively exclude, displace, and inhibit the selected EAEC in different capacities corresponding to LAB-EAEC strain specificity.

Globally, EAEC isolates are commonly known for their resistance to a wide range of antibiotics (Amin *et al.*, 2018; Eltai *et al.*, 2020; Guiral *et al.*, 2019; Kumar *et al.*, 2016). Several initiatives have been taken to address the multidrug resistance problem in most countries, including the Republic of South Africa. Some of these initiatives include antibiotic stewardship or rational use of antibiotics in communities and hospitals, education, the transformation of societal norms, and the quest for novel diagnostic techniques and biological therapeutics. Exploration of natural therapeutics, including probiotics, has recently received tremendous attention in research. Probiotics have been categorised as host-immune-modulating biologicals (Kumar *et al.*, 2016; Mallappa *et al.*, 2019; Minj *et al.*, 2021;

Rocchetti *et al.*, 2021). The effects of probiotics on different gastrointestinal diseases involving *Clostridium difficile* infection (Kalakuntla *et al.*, 2019; Lau and Chamberlain, 2016; Shen *et al.*, 2017; Wilkins and Sequoia, 2017), irritable bowel syndrome caused by *Helicobacter* (Eslami *et al.*, 2019; Homan and Orel, 2015; Kafshdooz *et al.*, 2017; Shi *et al.*, 2019), rotavirus-related diarrhoea (Lopetuso *et al.*, 2017; Mazurak *et al.*, 2015; Simon *et al.*, 2021; Yang *et al.*, 2015), and multidrug resistant *Shigella* spp (Kotloff, 2022; Richards, 2017; Riddle *et al.*, 2017) have been explored. Generally, probiotic LAB imitates commensal microflora by antagonism of pathogens, competitive interactions, and production of antimicrobial factors (Bron *et al.*, 2017; Liu *et al.*, 2020b; Monteagudo-Mera *et al.*, 2019; Nataraj and Mallappa, 2021) plantaricin by *L. plantarum* or bacteriocin-like substances including acidolin, acidophilin, and lactocidin.

In most *in vivo* and even some *in vitro* studies, probiotics are noted to exert their therapeutic impacts against enteropathogens via non-immune mechanisms such as improved gut motility, increased mucus secretion, and maintenance of the gut mucosal barrier (Conte *et al.*, 2020; Cristofori *et al.*, 2021; Pique *et al.*, 2019; Raheem *et al.*, 2021; Vitetta *et al.*, 2018). Additionally, there might also be competition for limited nutrients, obstruction of pathogens' ability to colonise and manipulation of the composition and activities of the gut microbiota (Ducarmon *et al.*, 2019; Hossain *et al.*, 2017; Mathipa and Thantsha, 2017; Sehrawat *et al.*, 2021). Despite the few resilience against the selected probiotic LAB, the general reductions in EAEC counts in the presence of the former from this study could be attributed to competitive exclusion, displacement, and inhibition against the adhesion of the affected EAEC strains as previously reported between *E. coli* and *L. plantarum* by producing antimicrobial inhibitory compounds like acetic acid, bacteriocins, lactic acid, and toxic oxygen metabolites (Anand *et al.*, 2016; Giaouris *et al.*, 2015; Suchodolski and Jergens, 2016) or by mannose-specific attachment places (Anand *et al.*, 2016; Etienne-Mesmin *et al.*, 2019). Therefore, failure to impede (prevent) the adhesion and subsequent colonisation of enteropathogens, including EAEC, leads to disruption of intestinal mucosa barrier and function.

### 8.2.3 Maintenance of intestinal epithelial barrier integrity and function from the disruptive effects of D-EAEC: the role of the potential probiotics from *ogi*

EAEC has globally been noted to be the underlying cause of inflammatory bowel syndrome in different clinical situations (Guiral *et al.*, 2019; Jenkins, 2018; Modgil *et al.*, 2021). EAEC was confirmed to be the most frequent bacterial pathogen occurring among diarrhoea patients of all ages in an epidemiological study carried out in Baltimore, MD, and New Haven, CT (Nataro *et al.*, 2006; Steiner *et al.*, 2006). EAEC pathogenesis is understood to be initiated by its adhesion to the intestinal epithelium (mucosa), followed by production (and secretion) of cytotoxins and enterotoxins which damage intestinal explants, eventually leading to their cytotoxicity (cell apoptosis). The adhesion of EAEC to human intestinal epithelial cells (IECs) is facilitated by the aggregative adherence fimbriae (AAF) associated with Dr adhesins of uropathogenic and diarrhoeagenic *E. coli* (Elias and Navarro-Garcia, 2016; Jønsson *et al.*, 2015; Lara *et al.*, 2017). Several bacterial pathogens that colonise the intestinal mucosa directly or indirectly cause disruptions in the epithelial barrier function which is associated with induction of inflammatory cytokines (e.g., IL-8) secretion (discharge) in addition to reductions in transepithelial electrical resistance (TEER) in polarised epithelial explants. Disrupted epithelial explants are mainly associated with the delocalisation of intercellular tight junction proteins requiring AAF/II adhesin expression. Epithelial barrier disruption may contribute to the free passage of fluids and electrolytes, as witnessed in the “leaky-flux” model (Anbazhagan *et al.*, 2018; Barmeyer *et al.*, 2017; Konig *et al.*, 2016; Lobo De Sa *et al.*, 2021). This situation can lead to loss of mucosal proteins and subsequent malabsorption of luminal nutrients mediating access to the basolateral chamber by enteropathogens that desire this access course.

This study showed that regardless of their diarrhoeagenic status, all the three EAEC strains induced IL-8 secretion even though the margin of IL-8 induced by the ND-EAEC was substantially lower (118.3 pg/mL) than either of the two D-EAEC 3591-87 and K2 (378.7 and 412.2 pg/mL, respectively) (Figure 6.1). The control (non-infected) polarised Caco-2 cell monolayers (PCC-2CMLs) (28.5

pg/mL) together with those infected with only the four selected LAB (42.0 – 56.1 pg/mL) from this study demonstrated relatively lower IL-8 induction levels compared to those that were infected with either of the two D-EAEC strain. This trend of results is like the TEER findings. The control (non-infected) PCC-2CMLs had its TEER (% of initial TEER) value highest (104.6 %) which was not different from monolayers LAB-challenged monolayers (95.6 – 98.5 %) (Figure 6.4). The two D-EAEC 3591-87 and K2 drastically reduced these TEER values to 65.5 and 58.6 %. Even though the ND-EAEC also reduced the initial TEER value to 86.0 %, the impact was less severe than the two D-EAEC strains. The three selected LAB demonstrated varying competencies in maintaining the intestinal epithelial structure and function by limiting the induction of IL-8 secretion and boosting the TEER levels (Figure 6.1 and Figure 6.4). Thus, the ability of the potential LAB to alleviate the disruption caused by the EAEC to the intestinal epithelium was strain-strain-specific. The results from the current study further demonstrated that inflammatory cytokine (IL-8) release and TEER reductions are heavily dependent on the pathogen infection dose (Figure 6.2 and Figure 6.5, respectively). In the same way, these properties are dependent on bacterial infection mode and treatment time (Figure 6.3 and Figure 6.6). The permeability of PCC-2CMLs to large molecular markers, including phenol red (3H-2,1-Benzoxathiole 1,1-dioxide or phenolsulfonphthale), permits EAEC translocation across (section 8.2.4) indicates that the EAEC-induced barrier defect may be sufficiently large to allow proteins passage between the apical and basolateral compartments via the paracellular spaces. Caco-2 cell permeability might render the passage of bacterial toxins or even bacterial cells from the apical to the basolateral chamber (intestinal submucosa). Additionally, the disruption of the epithelial barrier may lead to the leaching of host proteins and ions into the lumen, further aggravating the diarrhoeal disease or other related consequences, which might necessitate other investigations. Scanning electron microscopy micrographs under low magnification (which could have been best represented with immunofluorescence confocal laser scanning microscopy) demonstrated limited morphological variations in the EAEC-challenged PCC-2CMLs. These variations are usually associated with delocalisation of tight junction proteins claudin-1 and occludin due to EAEC infection (Chervy *et al.*,

2020; Rosa *et al.*, 2017). Claudins have been regarded as "gatekeepers" of permeability (Fujii *et al.*, 2016; Irudayanathan and Nangia, 2020; Jin and Park, 2018; Zihni *et al.*, 2016) and are noted for reconstructing tight junctions even within cells that naturally lack tight junctions (Otani and Furuse, 2020; Otani *et al.*, 2019; Piontek *et al.*, 2020; Zihni *et al.*, 2016). Consequently, destruction of claudin-1 of intercellular tight junctions due to EAEC infections can substantially affect epithelial barrier permeability and function. Claudin-1 constitutes a central theme during enteric infection since various enteropathogens like *Arcobacter butzleri* (Ferreira *et al.*, 2016; Ramees *et al.*, 2017), *E. coli* C25, and enterohemorrhagic *E. coli* 0157:H7 (Barbara *et al.*, 2021; Bernard and Nicholson, 2022; Diez-Sainz *et al.*, 2021; Marlicz *et al.*, 2017), and *Salmonella enterica* serovar Typhimurium (Martz *et al.*, 2015; Rokana *et al.*, 2017; Splichalova *et al.*, 2019), trigger relocation of Claudin-1 which upsets paracellular permeability.

This study has shown that the EAEC isolates from unpasteurised fresh milk induced IL-8 secretion and reduces TEER and therefore can disrupt intestinal epithelial barrier and function like other pathogens. The study further illustrated that two potential probiotic LAB isolates, *L. plantarum* FS2 and *P. pentosaceus* D39 from *ogi*, like their reference counterpart, *B. bifidum* ATCC 11863, can prevent the disruption of intestinal barrier and function. They can also facilitate disrupted IECs from the destructive effects of D-EAEC. Nevertheless, the persistent disorder of the IECs in response to pathogen (EAEC) infections will compromise cellular permeability, bacterial cell translocation and eventually, cytotoxicity.

### 8.2.4 Amelioration of epithelial cell viability from the cytotoxic effects of D-EAEC: the role of the potential probiotics from *ogi*

Cytotoxicity is the toxicity caused by the activities of chemotherapeutic substances on living cells (Mukherjee, 2019). It also involves the disruption of actin, nuclear and other cytoskeletal proteins

(Maroncle *et al.*, 2006). It is used to describe how harmful a substance is to living cells, and accordingly, a cytotoxic substance can trigger cell damage or cell death by apoptosis or necrosis.

The mechanisms underlying the mechanisms of probiotic action were hypothesised numerously, including antagonism against enteropathogens, competition for adhesion sites and luminal nutrients, effect on transient intestinal time, immunomodulation etc. Out of these different mechanisms of action, it is unclear which is most desirable. A pro-inflammatory response might be needed for adequate clearance of intestinal infection (Czerucka and Rampal, 2019; Plaza-Diaz *et al.*, 2018; Shi *et al.*, 2019; Van Zyl *et al.*, 2020). However, extended NF-κβ activation followed by synthesis and production of CXCL10, IL-8, and RANTES was associated with IBD in animal models (Abba *et al.*, 2015; Chibbar and Dieleman, 2019). Interestingly, some bacterial strains were demonstrated to act on elements of the adaptive immune response to minimise inflammation. *Faecalibacterium prausnitzii* A2–165 (DSM 17677), for example, can reduce the expression of pro-inflammatory cytokines IFNc and IL-12 by peripheral blood mononuclear cells (Lapiere *et al.*, 2020; Laval *et al.*, 2015; Mohebali *et al.*, 2020).

One of the principal mechanisms for probiotic action is believed to be the competitive exclusion of the pathogens (Cremon *et al.*, 2018; Knipe *et al.*, 2021; Plaza-Diaz *et al.*, 2019; Wan *et al.*, 2019). The results from the current study support the theory that LAB are less effective in ameliorating active cytotoxic cells due to their inability restore the irreversible cytotoxic damage caused to the cellular skeleton and organelles by the enteropathogens (Brito *et al.*, 2019; Déciga-Alcaraz *et al.*, 2020). Probiotics must be able to compete efficiently with pathogens under the non-healthy conditions of the intestine (Alok *et al.*, 2017; Bajaj *et al.*, 2021; George Kerry *et al.*, 2018; Pais *et al.*, 2020; Zendeboodi *et al.*, 2020). They should be capable of regulating immune responses to pathogens to restore normalcy (Kalinkovich and Livshits, 2019; Levy *et al.*, 2017; Sylvia and Demas, 2018; Yoo *et al.*, 2022). These are the essential characteristics investigated in this study.

The cell viability results from this study generally indicate that the probiotic LAB isolated from *ogi* were more effective in protecting the host epithelial cell viability (4.4 – 24.7 %) when the PCC-2CMLs

were challenged with both the EAEC and LAB simultaneously (Figure 7.1) than when the Caco-2 cells were pre-exposed to the EAEC followed by the LAB [(-20.5) – 0.2 %] (Figure 7.2). Similarly, the cytotoxicity inhibitory effect by the probiotic LAB was generally more effective (20.0 – 28.3 %) when the Caco-2 cells were first inoculated with the potential probiotic bacteria followed by the EAEC (Figure 7.3) than when the former was initially exposed to the EAEC followed by the LAB strains [(-20.5) – 0.2 %] (Figure 7.2). In all, the presumptive probiotics were more effective in protecting the host cell viability (20.0 – 28.3 %) when the Caco-2 cells were initially infected with the promising probiotic LAB followed by the EAEC (Figure 7.3) strains than either of the two modes of infecting the Caco-2 cells (Figure 7.1 and Figure 7.2). Thus, the current study's results clearly show that the selected probiotic LAB failed to redeem (revive) the cytotoxic Caco-2 cells from the cytotoxic (apoptotic) effects of the selected EAEC.

The trend of the host epithelial cell viability results is almost like that of the epithelial cell permeability assay. Challenging the Caco-2 cells with the selected EAEC strains increased cellular permeability to different degrees; D-EAEC 3591-87 (5.8 – 5.9 %), K2 (6.8 – 7.7 %), and the ND-EAEC (1.7 – 1.8 %) (Figure 7.4, Figure 7.5, and Figure 7.6). When these setups interacted with the selected presumptive probiotic bacteria, Caco-2 cell permeabilities were restored in a strain-strain dependent manner based on the bacterial infection mode. Simultaneous coinfection of the Caco-2 cells with both EAEC and LAB restored (reduced) the cellular permeability (4.4 – 24.7 %) (Figure 7.4) more than when the cells were first challenged with EAEC strains (3 h) followed by LAB (3 h) [(-0.2) – 20.5] (Figure 7.5). Similarly, infecting the Caco-2 cells first with LAB (3 h) followed by EAEC (3 h) also restored (reduced) cellular permeability (20.0 – 28.3 %) (Figure 7.6) more than when the Caco-2 cells were first infected with EAEC (3 h) followed by LAB (3 h) [(-0.2) – 20.5]. Therefore, in all, infecting the Caco-2 cells first with the probiotic LAB (3 h) followed by EAEC (3 h) was best in maintaining the permeability of the epithelial cells (Figure 7.5). These findings further buttressed the importance of

using probiotics for prophylactic purposes was often more effective than using them for therapeutic purposes (Abraham and Quigley, 2017; Becker *et al.*, 2015; Khan *et al.*, 2019; Shamoon *et al.*, 2019).

The selected presumptive probiotic LAB demonstrated diverse competencies in maintaining epithelial cell integrity and function by restoring the host epithelial cell viability and permeability in a strain-strain dependent manner. *Lactobacillus plantarum* FS2 demonstrated the best potency in restoring epithelial cell viability [(17.1 to 20.9), (-13.3 to 0.6), (20.0 to 28.3) %] (Figure 7.1, Figure 7.2, and Figure 7.3) whilst restoring Caco-2 cell permeability by -3.6 to -0.8, -1.0 to -0.4, and -5.2 to 0.1 % (Figure 7.4, Figure 7.5, and Figure 7.6). *P. pentosaceus* D39 also restored epithelial cell viability by 10.2 to 17.8, -20.5 to 0.2, and 20.7 – 22.9 % (Figure 7.1, Figure 7.2, and Figure 7.3) whilst restoring (reducing) the epithelial cell permeability by (-3.2 to -0.5), (-1.9 to 0.3), and (-4.4 to -0.7) % % (Figure 7.4, Figure 7.5, and Figure 7.6). The positive probiotic control, *B. bifidum* ATCC 11863, also ameliorated epithelial cell viability by 4.4 to 24.7, -20.5 to 0.6, 20.0 to 28.3 % (Figure 7.1, Figure 7.2, and Figure 7.3) whilst returning Caco-2 cell permeability by -3.7 to -0.7, -0.5 to 0.4, and -4.6 to -0.8 % (Figure 7.4, Figure 7.5, and Figure 7.6). Thus, the effects of the potential probiotic LAB in maintaining (restoring) host epithelial cell viability and permeability demonstrated strain-strain specificity as reported elsewhere (Bubnov *et al.*, 2018; Leblanc *et al.*, 2017; Wan *et al.*, 2016b; Wang *et al.*, 2018b; Zhang *et al.*, 2018).

The results presented from this study show that the two LAB strains, *L. plantarum* FS2 and *P. pentosaceus* D39, isolated from the West African traditionally fermented cereal, *ogi*, appear to be promising probiotic bacteria candidates with the *in vitro* experiments conducted so far. *P. pentosaceus* has been overlooked as a probiotic strain in preference for *Lactobacillus* and *Bifidobacterium spp*, so inadequate work has been done to establish its mode of action.

Thus, the two LAB hold high potential of being probiotic prophylactic and therapeutic management of inflammatory bowel disease (IBD) as demonstrated by other probiotic bacteria (Khan *et al.*, 2019; Scott *et al.*, 2021; Shigemori and Shimosato, 2017; Sireswar *et al.*, 2019; Vemuri *et al.*, 2017). Thus,

besides limiting EAEC adhesion to the IECs and maintaining intestinal barrier integrity and function by reducing intestinal inflammation, the two presumptive probiotic bacteria from *ogi* can reduce epithelial cell death and IL-8 response to the pathogen. The two potential probiotic LAB, *L. plantarum* FS2 and *P. pentosaceus* D39, isolated from *ogi*, can offer far-reaching applications, which might reduce the incidence of gut enteropathogens by reducing inflammation for the improvement of intestinal epithelial barrier structure and function. However, there is the need to engage in further in vitro studies and investigate the suitability of these potential probiotic bacteria unilaterally or in combinations to translate these interesting in vitro findings to the treatment of IBDs.

# CHAPTER 9.

# CONCLUSIONS AND RECOMMENDATIONS

## 9.1 Conclusions

Results from studies involving the characterisation of the bacterial surface properties show that the potential probiotic bacteria isolated from *ogi* demonstrated diverse capabilities to autoaggregate and co-aggregate with the selected enteroaggregative *Escherichia coli* (EAEC) strains previously isolated from the unpasteurised fresh milk. The results insinuate that besides human, animal and dairy sources, lactic acid bacteria (LAB) isolates from traditionally fermented food like *ogi* have good prospects of inhibiting diarrhoeagenic pathogens, including EAEC, by exercising their antimicrobial actions against them. Thus, the cell surface characteristics demonstrated by the two LAB follow typical probiotics, implying that the LAB isolates obtained from fermented *ogi* might be valuable for prophylactic and therapeutic management of gastroenteritis. This observation further suggests that traditionally fermented cereals can be considered alternative probiotic sources with unique possibilities for addressing inflammatory bowel syndrome.

The results from the adhesion studies indicate that the selected LAB competitively exclude, displaces, and inhibits enteropathogens (EAEC) from adhesion, illustrating that the former could be competent probiotics to be used for preventing and treating persistent diarrhoea under specialised conditions. Additionally, the results from this sub-study show that the two LAB, *L. fermentum*, FS2 and *P. pentosaceus* D39 from *ogi*, like the two reference probiotic bacteria (PBB), *L. acidophilus ATCC* 4356 and *B. bifidum ATCC* 11863 evaluated in this demonstrated significant probiotic properties. They did so by competitively excluding, displacing, and inhibiting the selected EAEC in a strain dependent manner. These processes' high levels of strain-strain specificities should be deemed paramount. This observation further suggests that consideration should be extended to the pathogens and the target

group on a case-by-case basis to characterise LAB for probiotic properties. It will also allow the selection of probiotics with unique competencies to address specific prophylactic or therapeutic needs.

Results from another sub-study involving abilities evaluation of the two LAB, *L. plantarum* FS2 and *Pediococcus pentosaceus* D39 (from *ogi*), to maintain the epithelial barrier integrity and function from the ravaging effects of the selected diarrhoeagenic enteroaggregative *E. coli* (D-EAEC) strains (from the unpasteurised fresh milk) demonstrated their potential to offer valuable biotechniques for the preventive and curative management of intestinal barrier dysfunction. Microbial populations from traditionally fermented foods may serve as an essential pool for specific beneficial microbes like the LAB with crucial traits that can qualify them as probiotic candidates for prophylactic and therapeutic control of several diseases for humans and animals. Valuable microbes such as these can exert diverse beneficial impacts like inhibition of pathogen proliferation and virulence within their host. They can also trigger the host immune response and function by enhancing the intestinal barrier integrity. With the aid of different mechanisms of probiosis, the probiotics may produce varying positive effects at various levels. When administered, the selected probiotics have demonstrated that they can counteract the chronicity of infectious diarrhoea. Accordingly, they can also lessen the period and severity of inflammatory bowel syndromes during therapy. Thus, developing knowledge and expertise in the human microbiome coupled with rational probiotics selection centred on their proven mechanisms of action can optimise and strategize them for different prophylactic and therapeutic objectives. Ultimately, the selected probiotics are anticipated to improve the stability and diversity of the microbial ecology for disease prevention to improve human health.

The last sub-study to evaluate the competence of the two LAB isolates, *L. plantarum* FS2 and *Pediococcus pentosaceus* D39 (from *ogi*), to alleviate cytotoxicity and epithelial barrier permeability. Results from this sub-study indicate that the permeability of the polarized Caco-2 Cell Monolayers (PCC-2CMLs) was compromised mainly by the D-EAEC strains. The decline in the permeability of the selected EAEC strains from unpasteurised fresh milk suggests that the two LAB lessened the

magnitude of intestinal barrier function caused by the EAEC. Intervention with the selected LAB significantly improved epithelial cell viability and barrier function in terms of permeabilities. The above observation could be attributable to the moderation in the expression of genes responsible for the attenuation or enhancement of EAEC-compromised intestinal barrier functions. So, the LAB, *L. plantarum* FS2 and *P. pentosaceus* D39 demonstrated potential probiotics that can be used as food additives to prevent gastroenteritis based on their proven effectiveness in their competitive exclusion, displacement, and inhibition results. There is, however, the need to conduct further *in vitro* and clinical trials with these LAB strains to evaluate their safety and suitability before their integration into food systems for consumption.

## 9.2 Recommendations

The following recommendations are suggested to improve this work to further contribute to the body of knowledge by relating environmental and foodborne diarrhoeagenic *E. coli* (DEC) to food safety and health.

The oral use of probiotics is more common nowadays than before. Nevertheless, issues relating to the use of live probiotics are still debatable. The reason is mainly because of the following possible risk factors: acquisition of antibiotic resistance factors (genes), meddling with gut colonisation in new-borns, or instances involving systemic infections due to translocation into underlying tissues, especially in vulnerable populations and paediatric patients. There are increasing interest in using non-viable (heat-killed or tyndallized) probiotic bacteria (bifidobacteria and LAB) cells to escape these threats. Cell-free supernatants, heat-treated probiotic cells and purified components have been demonstrated to confer beneficial impacts, including immunomodulatory effects, maintenance of intestinal barrier integrity, and protection against enteropathogens.

Similarly, heat-treated microbial cells demonstrated higher surface hydrophobicity than their live counterparts. This was attributed to the change in structural and configurational properties of the outer

cell-wall layers components and their dimensional distribution on the cell wall. In line with these competencies as established by other probiotics and LAB, future research should consider comparing the non-viable (heat-killed or tyndallized) forms of these LAB to live ones for preventing intestinal inflammation caused by EAEC strains. The heat treatment technique should be extended to studies involving their aggregation properties, cell surface hydrophobicity, and their adhesion to the mucus or intestinal epithelial layer.

Studies involving intestinal inflammation and enteropathogens like *E. coli* (EAEC) involve different spectrums of inflammatory, anti-inflammatory, and pro-inflammatory cytokines. In a typical example, whilst *S. thermophilus* NCIMB 41856 elevated an anti-inflammatory response, *E. coli* Nissle 1917 triggered a pro-inflammatory response (Bailey *et al.*, 2011; Hafez *et al.*, 2010). Thus, the activation of epithelial cells, as demonstrated by *E. coli* Nissle, 1917 to stimulate an nuclear factor kappa β (NF-κβ) and interleukin-8 (IL-8) response, was hypothesised to lead to a rise in intrinsic immune defences to improve intestinal barrier function (Pagnini *et al.*, 2010). Accordingly, a spectrum involving at least two cytokines (inflammatory, anti-inflammatory, and pro-inflammatory) should be considered in future studies involving the infliction of inflammation by diarrhoeagenic pathogens.

The virulence and pathogenicity of D-EAEC, like other (diarrhoeagenic) enteropathogens and probiotics' ability to exert their beneficial effects on their host, depend heavily on their ability to survive the ever-changing severe intestinal conditions, including acidic, alkaline and the presence of antibiotics. Therefore, screening the EAEC and LAB strains involved in the current studies is strongly recommended for their tolerance (susceptibility) to different acidic and alkaline conditions and a broad spectrum of antibiotics for the gut. Additionally, the adhesion of both bacteria strains and the competence of the LAB strains to disrupt the EAEC adhesion under these diverse conditions need to be profiled thoroughly.

Probiotic mixtures containing two or more probiotic bacteria have been demonstrated to be more effective in addressing gastrointestinal infections than those with single probiotic bacteria strains. This probiotic efficiency is mainly based on the collective contributions from the different probiotic bacteria strains with unique and diverse competencies. Thus, the various competencies from the other probiotics further complement the efficacy of the probiotic mix against diverse pathogens than those with a single PBB. Therefore, formulating a mixture with two or more probiotic species or strains will render different effects against different enteropathogens, offering a broader spectrum of actions compared to one containing a single probiotic bacteria strain. Accordingly, in future studies, it is recommended to profile the efficacy of the two potential probiotic LAB from *ogi* together with a few probiotics as a mixture against the selected diarrhoeagenic EAEC strains.

The application of whole-genome sequencing is regarded as the golden standard for bacteria identification and characterisation, amongst others. Applying this technique for the genetic (molecular) characterisation of heterogeneous pathogens will offer more information at diverse genome levels extending from their physiological and virulence factors to their phylogenies and phenotypic characteristics. This analytical technique also provides the benefit of assessing newly sequenced strains in other global databases. This analytical tool also provides the ideal platform for comparing the source and geographical location of such pathogens for understanding the emergence of such pathogens as EAEC. Similarly, the technique also offers vital information, including genes relating to antimicrobial and antibiotic resistances, toxic metabolites, and virulence factors that can pose health threats. Therefore, future studies should consider analyzing both EAEC and LAB strains using the whole genome sequencing analytical tool to better understand the potential health risks posed by the pathogens and the competencies and weaknesses of the potential probiotics.

# PUBLICATIONS AND PRESENTATIONS FROM THIS WORK

## 10.1 Peer Reviewed Publications

Agbemavor, W. S. K. and Buys, E. M. 2021. Presumptive probiotic bacteria from traditionally fermented African food challenge the adhesion of enteroaggregative *E. coli*. *Journal of Food Safety*, e12905: e12905.

Agbemavor, W. S. K. and Buys, E. M. 2022. Presumptive probiotics ameliorate the inflammatory response of human intestinal epithelium challenged by enteroaggregative *Escherichia coli*. *Food Research International*, submitted.

## 10.2 Conference presentations

The conference presentations consist of both oral and poster presentations detailed as detailed follows.

### 10.2.1 Oral presentations

Agbemavor, W. S. K. and Buys, E. M. 2019. Adhesion and competitive exclusion of probiotic bacteria against enteroaggregative *E. coli*. 23rd South African Association for Food Science and Technology Biennial Conference. Johannesburg, the Republic of South Africa. (1st – 4th September 2019). Available at https://saafost2019.org.za/provisional-programme-tuesday-3-september-2019/.

Agbemavor, W. S. K. and Buys, E. M. 2018. Adhesion and competitive exclusion of probiotic bacteria against enteroaggregative *E. coli*. 2nd International Conference for Food Safety and Security. Pretoria, the Republic of South Africa. (15th – 17th October 2018). Available at https://www.fsas2018.co.za/index.php

Agbemavor, W. S. K. and Buys, E. M. 2018. The effect of probiotic bacteria on the adhesion of enteroaggregative *Escherichia coli* to epithelial cells. South African Society of Dairy Technology Dairy Students Evening. Pretoria, South Africa. Pretoria, the Republic of South Africa. (30th August 2017).

### 10.2.2 Poster presentations

Agbemavor, W. S. K. and Buys, E. M. 2017. Aggregative and bactericidal effects of probiotic bacteria on enteroaggregative *E. coli*. 22nd South African Association of Food Science and Technology Biennial Conference. Cape Town, the Republic of South Africa. Available at https://www.saafost.org.za/congress/congress-2017/

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