CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Tropical AgriSciences
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Selective combinatory effect of antibiotics with phytochemicals and synthetic analogues of plant-derived compounds against diarrhoea-causing and beneficial intestinal bacteria

DISSERTATION THESIS

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Declaration

I, Hayford Osei-Owusu, hereby declare that this thesis entitled "Selective combinatory effect of antibiotics with phytochemicals and synthetic analogues of plant-derived compounds against diarrhoea-causing and beneficial intestinal bacteria" was written independently, all texts in this thesis are original, and all the sources have been quoted and acknowledged by means of complete references and according to Citation rules of the FTA. I also confirm that this work has not been previously submitted, nor is it currently submitted, for any other degree, to this or any other university.

In Prague, June 17, 2024

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Ing. Hayford Osei-Owusu

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Abstract

Despite being treated and preventable, diarrhoea-associated morbidity and mortality remains an important public health concern among children and adults, especially in developing countries. In addition, antibiotic-associated gut dysbiosis is a significant health problem globally. The development of combinatory agents that increase the selective inhibitory effect (synergism) against diarrhoea-causing pathogens and, at the same time, have a lowered impact (antagonism) or no adverse action on the gut microbiota is therefore suggested as a new strategy effective for diarrhoea treatment and prevention. In this study, the *in vitro* selective combinatory effect of ciprofloxacin with either nitroxoline, sanguinarine, or zinc pyrithione (representing various classes of alkaloid-related compounds) against selected standard diarrhoeagenic (*Bacillus cereus*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Shigella flexneri* and *Vibrio parahaemolyticus*) and gut beneficial (*Bifidobacterium adolescentis*, *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium breve*, *Lacticaseibacillus casei* and *Lacticaseibacillus rhamnosus*) bacteria was assessed using broth microdilution assay. Also, the combined effect of tetracycline together with the above-mentioned alkaloid-related compounds was evaluated against strains of diarrhoea bacteria, including *E. faecalis*, *Escherichia coli*, *L. monocytogenes*, *S. flexneri*, *V. parahaemolyticus*, and *Yersinia enterocolitica in vitro*. The chequerboard method in 96-well microtiter plates was used to determine the sum of the fractional inhibitory concentration indices (FICIs). The preliminary screening results indicated that combinations of alkaloid-related compounds with ciprofloxacin produce strong synergistic interactions. Further experiments showed that the combinations of ciprofloxacin with nitroxoline, sanguinarine and zinc pyrithione exhibited synergistic effect against the diarrhoeic bacteria with FICI values ranging from 0.071 to 0.5 whereas antagonistic effect was achieved at FICI values ranging from 4.012 to 8.023 towards the *Bifidobacterium* strains. Furthermore, the aforementioned combinations exhibited no interaction on *Lacticaseibacillus* strains tested. Ciprofloxacin/zinc pyrithione produced the greatest synergistic action against *S. flexneri*, whereas strong antagonistic interaction was observed towards *B. breve* for ciprofloxacin-nitroxoline combinations. Among combinations of alkaloid-related compounds with other antibiotics, tetracycline, when combined with either nitroxoline, sanguinarine, or zinc pyrithione, demonstrated synergistic effects against most of the pathogenic bacteria tested, with FICI values ranging

from 0.086 to 0.5. Tetracycline-nitroxoline combinations produced the greatest synergistic action against *S. flexneri* at a FICI value of 0.086. These findings indicate that certain combinations of the agents tested in this study can thus be used for the development of new anti-diarrhoeal medications that may have lower or no effect on the gut microbiota. Nevertheless, studies focusing on their *in vivo* anti-diarrheal activity and safety are required before any consideration for utilization in human medicine.

Key words: alkaloid-related compounds, antagonism, bacteria, diarrhoea, dysbiosis, gut microbiota, mortality, synergism.

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List of abbreviations

%	Percent
µg	Microgram
ATCC	American Type Culture Collection
Ca^{2+}	Calcium ions
CCM	Czech Collection of Microorganisms
CFU	Colony-forming unit
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CytK	Cytolysin K
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSMZ	German Collection of Microorganisms and Cell Cultures
EFSA	European Food Safety Authority
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
FICI	Fractional inhibitory concentration index
gm	Gram
GRAS	Generally Recognized As Safe
Hbl	Hemolysin BL
IBS	Irritable bowel syndrome
kg	Kilogram
KP	Kanagawa phenonemon
LAB	Lactic acid bacteria
LD_{50}	Median lethal dose
mg	Milligram
Mg^{2+}	Magnesium cation
MHB	Mueller-Hinton broth
MIC	Minimum inhibitory concentration

mL	Milliliter
Mn^{+2}	Manganese ions
NaCl	Sodium chloride
NCTC	National Collection of Type Cultures
Nhe	Non-hemolytic enterotoxin
NTX	Nitroxoline
ORS	Oral rehydration salt
QPS	Qualified Presumption of Safety
SCCS	The Scientific Committee on Consumer Safety
SCFAs	Short-chain fatty acids
ShET	*Shigella* enterotoxin
SNG	Sanguinarine
T3SS	Type III secretion system
TDH	Thermostable direct hemolysin
TET	Tetracycline
TRH	TDH-related hemolysin
UK	United Kingdom
UNICEF	United Nations Children's Fund
USA	The United States of America
UTIs	Urinary tract infections
UV	Ultraviolet
WCH	Wilkins-Chalgren broth
WHO	World Health Organization
ZPT	Zinc pyrithione
μL	Microliter
Σ	Sum

1. Introduction

The human gut is a complex ecosystem, with diverse microbial community (e.g., viruses and bacteria) known as the gut microbiota (Akhlash 2021). It is mostly composed of two dominant bacterial phyla Bacillota and Bacteroidota that represent over 90% of the total community, and by other subdominant phyla including Pseudomonadota, Actinomycetota, and Verrucomicrobiota (Magne et al. 2020; Cani et al. 2021, Oren & Garrity 2021). Under standard conditions, these microorganisms live in symbiotic relationship with the host. However, disruption in the composition and function of the microbial community, defined as gut microbiota dysbiosis, is associated with gastroenteric disorders including diarrhoea (Illiano et al. 2020; Li et al. 2021**)**. Because increased bowel movement and fluid secretion destabilise the gut environment, diarrhoea represents a significant event in gut dysbiosis condition (Chung & Le 2022). Antimicrobial therapy using antibiotics of different classes, such as fluoroquinolones, and tetracyclines is currently conventional approach to treat diarrhoea infections (Diniz-Santos et al. 2006). However, extensive antibiotic use can have some negative effects on the gut microbiota, including altered metabolic activity, reduced species diversity, increased gut susceptibility to invasion and colonization of bacterial pathogens, and stimulated development of bacterial antibiotic-resistance, that can lead to antibiotic-associated diarrhoea and recurrent *Clostridioides difficile* infections (Ramirez et al. 2020). For example, it has been documented that administration of gentamicin, meropenem, and vancomycin resulted in an increase in the prevalence of *Enterobacteriaceae* and a decrease in bifidobacteria in adult (Palleja et al. 2018). Phytochemicals have been reported to possess active biological properties that can positively modulate the composition of gut microbiota through selective inhibition of certain microbes in the intestine (Dingeo et al. 2020; Santhiravel et al. 2022). Recently, the development of combined antimicrobial agents that can increase their selective inhibitory effect (synergism) against intestinal bacterial pathogens and, simultaneously, have a lowered impact (antagonism) or no negative action on the gut microbiota was suggested as a new strategy efficient for the treatment of diarrhoeal conditions.

Combination therapy based on two or more antimicrobial agents is an emerging pharmacological approach effective for treating various bacterial infections since these agents can produce different mechanistic actions that can reduce the dosage of individual antimicrobials and expand the spectrum of antimicrobial activity against those infections (Windiasti et al. 2019). For example, risorine capsule (Cadila Pharmaceuticals Ltd, India), is a combination medicine used in practice for the treatment of tuberculosis. It composes of natural substance (piperine) and commercial antibiotics (rifampicin, and isoniazid) (Chawla 2016). Several studies have demonstrated that conventional antibiotics as well as phytochemicals and their derivatives appeared to be promising for research on their selective and synergistic growth-inhibitory activity against diarrhoea-causing bacteria. For example, ciprofloxacin, a broad-spectrum fluoroquinolone antibiotic commonly used against diarrhoea infections (Ericsson et al. 1987; Heck et al. 1994), has shown a considerable *in vitro* growth-inhibitory effect against clostridia (*C. difficile* and *C. perfringens*) when combined with metronidazole (Werk & Schneider 1988). Furthermore, research conducted and published by Kudera et al. (2020) indicated the selective antibacterial activity of ciprofloxacin against diarrheic bacteria (e.g., *Shigella flexneri*) with a lower effect on beneficial strains (e.g., *Bifidobacterium adolescentis*). However, research focused on the identification of anti-diarrhoeal agents producing both combinatory and selective antimicrobial actions are completely missing. Among the classes of antimicrobials that have been extensively studied, active phytochemical compounds, alkaloids, seem to be prospective candidates producing synergistic effects. For example, tetracycline, a broad-spectrum bacteriostatic antibiotic, in the class of tetracyclines commonly used for the treatment of several infectious diseases, including cholera (Britannica 2012) has demonstrated synergistic effect with chanoclavine, an alkaloid compound isolated from the seeds of *Ipomoea muricata*, against clinical isolate strains of *Escherichia coli* (Dwivedi et al. 2019). Above mentioned data suggest that determination of both combinatory and selective inhibitory effects of commonly used antimicrobial agents (e.g. antibiotics) with plant-derived antimicrobial agents against diarrhoea-causing and gut beneficial bacteria is promising approach for development of more efficient and safer preparations for treatment of infectious diarrhoea.

2. Literature Review

2.1 Gut microbiota

The gut microbiota refers to the entire population of microbial organisms residing in the human gastrointestinal tract including archaea, bacteria, fungi, protozoans, and viruses (Sekirov et al. 2010; Jandhyala et al. 2015) whereas the collective genome of these microorganisms inhabiting the gut is termed as gut microbiome (Berg et al. 2020). It has been reported that the dominant gut microbial communities are compose of eubacterial phyla Bacteroidota, Cyanobacteriota, Bacillota, Fusobacteriota, Pseudomonadota, Verrucomicrobiota, and archaebacterial phylum Euryarchaeota (Ghosh et al. 2021; Oren & Garrity 2021). Among these phyla, Bacteroidota and Bacillota predominate the gut (Pushpanathan et al. 2019). It has been mentioned that the gut microbiota maintains a symbiotic relationship with the host and regulates several important functions including host metabolism, immunity, and intestinal barrier function (Lobionda et al. 2019). The state of microbial imbalance in the gut could be due to the gain or loss of community members or changes in relative abundance of microbes (Messer & Chang 2018). Therefore, disruption of the gut microbial community which results in an imbalance of the microbiota (dysbacteriosis), triggers changes in their functional composition and metabolic activities, and may contribute to many diseases, including inflammatory bowel disease, irritable bowel syndrome (IBS), and diarrhoea (Zuo & Ng 2018; Illiano et al. 2020; Li et al. 2021).

2.2 Diarrhoeal disease

Diarrhoea is one of the most common infant diseases prevalent in both developing and developed countries (Manetu et al. 2021). It is a major cause of morbidity and mortality, contributing to over 4 to 5 million human deaths annually (Agunu et al. 2005). Furthermore, it remains a problem, especially for children younger than 2 years of age living in South Asia and sub-Saharan Africa (Carvajal-Vélez et al. 2016), and countries in this region accounts for almost 90% of global diarrhoeal deaths in children (Manetu et al. 2021). It has also been documented that incidence of death resulting from this infection

among adults which are above 70 years is three times more than children under five (Srivastava et al. 2022). According to United Nations Children's Fund (UNICEF) and World Health Organization (WHO) (2009), diarrhoea is characterized by loose or watery stools at least three times per day or more frequently than normal for an individual. Diarrhoea is usually a symptom of an intestinal infection, which can be caused by a variety of infectious agents including, bacteria, viruses and parasites. *Campylobacter jejuni*, *Clostridium difficile*., Diarrhoeagenic *Escherichia coli* spp., *Salmonella* spp., *Shigella* spp., *Vibrio* spp., and *Yersinia* spp. are the commonest representatives of bacteria associated with this infection (Njume & Goduka 2012; Who 2017; Roshan et al. 2018). Additionally, the incidence of diarrhoea-causing pathogens varies between developed and developing world settings. In developed countries, about 70 % of diarrhoea cases are of viral, 10–20 % of bacterial and less than 10% of protozoal origin whereas in developing countries 50–60 % of cases are of bacterial, 35 % of viral (Cooke 2010), and about 12% of intestinal parasites origin (Yemata et al. 2020). Diarrhoea is spread through contaminated food or drinking-water, or from person-to-person as a result of poor hygiene (WHO 2017). Oral rehydration therapy is the mainstay of treatment for diarrhoea, including illnesses for which antibiotics are indicated, such as cholera and shigellosis (Santosham & Reid 1986; Quraishi et al. 2018). For example, ciprofloxacin is a drug of choice for the treatment of *S*. dysentery (Who 2005) and doxycycline is a first line for the treatment of *Vibrio cholerae* infection (Gtfcc.org 2022). However, misuse and extensive use of antibiotics is associated with negative effects, such as the development of multidrug bacterial resistance (Sanhueza et al. 2017) and dysbiosis of gut microbial community (Francino 2015). Khaneghah et al. (2020) has reported that the ecosystem of the gastrointestinal tract is a repository of beneficial bacteria (e.g., *Bifidobacterium* and *Lacticaseibacillus*), which are interacting with the epithelia of intestinal cells and strengthening the intestinal barrier against invasion and adherence of diarrheic pathogens. Under these circumstances, alternative antimicrobials, and strategies, such as selective combination therapy, are promising to prevent the emergence of multidrug resistance in bacteria, intestinal dysbiosis, and reduce the prevalence of diarrhoea.

2.3 Diarrhoea-causing bacteria

Several studies have reported diverse pathogenic bacteria, that are associated with diarrhoea in public health settings (Tribble 2017; Afum et al. 2022). These infectious agents belong to the group of Gram-negative (e.g., *E. coli*, and S*higella* spp.) and Gram-positive (e.g., *C. difficile*., and *L. monocytogenes*) aerobic or anaerobic bacteria that reside mostly in the intestine to induce diarrhoea (Bagdasarian et al. 2015; Beyene et al. 2022). The most important bacteria causing diarrhoea and the leading aetiology are described below.

2.3.1 *Bacillus cereus*

It is a Gram-positive, aerobic-to-facultative, motile, and spore-forming rod bacteria primarily associated with gastrointestinal diseases, namely emetic and diarrhoeal syndromes (Bottone 2010; Jeßberger et al. 2014). It is ubiquitous in the environment (e.g., in soil, dust and plants) and this pathogen is estimated to be the causative agent for about 1.4%–12% of all food poisoning cases worldwide (Dietrich et al. 2021). In addition, this species has been reported to cause severe local and systemic infections such as bacteraemia, bacterial pneumonia, brain abscess, endocarditis, endophthalmitis, meningitis, necrotizing skin and soft-tissue infections, ocular keratitis, osteomyelitis, and pyelonephritis (Wright 2016). Previous literature has reported that the ability of *B. cereus* to cause disease whether intestinal or non-intestinal is associated with tissue-destructive and reactive exoenzyme production (Bottone 2010). Furthermore, it has been documented that diarrhoeal type of food poisoning (characterized by abdominal cramps and watery or profuse diarrhoea), is caused by enterotoxins produced during vegetative growth of the bacteria in the small intestine whereas the emetic type (characterized by nausea, and abdominal cramps), that result in vomiting is produced by growing cells in the food (Lund & Granum 1997; Nguyen & Tallent 2019). Although this bacterial species produces various enterotoxins including, cytolysin K (CytK), enterotoxin FM, enterotoxin T, hemolysin BL (Hbl), hemolysin II, and non-hemolytic enterotoxin (Nhe), diarrhoea is caused by the production of three different heat-labile enterotoxins CytK, Hbl, and Nhe in the gut (Zeighami et al. 2019). In addition, the total infective dose reported to cause

food poisoning, ranges between 10^5 and 10^8 cfu/g viable cells or spores. Hence, any food containing more than 10^3 cfu/g of *B. cereus* is considered unsafe for consumption (Granum & Lund 1997). Oral rehydration therapy is the treatment for *B. cereus* infection, and intravenous fluid hydration is also recommended in severe cases (McDowell & Sands 2023).

2.3.2 *Clostridium difficile*

C. difficile is a fastidious Gram-positive, anaerobic, spore-forming and toxin-producing bacillus that causes infections of the gastrointestinal tract, and it is responsible for about 15% to 20% of antibiotic-related cases of diarrhoea and almost all cases of pseudomembranous colitis (Schroeder 2005; Schäffler & Breitrück 2018). This bacterium has been frequently reported to cause nosocomial diarrhoea, which has been associated with epidemics in hospitals and long-term care facilities, increased healthcare utilization, and morbidity (Evans & Safdar 2015). The mortality associated with *C. difficile* infectious diarrhoea is estimated between 17% and 25% among the elderly and its infection is spread by the faecal-oral route. Furthermore, this pathogen is ubiquitous and widely distributed in nature, and produces infectious spores that are resistant to disinfection and harsh environments, thereby enhancing the spread of its infections over distance and nosocomial transmission (Dilnessa et al. 2022). It has previously been reported that patients treated by antibiotics such as cephalosporins, clindamycin, and fluoroquinolones have disrupted intestinal microbiota which allows overgrowth of *C. difficile* (Evans & Safdar 2015). This condition may increase the development of *C. difficile* infection, impairment in humoral immunity, renal disease, and hypoalbuminemia (Di Bella et al. 2016). Also, in the absence of antibiotics, cytotoxic chemotherapeutic agents have been reported to cause *C. difficile*-associated diarrhoea, (Anand & Glatt 1993). It has been mentioned that this bacterium produces exotoxins (toxin A and B) in addition to binary toxin, whereby toxin A is identified to play critical role in the pathogenesis of *C. difficile* diarrhoea because it is associated with extensive tissue damage and fluid accumulation. Occasionally, events that must occur before development of *C. difficile* diarrhoea include, alteration of the normal faecal flora, colonic colonization with toxigenic *C. difficile* and

growth of the organism with elaboration of its toxins (Poutanen & Simor 2004). Antimicrobial therapy recommended for management of *C. difficile* diarrhoea are fidaxomicin, metronidazole and vancomycin (McDonald et al. 2021).

2.3.3 *Escherichia coli*

E. coli is a Gram-negative, rod-shaped, non-sporulating, facultative anaerobic bacterium that lives in the intestines of warm-blooded animals and human (Delmas et al. 2015). It has been reported that this bacterium resides in the gut microbiota, which consists of more than 500 species estimated at 10^{10}-10^{11} cells per gram of large-intestinal (caecum and colon) content. In addition, it is considered as the predominant aerobic microbes in the gastrointestinal tract (Tenaillon et al. 2010). Although most of *E. coli* strains coexist in good health and with mutual benefit but may cause disease in immunocompromised hosts or where the normal gastrointestinal barriers are breached (Kaper et al. 2004). Previous literature has documented different pathotypes of diarrheagenic *E. coli*, including diffusely adherent *E. coli*, enteroaggregative *E. coli*, enterohemorrhagic (Shiga toxin-producing) *E. coli*, enteroinvasive *E. coli*, enteropathogenic *E. coli*, and enterotoxigenic *E. coli* which are the primary cause of diarrhoea, vary regarding their preferential host colonization sites, virulence mechanisms, clinical symptoms and consequences (Le Bouguénec & Servin 2006; Gomes et al. 2016). Eybpoosh et al. (2021) have reported that the incidence of *E. coli* pathotypes may differ by geographic region, stating that enterohemorrhagic *E. coli* serotypes (O157:H7) are prevalent in the United States and Canada and non-O157:H7 serotypes are commonly found in Latin America and Europe, whereas enterotoxigenic *E. coli* are pervasive in the developing world. It has been mentioned that three general clinical syndromes can result from infection with one of the above mentioned pathotypes, namely enteric/diarrhoeal disease, urinary tract infections (UTIs) and sepsis/meningitis (Nataro & Kaper 1998). Nataro and Kaper (1998) have further described that the pathogenetic strategy for diarrheagenic *E. coli* to cause diarrhoea in a compromise host is as a result of their ability to produce enterotoxins, invade the intestinal epithelium cells as well as adhesion with specific intestinal mucosa membrane. It has previously been reported that pathogenic

strains of *E. coli* have specific adhesion factors which allow them to colonise the intestine, namely fimbrial and afimbrial (that involved the outer membrane proteins such as intimin) adhesins. In addition, this bacterium produces endotoxins and exotoxins whereby the latter is categorised into enterotoxins, cytotoxins, and neurotoxins (Castro et al. 2021). Out of these, enterotoxins are implicated to cause watery diarrhoea (Martín-Rodríguez et al. 2022) and as a result of cytotoxins production, inflammatory diarrhoea is occurred which is characterised by bowel movements containing blood and mucus (Navaneethan & Giannella 2008). Prevention of dehydration by correcting fluid and electrolyte imbalances using oral or parenteral rehydration therapy is a recommended approach to treat diarrhoea caused by *E. coli* (Nataro & Kaper 1998). In addition, fluoroquinolones including ciprofloxacin, levofloxacin, norfloxacin, and ofloxacin have been the drugs of choice for the treatment of *E. coli*-associated diarrhoea in adults (Glandt 1999; Diemert 2006).

2.3.4 *Listeria monocytogenes*

L. monocytogenes is a Gram-positive bacillus, non-spore forming, motile rod-shaped, and facultative anaerobic bacterium responsible for listeriosis in humans (McLauchlin et al. 2004; Mehmood et al. 2017). This organism is ubiquitous in the environments, and it is readily inactivated at pasteurization temperature to cause infection after ingestion of contaminated food (Michel et al. 1998; Schuppler & Loessner 2010). It has been reported that the prevalence of asymptomatic stool carriage of *L. monocytogenes* in healthy adults is about 1%–5% (Ooi & Lorber 2005). Although its main route of spread is through faecal-oral transmission, it can also infect new-borns through the placenta and birth canal (Shi et al. 2021). Encephalitis, endocarditis, granulomatous intrauterine infection, hepatic necrosis, meningoencephalitis, myocarditis, septicaemia in infants, spontaneous abortion in pregnant women, skin problems, and gastrointestinal infections (e.g., diarrhoea) are common symptoms associated with listeriosis in humans (Ooi & Lorber 2005; Abbasi et al. 2019). The mechanism by which this bacterium causes diarrhoea is not elucidated. It is however reported to be possible by its direct invasion of the epithelial cells of the intestinal mucosa through the enterocytes lining the absorptive

epithelium of the microvilli and translocation across the M-cells of Peyer's patches (Schuppler & Loessner 2010). Diarrhoea caused by *L. monocytogenes* is non-bloody, and watery stool with an average of 10 to 12 stools in 24 hours. In addition, out of the reported thirteen serotypes of this species, only serotypes 4b, 1/2a, and 1/2b are identified to cause over 98% of the human listeriosis, whereby serotype 4b has been incriminated for causing invasive listeriosis (Mehmood et al. 2017). Although several antibiotics have been demonstrated to be effective against infections of this pathogen, combination therapy of ampicillin/gentamicin or ampicillin alone remains the treatment of choice (Temple & Nahata 2000).

2.3.5 *Shigella flexneri*

Shigella species are Gram-negative, non-spore forming, non-motile, rod-shaped, and facultative anaerobic bacteria closely related biochemically and antigenically to *E. coli* (Torres 2004). These bacteria belong to the family of Enterobacteriaceae that are associated with healthcare-related infections, including bacillary dysentery, also known as shigellosis (Jafari-Sales & Shariat 2021; Britannica 2022). Shigellosis is a major public health problem worldwide (Njuguna et al. 2013), and it is one of the common childhood infections, especially those under 5 years in developing countries. It is also common in persons who travel from industrialized to less developed countries (Abu-Elyazeed et al. 2004). It has been estimated that about 113 million episodes of shigellosis occur annually, causing more than 400,000 deaths in the developing world (Kotloff et al. 1999). It has been reported in literature that shigellosis is normally characterised by passing watery diarrhoea with bloody mucous in stool, and the patient may also show clinical symptoms of abdominal cramp, fever, and tenesmus (Nisa et al. 2020). *Shigella* infection is spread, but generally restricted to the intestinal lining, where it leads to colonic inflammation, mucosal ulceration, and a loss in intestinal barrier function (Schnupf & Sansonetti 2019). Additionally, this bacterium is found in the intestinal tract of infected people and transmitted through the faecal-oral route or through ingestion of contaminated food and water and also via person-to-person contact (Ranganathan et al. 2019; Jafari-Sales & Shariat 2021). *Shigella* maintains a large number of proteins integral to metabolism, T3SS

structure, effector function, transcriptional and posttranscriptional regulation, growth, and virulence, which all undergo tyrosine phosphorylation (Duchen et al. 2021). There are four main species of *Shigella*, namely, *S. boydii, S. dysenteriae, S. flexneri,* and *S. sonnei* and more than 40 serotypes identified according to characteristics of the biochemical and serological properties (Gupta et al 2004). Among these subgroups, *S. flexneri* which is classified into at least twenty serotypes has been identified as the most frequently isolated species worldwide (Who 2006; Zhu et al. 2017). This species is common and accounted for 60% of cases in the developing countries. Furthermore, *S. sonnei* causes 77% of cases in the developed world as compared to only 15% of cases in the developing world, and *S. dysenteriae* is usually the cause of epidemics of dysentery (Who 2006). It has previously been discussed that *Shigella* has a low infectious dose of 10 to 100 bacteria that is sufficient to cause infection (DuPont et al. 1989; Schnupf & Sansonetti 2019). The bacterium transits across the colonic epithelial layer through M cells, and then invades epithelial cells of the colon from the basolateral face (Killackey et al. 2016). Inside the host cell, the bacterium evolves effector proteins that alter the metabolism of the target cell. This can lead to the lysis of vacuolar membranes or reorganization of actin polymerization to facilitate the intestinal epithelium colonisation (Ashida et al. 2015). It has been documented that diarrhoea is an early symptom of shigellosis when the bacterium reaches the small intestine (Killackey et al. 2016).

S. flexneri produces enterotoxins 1 and 2 (ShET1 and ShET2) which are encoded on the chromosome and plasmid, respectively (Faherty et al. 2012). The ShET2 is more widespread and detectable in 80% of all four *shigella* species whereas ShET1 is present on the chromosome of *S. flexneri* serotype 2a. These enterotoxins induce the diarrhoeal prodrome that often precedes bacillary dysentery . In addition, *S dysenteriae* serotype 1 expresses Shiga toxin, cytotoxin that inhibits protein synthesis in susceptible mammalian cells. This toxin role in human diarrhoea is uncertain, however, experimental infection of rhesus monkeys with *S. dysenteriae* 1, and with a Shiga toxin-negative mutant, indicates that cytotoxin causes capillary destruction and focal haemorrhages that worsens dysentery. Shiga toxin is associated with haemolytic-uremic syndrome, a complication of infections with *S dysenteriae* (Hale et al. 1996). Also, other life-threatening problems, such as bacteraemia, reactive arthritis, and toxic megacolon are reported (Gupta et al

2004). In adults and children, oral azithromycin and ciprofloxacin are commonly recommended for the treatment of shigellosis as first-line therapy. In case of severe infection and patients having weak immune system, parenteral ceftriaxone is advised (Nisa et al. 2020).

2.3.6 *Vibrio parahaemolyticus*

It is a Gram-negative, rod-shaped, non-spore forming, facultative anaerobic, oxidase positive and motile bacterium with a single polar flagellum ubiquitous in the sea and in estuaries (Yeung & Boor 2004; Ramamurthy & Nair 2014). This bacterium belongs to the family *Vibrionaceae*, that is considered as one of the important pathogens of seafood-borne diarrhoeal diseases in humans (Pazhani et al. 2021). Incidence of vibriosis is associated with *V. parahaemolyticus* infections (Amato et al. 2022). Previously, cases of this species were restricted to Japan. Nowadays, its infection is reported in diverse geographical locations, including most Asian countries, Africa, Europe, New Zealand, and the USA where there are about 30,000 infections each year (Baker-Austin et al. 2018; Letchumanan et al. 2019). This pathogen causes acute gastroenteritis, which is characterised by abdominal pain, diarrhoea, fever, nausea, and vomiting, especially in areas where consumption of seafood is high. Also, it involves in causing fulminant necrotising fasciitis, wound infections, and septicaemia (Makino et al. 2003; Ghenem et al. 2017). Its infection does not spread via person-to-person transmission or the faecal–oral route but rather, by consumption of raw or undercooked contaminated seafood (Baker-Austin et al. 2018). It has been suggested that infection caused by this pathogen disrupts lining of the intestine, which creates epithelial degeneration and denudation of the intestinal sites, resulting in watery diarrhoea (Ritchie et al. 2012). *V. parahaemolyticus* is classified by serotyping and the serotypes of this pathogen are determined by the combination of somatic (O) and capsular (K) antigens. Following this determination, about 13 O serotypes and 71 K serotypes have been reported (Li et al. 2016). Furthermore, three serotypes, including O3:K6, O4:K68, and O1: K untypeable (KUT) strains have been recognised to cause *V. parahaemolyticus* pandemic (Zamora-Pantoja et al. 2013). Also it has been documented that between 2007

and 2012, this species was the leading cause of diarrhoea infection in the southern China region, and the predominant isolates were serotype O3:K6 strains (Baker-Austin et al. 2018).

It has previously been reported that a protein secreted by *V. parahaemolyticus*, known as thermostable direct hemolysin (TDH), has been the main pathogenic factor suspected to be an enterotoxin involved in most cases of *V. parahaemolyticus* diarrhoea (Raimondi et al. 2000). The TDH is the major virulence gene of this bacterium which is present in most of the clinical Kanagawa phenonemon (KP)-positive strains (Li et al. 2014). Cardiotoxicity, cytotoxicity, and enterotoxicity, activities have been confirmed in TDH. In addition, TDH-related hemolysin (TRH) which is produced by KP negative strains is hemolytic, heat labile and it is also assumed to play a role in diarrhoea infections. It has been described that the human host shows a strong systemic response and produces mucosal B-cells against these antigens, after the bacterium had produced TDH and lipopolysaccharides released from a dead cell (Li et al. 2014). Similarly, *V. parahaemolyticus* encrypts two type 3 secretion systems (T3SSs) (Ritchie et al. 2012), which are located in different chromosomes. The type 3 secretion system 1 (T3SS1) is essential for cytotoxicity whereas the type 3 secretion system 2 (T3SS2) is commonly responsible for enterotoxicity and intestinal fluid accumulation **(**Li et al. 2014; Letchumanan et al. 2019). Most cases of *V. parahaemolyticus* infection are self-limiting and can therefore be treated by oral rehydration. However, occasional treatment with antibiotics like ciprofloxacin, doxycycline, and erythromycin is required (Yeung & Boor 2004).

2.3.7 *Yersinia enterocolitica*

Y. enterocolitica is a rod-shaped facultative anaerobic, nonspore-forming Gram-negative bacterium found in nature, especially in animals, food, and water (Shoaib et al. 2019). This species belongs to the family *Enterobacteriaceae* (Hertwig et al. 2013). It is a causative agent of intestinal yersiniosis, a diarrhoeal disease that is characterised by acute febrile diarrhoea, abdominal pain (mimicking appendicitis), and mesenteric lymphadenitis that can result in systemic infections (Guerrant et al. 1990; Rakin et al.

2014). Detection of post infection sequelae as a consequence of *Y. enterocolitica* infection, including reactive arthritis and erythema nodosum have been reported (Fàbrega et al. 2015). Additionally, enterocolitis with an inflammatory diarrhoea in affected children is mostly observed whereas older children and adolescents usually show pseudo-appendicitis syndrome, empyema, axillary abscesses, and endocarditis (Sabina et al. 2011). Transmission is through infected/contaminated food handlers, nosocomial infections, and infected blood (Mancini et al. 2022). Pigs are considered the main reservoir of pathogenic *Yersinia* strains (EFSA 2010). Shoaib at al. (2019) have further reported that about 35-70% of swine herds and 45-100% of individual pigs harbour *Y. enterocolitica*. The incidence of yersiniosis due to pork consumption in humans has been estimated at 2.8 cases per 100 000 inhabitants per year in Europe (Rakin et al. 2014). Also, out of an estimated 177 000 cases of yersiniosis that occur in the US each year, 90% are foodborne (Shoaib et al. 2019). *Y. enterocolitica* infective dose for human infections is about 10^8-10^9 CFU mL^{-1} (Mancini et al. 2022). This bacterium is composed of a biochemically and genetically heterogeneous group of organisms that are divided into 6 biotypes, namely, 1A, 1B, 2, 3, 4, and 5. In addition, *Y. enterocolitica* has over 70 distinct serotypes, on the basis of antigenic variations in cell wall lipopolysaccharide (Sabina et al. 2011; Rakin et al. 2014). The biotypes 1B, 2, 3, 4 and 5 are frequently isolated in human infections, whereas non-pathogenic 1A strains are ubiquitous in the environment (Rakin et al. 2014; Mancini et al. 2022). In contrast, it has previously been reported that a significant proportion of *Y. enterocolitica* isolates obtained from patients with diarrhoea belong to biotype 1A (3, 6, 17, 32, 34, 39, 46, 47) (Grant et al. 1998). *Y. enterocolitica* infection is geographically distributed with different strain-specific prevalence (Hering et al. 2016). For instance, serotype O:8 is common in Japan and, in the USA, whereas O:3 is predominantly found in Australia, Canada, China, and Europe. Additionally, serotype O:9 is prevalent in the Netherlands and in Scandinavia (Sabina et al. 2011). It has also been reported that the human pathogenic strains commonly isolated worldwide are serogroups O:3, O:5,27, O:8 and O:9 (Fàbrega & Vila 2012). In animal models, rabbits inoculated with serotype O:9 *Y. enterocolitica* strains resulted in diarrhoea and deaths (Bottone 1997). Pathogenesis of this bacterium is partly understood. Nevertheless, most of the isolates have pathogenic properties that enable them to penetrate the intestinal wall,

which is controlled by 70-kb virulence plasmid genes. They also have the ability to produce heat-stable enterotoxin which is controlled by set of chromosomal genes (Grant et al. 1998). The bacterium starts to colonise the intestinal tract, specifically the distal small intestine (terminal ileum) and proximal colon which are considered as the main sites for infection (Shoaib et al. 2019). Then, it uses virulent gene to traverse the intestinal lumen, attach, and penetrate the mucus barrier overlying the mucosal epithelial cells where it finally adheres to intestinal cells. Consequently, it binds and penetrates M cells of Peyer's patches (Autenrieth & Firsching 1996). Once internalized, the bacterium is transported across the epithelial barrier and expelled from the basolateral side of the M cell. Inside of the cell, *Y. enterocolitica* replicates in native murine macrophages and moves within the migrating phagocytes to mesenteric lymph nodes, causing an inflammatory response that triggers abdominal pain (Fàbrega & Vila 2012). Most of the gastrointestinal infections caused by *Y. enterocolitica* are self-limiting and do not require specific treatment as the colon will heal on its own. However, in case of severe enteritis, treatment by antibiotics including aminoglycosides, co-trimoxazole, fluoroquinolones, tetracycline, and third generation cephalosporins is necessary (Mills 2014).

2.4 Probiotic/beneficial bacteria

Probiotics are defined as live microorganisms, which when consumed in sufficient amounts confer a health effect on the host (Guarner & Schaafsma 1998; Fao/Who 2001). The majority of probiotics belong to a significant group of natural non-pathogenic bacteria, including bifidobacteria, *Lacticaseibacillus*, and streptococci, which harmlessly existed in the human gastrointestinal microbiota (Gionchetti et al. 2000). Furthermore, previous literature has reported that bifidobacteria and *Lacticaseibacillus* reside in the large and small intestinal tract, respectively (Levison 2022). They are essential for health and have been used in various dietary supplements and functional foods for decades, whereby their effect on human health is extensively discussed and investigated (Saarela et al. 2002; Fontana et al. 2013; Kerry et al. 2018). The US Food and Drug Administration (FDA) has considered probiotic microorganisms, primarily lactic acid bacteria (LAB) in the category of "Generally Recognized As Safe (GRAS)" (Dahiya & Nigam 2022).

According to the European Food Safety Authority (EFSA), several probiotic LAB strains can be regarded to have "Qualified Presumption of Safety (QPS)" status (Yadav et al. 2022). The International Dairy Federation has recommended that probiotic products should contain at least 10^7 cfu/g of *L. acidophilus* and 10^6 cfu g^{-1} of bifidobacteria in fermented milk products at the time of sale (Roy 2011). It has been reported that the concentration of probiotics required to achieve clinical effect is commonly quoted as $\geq 10^6$ cfu/ml and $\geq 10^8$ cfu/g in the small bowel and the colon respectively (Minelli & Benini 2008). Probiotics when administered at standard doses of 10^7-10^8 cfu/day against antibiotic-associated diarrhoea, *C. difficile*-associated diarrhoea and diarrhoea caused by bacteria or virus, reduced the stool frequency and mean duration of infection in adults and children (Minelli & Benini 2008). Another study reports that the minimum effective dose of probiotic which can affect the intestinal environment to provide human health is 10^6-10^9 live microbial cells per day (Linares et al. 2016). Several action mechanisms of probiotics against intestinal infections have been elucidated. Probiotics may manipulate intestinal microbial communities and suppress growth of pathogens by inducing the host's production of β-defensin and Immunoglobulin A (Hemarajata & Versalovic 2013). Also, probiotic organisms may strengthen the intestinal barrier by maintaining tight junctions and inducing mucin production. In addition, probiotic-mediated immunomodulation may occur via mediation of cytokine secretion through signalling pathways, which stimulates the proliferation and differentiation of immune cells such as T cells or epithelial cells (Hemarajata & Versalovic 2013). Its ability to inhibit pathogens by producing various inhibitory substances, including acetaldehyde, acetoin, bacteriocins, carbon dioxide, diacetyl, hydrogen peroxide, organic acids, and short-chain fatty acids (SCFAs), suggested to have potential antimicrobial effects (Ammor et al. 2006; Tharmaraj & Shah 2009). Furthermore, probiotics compete for inhibition on the intestinal epithelial surface by blocking the adhering sites to reduce pathogens interaction (Yadav et al. 2022). Lastly, probiotic microorganisms demonstrate antagonistic mechanisms by competing and inhibiting pathogens that consume and deprive the host of essential nutrients (Terpou et al. 2019).

2.4.1 Bifidobacteria

They are gram-positive, non-motile, and non-spore-forming anaerobic bacteria with pleomorphic rods (Lim & Shin 2020). These species are ubiquitous in the human orogastrointestinal tract and vagina (Esaiassen et al. 2017), whereas isolates found in the gastrointestinal tract of various mammals, birds and insects are also reported (Pino et al. 2022). The genus *Bifidobacterium* belongs to the phylum Actinomycetota, and the of family *Bifidobacteriaceae* (Bottacini et al. 2014). Currently, they comprise of 94 identified (sub)species which are commensal in the gastrointestinal tract of humans and animals (Saturio et al. 2021). Bifidobacteria constitute over 80% of the intestinal microbiota of breast-fed infants and about 3 to 6% of the adult faecal flora (Esaiassen et al. 2017). They are mainly present in the colon of the intestine which form significant part of the natural human intestinal microbiota (Liong & Shah 2005). Following birth, this bacterium rapidly colonises and predominates the intestinal microbiota of infant (Colston et al. 2022). Their level of population decreases in elderly because of age, change in diet, and reduced secretion of gastric juices (Shah 2011). *B. adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. dentium*, *B. longum*, *B. pseudocatenulatum*, and *B. pseudolongum* are some of the species commonly found in humans, whilst *B. animalis* subsp. *lactis* is mostly included in food supplements and functional foods (Saturio et al. 2021). Additionally, *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, and *B. longum* have obtained the QPS status by EFSA as safe biological agents which can be added and consumed in various foods (Hidalgo-Cantabrana et al. 2017). Inside the intestine, the interactions between bifidobacteria and epithelial cells are mediated by proteins or glycoproteins which bind fatty acid fractions of lipotechoic acids. In addition, certain strains including *B. infantis* are reported to secrete polysaccharides that initiate adhesion of intestinal epithelial cells (Shah 2011). *Bifidobacterium* species metabolise and ferment human milk oligosaccharides by using glycosyl hydrolases to produce SCFAs, that improve maintenance of intestinal barrier integrity and anti-inflammatory functions. Also, it involves in the metabolism of aromatic amino acids, including phenylalanine, tryptophan, and tyrosine that produce aromatic lactic acids (4-hydroxyphenyl acetic , indolelactic , and phenyllactic acids, which have antibacterial and anti-inflammatory

activities (Sadeghpour & Hu 2023). The potential of bifidobacteria to cause diseases remains unclear and data on the incidence of invasive infections are very limited. However, research study has established that *Bifidobacterium* spp. causes bacteraemia in immunocompromised patients or patients with a compromised intestinal barrier (Esaiassen et al. 2017). Also, a reduction in their abundance in infants has been associated with diabetes, increase prevalence of obesity, and metabolic disorder as well as *C. difficile*-associated diarrhoea in the elderly (Arboleya et al. 2016; Stuivenberg et al. 2022).

On the other hand, the role of bifidobacteria in the prevention of gastrointestinal disorders has been described. In mice model experiment, bacterial translocation in Peyer's patches was decreased because of higher colonisation of bifidobacteria in both caecum and colon which led to a poorer bacterial contamination of blood, liver and lungs (Romond et al. 2008). The administration of bifidobacteria strains has prevented and improved intestinal diseases. For example, it has been demonstrated that children fed with an infant formula containing strain *B. animalis* subsp. *lactis* BB-12 showed shorter and fewer episodes of diarrhoea (Weizman et al. 2005). The earliest study conducted by Wang & Feng (2019) has revealed that combinatorial treatment of acute diarrhoea in children with *bifidobacterium* and *Saccharomyces boulardii* effectively improved the clinical efficacy, shortened diarrhoea duration and hospital stay, and improved their immune function. Furthermore, a report has suggested that combination of *B. longum* subsp. *infantis* CECT 7210 and *B. breve* K-110 was effective against rotavirus diarrhoea among infants (O'Callaghan & van Sinderen 2016).

2.4.2 *Lacticaseibacillus*

The genus *Lacticaseibacillus* is classified in the phylum Bacillota and belongs to the family *Lactobacillaceae* which is represented by about 261 species (Widyastuti et al. 2021). Microorganisms belonging to the *Lacticaseibacillus* genus is phenotypically heterogeneous group of Gram-positive, facultatively anaerobic, catalase-negative, non-spore-forming, and rod-shaped that produces lactic acid as a major end-product of the metabolism (Forouhandeh et al. 2021). They are commonly isolated from the environments associated with fermented food (yogurt, cheese, olives, pickles, salami,

etc), oral cavities, gastrointestinal and vaginal tracts of humans and animals, which are widely used in fields related to food, feed, pharmaceuticals and biotechnology (e.g., dairy starters, probiotics, vaccine carriers and silage inoculants) (Walter 2008; Huang et al. 2018). Some of the commonest species, include *L. acidophilus*, *L. casei*, *L. fermentum*, *L. gasseri*, *L. plantarum*, *L. rhamnosus*, and *L. ultunensis* (Antoun et al. 2020). Based on their metabolism, *Lacticaseibacillus* species have been divided into three groups, namely obligate homofermentative group (e.g., *L. acidophilus* and *L. salivarius*) which ferment carbohydrates to produce lactic acid as the main by-product, the facultatively (e.g., *L. casei* and *L. plantarum*) and obligately (e.g., *L. reuteri* and *L. fermentum*) heterofermentative group that ferment carbohydrates to produce lactic acid, acetic acid/ethanol and carbon dioxide as by-products (De Angelis & Gobbetti 2016; Dempsey & Corr 2022). *L. acidophilus*, *L. brevis*, *L. casei*, *L. delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* subsp. *lactis*, *L. fermentum*, *L. gasseri*, *L. helveticus*, *L. johnsonii*, *L. paracasei* subsp. *paracasei*, *L. plantarum*, *L. reuteri* and *L. rhamnosus* are reported to be the main probiotic *Lacticaseibacillus* species (Dempsey & Corr 2022). *Lacticaseibacillus* are among the most common bacteria inhabited the stomach, duodenum, and jejunum of humans (Walter 2008). They are estimated to represent 6% and about 0.3% of the total bacterial cell numbers in the human duodenum and colon respectively (Heeney et al. 2018).

Several action mechanisms underlying the activities of *Lacticaseibacillus* on health-promoting and on gut barrier effect have been described. It has been shown that this species modulates the composition and function of gut microbiota through the production of metabolites that can serve as growth substrates (e.g., butyrate and exopolysaccharide) for some commensal bacteria. In addition, they produce bioactive components with anti-infective properties including bacteriocins, biosurfactants, and short chain fatty acids that inhibit pathogen adhesion to the mucosal surface to prevent infections (Ren et al**.** 2021). Furthermore, *Lacticaseibacillus* improve gut mucus barrier by increasing the production of mucins and defensins. They also positively influence the intestinal epithelial barrier by modifying the immune signalling of epithelial cells, altering the expression and distribution of tight junctions associated proteins, and inhibiting epithelial cell apoptosis

(Ren et al**.** 2021). Conversely, members of *Lacticaseibacillus* have been implicated in various diseases processes, including bacteraemia, endocarditis, empyema, meningitis, pleuropneumonia, sepsis and UTIs (Rossi et al. 2019). *Lacticaseibacillus* have been considered to produce probiotic foods, food supplements, and pharmaceutical preparations, thereby designated as "live biotherapeutic products" proposed to treat certain medical disorders (Rossi et al. 2022). These bacteria have demonstrated therapeutic efficacy in gastrointestinal infections such as diarrhoea (Liévin-Le Moal 2016; Szajewska et al. 2019). Furthermore, in a randomised double-blind placebo-controlled trial, consumption of a probiotic drink containing *L. casei*, *L. bulgaricus*, and *Streptococcus thermophilus* was reported to reduce incidence of antibiotic associated diarrhoea and *C. difficile* associated diarrhoea in adult (Hickson et al. 2007).

2.5 Diarrhoea treatment

Mostly, prevention and management of diarrhoea is based on the recommendation of health experts through conventional or traditional procedures. The WHO has recommended the use of zinc tablets in association with Oral rehydration salt (ORS) to treat diarrhoea infections (Who 2017). An experiment has demonstrated that combination of both ORS and zinc supplements was effective for diarrhoea (Dalfa et al. 2018). Furthermore, besides the use of antibiotics and probiotics for diarrhoea, other methods including pharmacotherapy (Gaginella 1983), phytotherapy (Doustfatemeh et al. 2017), surgery (Cuschieri 1986), thermotherapy (Bandres et al. 1988), and vaccination (Venkatesan & Van de Verg 2015) have proven effective in this area. Public health interventions, such as access to clean water, good hygiene, hand washing, and safe food preparation are also important to prevent diarrhoea (WHO 2005). With reference to this, antibiotics (e.g., azithromycin, ciprofloxacin, and levofloxacin) (Tribble 2017) are mostly prescribed for diarrhoea in healthcare systems worldwide. On the contrary, following the cessation of antibiotics treatment, the microbial community composition is reduced and shifted that may promote colonisation by opportunistic pathogens in the gut microbiota (Tanır et al**.** 2023). Previous literature has reported that ceftriaxone when ingested can alter gut microbiota composition and cause diarrhoea in up to 50% of children (Cunha

1998). Therefore, it is proposed that combinations of non-antibiotics rather than antibiotics alone, is considered alternative ideal for gastrointestinal infections, including antibiotic-associated diarrhoea and combating bacterial resistance because of their multiple antimicrobial mechanisms.

2.6 Selective and combinatory antimicrobial activity

In recent times, there has been an increased interest in the research that focus on selective growth inhibitory effect of antibacterial agents on gut microbiota. Previous studies have shown significant results in this area, where pathogenic bacteria were selectively inhibited with lower effect on the beneficial ones *in vitro*. For example, experiments have proven *in vitro* selectivity effect of 8-hydroxyquinoline, a quinolone alkaloid of plant origin, toward some clostridia causing diarrhoea (e.g., *C. difficile*) and gut beneficial bifidobacteria (e.g., *B. longum*) (Novakova et al. 2013; Novakova et al**.** 2014). Similarly, selective inhibitory effect of biochanin A against selected *Clostridium* strains (e.g., *C. difficile*.) and bifidobacteria (e.g., *B. breve*) has been described (Sklenickova et al. 2010). Last but not least, the studies of Skrivanova et al. (2016) which was conducted using media containing chicken ileal digesta have shown *in vitro* selective growth-inhibitory effect of the earlier mentioned alkaloid against *C. perfringens* over *bifidobacterium* species (e.g., *B. animalis*).

The combination of antimicrobial agents is another pharmacological strategy to increase treatment efficacy and to control resistance evolution. Additionally, their combinatorial effects may be amplified or weakened on microbial cells, that is, the agents may demonstrate synergistic or antagonistic interactions (Bollenbach 2015). Synergy is the enhanced activities of one agent with another when combined at the optimal ratio that exceed the sum of their individual effects (Ocampo et al. 2014; Murugaiyan et al. 2022), whereas antagonism occurs when one of the agents counteracts the action of the other that increases the MIC of the combined agents which is less effective than the single agents (Doldán-Martelli & Míguez 2015). Occasionally, combination of bacteriostatic agents with bactericidal agents induces antagonism (Singh & Yeh 2017), as the work of Booker et al. (2004) observed against antistaphylococcal effect of vancomycin in combination

with clindamycin. Antimicrobial agents' combination can be of the same class or with different class of agents, thus, for example, antibiotics with antibiotics (Bassetti & Righi 2015), antibiotics plus phytochemicals and their derivatives (Khameneh et al. 2021), antibiotics plus probiotics (Muhsin & Hassan 2019), antibiotics plus antimotility agents (Stagliano et al. 2022), or combination of non-antibiotic agents (Yu et al. 2022) to achieve clinical results. This phenomenon has seen significant success in an attempt to develop drugs, which are synergistically effective for bacterial-related infections such as diarrhoea. For instance, ciprofloxacin when combined with metronidazole potently demonstrated *in vitro* growth-inhibitory effect against *C. difficile* and *C. perfringens* (Werk & Schneider 1988). Additionally, in a clinical study, berberine tannate in combination with sulfadimidine and neomycin has proven effective in the treatment of acute diarrhea in children (Chauhan et al. 1969). Also, in a placebo-controlled clinical trial, *C. difficile*-associated diarrhoea patients responded well to the combinatorial treatment of metronidazole or vancomycin with *S. boulardii* (McFarland 2009). In clinical practice, pharmaceutical drugs, marketed as Avycaz (ceftazidime-avibactam, Forest Pharmaceuticals, Inc.) and Zerbaxa (ceftolozane-tazobactam, Merck) are mentioned to be effective combinatorial therapy for complicated intra-abdominal infections and UTIs (Mosley et al. 2016). With reference to the above, selective combinatorial drugs developed to inhibit the growth of pathogenic bacteria whilst simultaneously preserving the beneficial ones in the gut microbial community are not available in the market. Hence, research into potential candidates, not only for antibiotics but also for plant-based antimicrobials agents for future use is imperative.

2.6.1 Determination of combined antimicrobial effect

There are several methods available for determination of bacterial susceptibility to antimicrobial agents. These include broth (macro and micro) dilution, agar dilution, and agar diffusion methods (NCCLS 1999; EUCAST 2000). Broth and agar dilution testing methods are performed and are quantitively analysed which provide numerical minimum inhibitory concentration (MIC) values, whereas agar diffusion testing results are qualitatively analysed and categorised as sensitive, intermediate, and resistant

(Temmerman et al. 2020). It has previously been reported that accuracy, automation, cost, flexibility, individual preference, practicality, and reproducibility are some of the basic factors concern in the selection of testing methods (Woah. org 2023). A number of dilution and disc diffusion techniques, including microdilution chequerboard (Petersen et al. 2006), e-test (Epsilometer test) (Sueke et al. 2010), and time-kill assay (Sopirala et al. 2010) have been widely used to evaluate antimicrobial combination interactions between two or more agents in either liquid or solid media. Among these, the microdilution chequerboard has been one of the commonly used methods for measuring the effect of drug combinations (Rand et al. 1993). In this technique, two antimicrobials are tested in double serial dilutions, and the concentration of each drug is tested both alone and in combination in a 96-well microplate. It allows for determination of MIC of antimicrobial agents using visible turbidity or optical density readings. In this vein, it is possible to evaluate the effect of the individual agent, and at the same time, effect produced by their combination against the tested organism. The nature of the interaction between the two antimicrobials is determined either algebraically or geometrically (Bellio et al. 2021). Although this technique is time-consuming, laborious and error-prone series of calculations and dilutions, the test can be automated or manually performed (Brennan-Krohn & Kirby 2019). Furthermore, chequerboard technique measures antimicrobial inhibitory activity whereas time-kill method assesses bactericidal activity in synergy testing (White et al. 1996).

2.6.2 Calculation of combined antimicrobial effect

The chequerboard test requires evaluation of the fractional inhibitory concentration index (FICI) of each agent (Bassolé et al. 1983). The FICI is the MIC of drug in combination divided by the MIC of drug acting alone. For two interacting drugs A and B, the sum of the FIC's ($\sum FIC = FIC_A + FIC_B$), where $FIC_A = MIC_{A\ (\text{in the presence of B})}/MIC_{A\ (\text{alone})}$, and $FIC_B = MIC_{B\ (\text{in the presence of A})}/MIC_{B\ (\text{alone})}$ expresses the extent of the interaction (Hall et al. 1983). Usually, MICs or concentrations of maximal inhibition (C_{max}) are used as the reference concentrations. Chequerboard test produces various combinations, and by convention, the FICI values of the most effective combination are

used in its calculation. The FICI values used for the definition of the nature of the interaction varies between publications and makes comparison between studies difficult (Bassolé et al. 1983). According to the European Committee on Antimicrobial Susceptibility Testing (EUCAST), the FICI is interpreted as follows: synergistic effect if FICI ≤ 0.5; additive effect if FICI > 0.5 and ≤ 1; indifferent if FICI > 1 and ≤ 2; and antagonism if FICI ≥ 2 (EUCAST 2000). However, more strict evaluation of combinatory antimicrobial effects has previously been proposed. For example, according to the Odds (2003), synergistic effect occurs if FICI ≤ 0.5, no interaction if FICI > 0.5–4.0 and antagonism if FICI > 4.0. This is because, conclusions that interactions are 'additive', 'indifferent' or show 'partial synergy' applied to FICI data slightly above or below the critical theoretical cut-off of 1.0 seem to put a positive spin on findings that within the limits of experimental error, indicate only 'no interaction' between agents. In a combination therapy, synergistic effect is generally described as when the combined action of two or more drugs are larger than the additive effect of each individual drug whereas the combination is antagonistic when their outcome is smaller than the additive effect of each individual drug (Neubig et al. 2003; García-Fuente et al. 2018). It has therefore been reported that synergistic drug combinations are frequently used in clinics because they contribute to better efficacy at lower doses (Lv et al. 2022).

2.6.3 Isobologram analysis

Isobologram (Figure 1) is another method commonly used for the quantitative evaluation of drug combinations. This is the geometric form of interpreting combination effects of drugs (A and B) which are represented by points in a Cartesian plane, thus "A dose-axis" and "B dose-axis" responses. No interaction relationship, is the equation of the straight-line joining A and B. For synergistic dose combination, the point representing the combination lies below the line whilst antagonistic effect point lies above the line. The line joining all doses and dose combinations producing any given quantitative effect is termed the isobole for that effect. The isobole is concave when the dose combinations are producing synergistic effect. Similarly, the isobole is convex when the combination produces antagonism (Berenbaum1989).

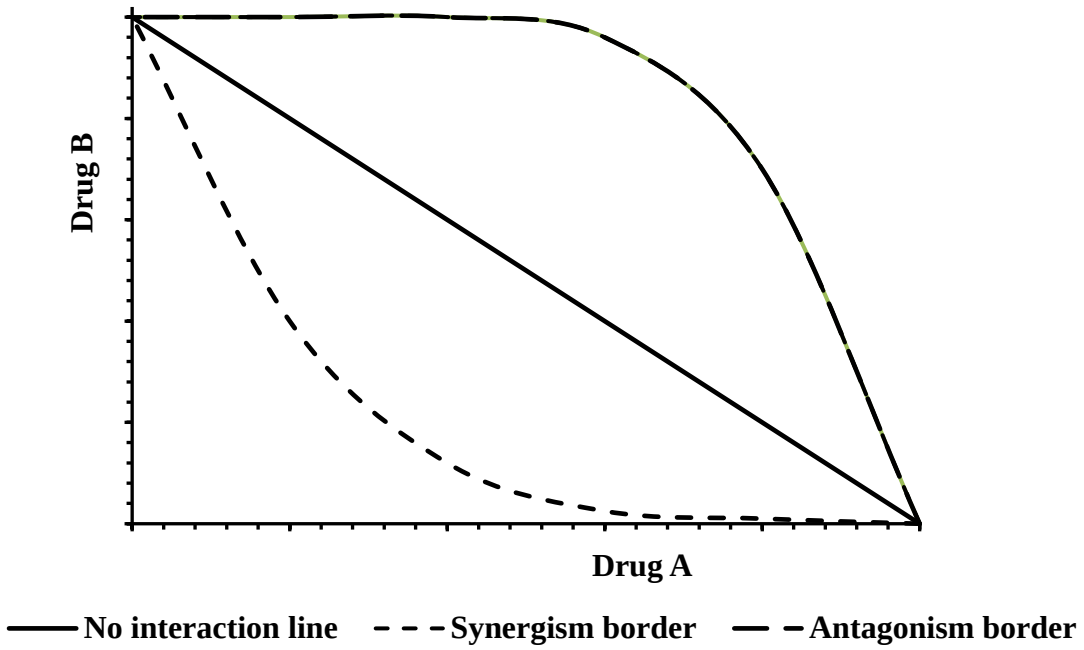


Figure 1. Isobologram for two-drug interactions**.**

2.7 Phytochemical

Phytochemical is a broad term for plant chemical which refers to a wide variety of compounds that occur naturally in plants (Huang et al. 2016). It plays important roles in plant growth and development. These include protecting plants from harmful agents such as insects, microbes, ultraviolet (UV) irradiation and extreme temperatures (Saxena et al. 2013; Martinez et al. 2017). In addition, it promotes germination, pollination, and seed dispersal through the actions of attracted birds and insects. Furthermore, phytochemical provides various colours and flavours to plants when consumed (Martinez et al. 2017). The phytochemical produced in plants is stored in different parts, such as the flowers, fruits, leaves, roots, stems, and seeds (Saxena et al. 2013). This plant-based bioactive compounds can be classified based on their chemical structure, biosynthetic origin or solubility in various solvents (Chanda & Ramachandra 2019). Historically, extensive research on isolation, identification, and quantification of phytochemicals has

been conducted with aim to establish their biological effect via *in vitro* and *in vivo* studies as well as in human clinical trials (Saxena et al. 2013). As a result of this effort, significant biological properties of phytochemicals have previously been described, including their antibacterial (Sadeek & Abdallah 2019) and antifungal (Gizaw et al. 2022) effects. The synthesis and accumulation of phytochemical depend on related factors, such as the environment, and genetics, whereby, changes in the constituent and composition may affect their bioavailability and biological activity (Li et al. 2012). Because of its diverse forms and structures, the exact classification of phytochemical has not been fully elucidated (Koche et al. 2018). However, depending on their role in plant metabolism, phytochemicals are classified as primary or secondary metabolites (Rex et al. 2018). Secondary metabolites in plants are categorised into three main groups based on their biosynthetic pathways: (a) phenolic compounds (b) terpenoids and steroids, and (c) nitrogen-containing compounds such as alkaloids (Jean & Hatton 1999; Rabizadeh et al. 2022).

2.7.1 Phenolic compounds

Although phenolic compounds are diverse, ubiquitous and widespread in nature, they are mostly found in plants (Anantharaju et al. 2016). These compounds are synthesised by means of the shikimic acid and the acetate malonate pathways (Kougan et al. 2013). They are structurally characterised by the presence of at least one aromatic ring substituted by one or more hydroxyl group, free or engaged in another function (Jean & Hatton 1999). Based on their chemical structure, they can be grouped into simple phenolic and polyphenolic compounds. Simple phenolic compounds have C_6 general skeleton representation (e.g., simple phenols and phenolic acids) whereas polyphenols have C_{15} general skeleton representation (e.g., flavonoids and tannins) (Al Mamari 2022). Many phenolic compounds have shown broad spectrum of biological activities with clinical potentials against various diseases caused by bacteria, fungi, virus, and parasites. In addition, it is reported that this compound produces antioxidant and anticancer effect (Lin et al. 2016; Ecevit et al. 2022). Furthermore, several studies have documented that phenolic compounds and their derivatives possess antimicrobial properties by inhibiting

microbial growth. It is well established that Whitfield's ointment (e.g., Gold Cross, Barton, Australia), available as an over-the-counter preparation is used for the topical treatment of dermatophytosis, such as athlete's foot caused by *tinea pedis*. This product is commonly made up of 3% salicylic acid, 6% benzoic acid and a suitable base (*e.g.* lanolin or vaseline). Similarly, the literature has reported that tannins found in plants such as *Quercus* spp. and *Uncaria gambir* are effective antidiarrhoeal agents due to their astringency and antiseptic effects (Kokoska et al. 2019). Cesinex (Hall Bioscience corporation, USA), a medical food made up of tannic acid and dried egg albumen, is prescribed in the USA for diarrhoea treatment. This is because of its ability to improve epithelial barrier properties, inhibit intestinal fluid secretion, and high antioxidant properties (Ren et al. 2012; Shiming et al. 2021). Also, the antibacterial activities of gallic acid, quercetin, caffeic acid, coumaric acid, tannic acid, and catechol have been observed against diarrhoea-causing *E. coli* in agar disc diffusion assay (Tyagi et al. 2015). In animal model experiment, tannins isolated from *Codiaeum variegatum* exhibited antidysenteric and antidiarrhoeic activities when treated with mice (Labu et al. 2015). Previously published work by Nitiema et al. (2012) has shown that coumarin was active against gastroenteritis bacterial strains such as *E. coli*, *Enterobacter aerogenes*, *S. typhimurium*, and *S. infantis*. In another study, epigallocatechin gallate, a catechin present in tea leaves, exhibited *in vitro* synergistic anti-staphylococcal activity when combined with oxytetracycline (Novy et al. 2013). Antibacterial action mechanisms of phenolic compounds have been attributed to their ability to damage bacterial membrane, inhibit virulence factors such as enzymes and toxins, and suppress of bacterial biofilm formation (Miklasińska-Majdanik et al. 2018).

2.7.2 Terpenoids and steroids

Terpenoids and steroids are the largest and structurally most diverse known group of secondary metabolites derived from natural sources (Jean & Hatton 1999**;** Yu et al. 2022). They are found in algae, animals (e.g., insects and marine organisms), bacteria, fungi, and plants (González-Burgos & Gómez-Serranillos 2012). Terpenes are hydrocarbon molecules that contain 10-15 carbons, whereas terpenoids are modified

terpenes in which the methyl groups are either moved or removed when oxygen atoms are added to the hydrocarbon molecules (Reyes et al. 2018). Terpenoids are derived from the condensation of dimethylallyl pyrophosphate and isopentenyl diphosphate. They are synthesised in the cytosolic mevalonic and the plastidic methylerythritol pathways (Reyes et al. 2018). Based on the number of isoprene units, terpenoids are classified as hemiterpenoids, monoterpenoids, sesquiterpenoids, diterpenoids, sesterterpenoids, triterpenoids, and polyterpenoids (Adedokun et al. 2023). It has previously been reported that these plant chemicals possess various biological activities including anticancer, antimicrobial, anti-inflammatory, antioxidant, and antiallergic effects (Masyita et al. 2022). The biological activities of terpenoids are because of their lipophilicity and tendency to partition into cell membranes, as well as the ability to interact with membrane-bound proteins or disrupt membrane integrity (Agatonovic-Kustrin & Morton 2018). Carveol, citronellol, eugenol, and geraniol have shown antibacterial activities against *E. coli*, and *S. enterica* serovar Typhimurium which are common causative agents of diarrhoea (Guimarães et al. 2019). In another experiment, tetracycline in combination with geraniol and thymol showed *in vitro* synergistic activity against diarrheic *E. coli* (Miladinović et al. 2015). Previously published data also indicated that penicillin/eugenol and penicillin/thymol combinations demonstrated synergistic effects against diarrhoeagenic *E. coli* with FIC values of 0.16 and 0.15 respectively (Gallucci et al. 2006). Colpermin capsule (Tillotts Pharma, Switzerland), which contains peppermint oil (*Mentha piperita* L), is being sold in the market for the relieve of symptoms associated with IBS including, bloating and diarrhoea (Inish 2024**)**.

2.7.3 Alkaloids

Alkaloids are organic compounds of natural origin which contain at least one nitrogen atom in their structure (Jean & Hatton 1999). These bioactive compounds are mostly found in plants, but less in microorganisms and animals (Dewick 2009). Alkaloids are commonly classified into three groups based on their biosynthetic origins and structural characteristics, namely, true alkaloids, protoalkaloids, and pseudoalkaloids. True alkaloids (e.g., atropine, nicotine, morphine, and quinine) contain nitrogen in a

heterocyclic ring as part of their structure. They are typically derived from amino acids and exhibit strong basicity due to the presence of the nitrogen atom. They are synthesized from amino acids such as tyrosine, tryptophan, lysine, or ornithine. Protoalkaloids (e.g., ephedrine, and mescaline) contain nitrogen, but the nitrogen atom is not part of a heterocyclic ring. These are also synthesis from amino acids, although they do not have a cyclic structure. Lastly, Pseudoalkaloids (e.g., caffeine, theobromine, and solanidine) do not originate from amino acids, though they may still contain nitrogen. They are synthesized from non-amino acid sources such as terpenoids, purines, or sterols. Despite this difference in origin, they often exhibit alkaloid-like biological activities (Jean & Hatton 1999; Cídlová et al. 2016; Dey et al. 2020). According to nature of their nitrogen-containing structure, they can be classified as indole (Hamid et al. 2017), isoquinoline (Qing et al. 2020), quinoline (Shang et al. 2018), pyridine (Lin et al. 2020), pyrrolidine (Poyraz et al**.** 2023), pyrrolizidine (Schramm et al. 2019), and tropane (Kohnen-Johannsen & Kayser O. 2019) alkaloids. Remarkable biological properties of alkaloids have attracted attention of many researchers. Its pharmacological application for prevention and treatment of diarrhoea has been reported. For example, isoquinoline alkaloid berberine derived from plants such as *Coptis* spp., *Berberis* spp. and *Hydrastis canadensis*, has been used in the form of capsules, tablets and tinctures which are usually sold in Asia [*e.g.* Huang Lian Su Tablets (*Coptis Sinensis* extract), Hubei Minkang Pharmaceutical, Yichang, China] and North America (*e.g.* Berberis Formula, Seroyal, Richmond Hill, Canada; Solaray Goldenseal Root Capsules, Nutraceutical Corporation, Park City, USA) to support digestive functions and relive gastrointestinal disorders including diarrhoea. In addition, berberine is being sold in pure form as a pharmaceutical drug in Asia to treat intestinal infectious diseases and diarrhoea (Berberine Hydrochloride Tablets, Northeast Pharmaceutical Group, Shenyang, China) (Kokoska et al. 2019). Previously, clinical trials have proven that combination of berberine, neomycin, and sulfonamides marketed as "Uneomcyin compound" (Unichem Laboratories, India) was effective in the treatment of infective diarrhoea in children (Chauhan et al. 1969). In a randomized double-blind placebo-controlled clinical trial, adult patients with IBS-diarrhoea responded well to the orally administered berberine hydrochloride (Chen et al. 2015). 8-Hydroxyquinoline, an alkaloid compound previously detected in *Sebastiania*

corniculata (Lee et al. 2010), has shown growth-inhibitory activity against diarrhoeagenic bacteria including *B. cereus*, *L. monocytogenes*, and *S. typhimurium* in agar diffusion assay (Yang et al. 2013). Furthermore, a comprehensive *in vitro* antimicrobial susceptibility testing of alkaloid compounds such as berberine, and 8-hydroxyquinoline revealed antibacterial activity against some diarrhoea-causing bacteria (e.g., *C. difficile*, *E. coli*, *S.* Enteritidis, and *Y. enterocolitica*) (Kudera et al. 2020). The structural modifications of alkaloids have become precursors of numerous semisynthetic and synthetic pharmaceutical drugs presently used in clinical practice (Cushnie et al. 2014; Awuchi 2019). For example, chloroxine (Endiaron, Sanofi-aventis, Prague, Czechia), a synthetic derivative of 8-hydroxyquinoline (Pubchem 2023), is an antibacterial drug which has been used for treatment of infectious diarrhoea and intestinal microbiota disorders caused by antibiotics (Wiki web 2023). The antibacterial action mechanism of alkaloids mentioned in literature includes inhibition of DNA topoisomerase II (Larghi et al. 2015), inhibition of protein synthesis and efflux pump activity (Kelley et al. 2013; Mabhiza et al. 2016), and inhibition of bacterial cell wall synthesis and metabolism (Li et al. 2014; Yan et al. 2021).

2.7.4 Antimicrobial effects of phytochemicals

Until now, plants have been widely studied by researchers and practitioners as source of bioactive compounds for drug development. Out of estimated 250,000 to 350,000 plant species identified so far, about 35,000 were critically investigated for their biological activities and used worldwide for medicinal purposes (Kong et al. 2003). It has been reported that about 25% of drugs used in modern medicine globally, are derived from plants (Shah & Bhat 2019).Out of 252 drugs classified by the WHO as basic and essential, 11% are exclusively of plant origin. Many of them are synthetic drugs obtained from natural precursors (Rates 2001). Several commercially drugs utilised in modern medicine were primarily used in crude form in traditional or folk healing practices, which possibly suggested the promising aspect of their antimicrobial activity. For example, the barks, leaves, roots, and stems of berberine-containing plants such as *Coptis chinensis*, *Berberis aristata* DC, *Berberis tinctoria* Lesch, and *Berberis petiolaris* Wall. ex G. Don

have been used in the form of extracts and decoctions in Ayurvedic and traditional Chinese medicine to treat diarrhoea (Neag et al. 2018; Aalim et al. 2022; Filli et al. 2022). Coptisine, an isoquinoline alkaloid found in *C. chinensis*, has been reported to reduce the degree of diarrhoea and mucosal injury to the ileum (Xiong et al. 2022). Antistaphylococcal activities of chelerythrine, a benzophenanthridine alkaloid isolated from the root of *Toddalia asiatica* L. has been found to inhibit bacterial cell membrane and protein biosynthesis. The root and bark of the plant have been used in traditional medicine to treat ailments including diarrhoea, and cholera (He et al. 2018). Over time, the synthetic and semi-synthetic derivatives of phytochemicals have been well known for their broad-spectrum biological activities and have played critical role as lead molecules for drug discovery in the pharmaceutical industries. In addition, various commercial products available on the market and their detailed clinical efficacy suggests that plants are prospective sources for the identification of new types of antimicrobial agents (Kokoska et al. 2019). Such of the drugs and medicinal preparations have been utilized in clinical applications for treatments of various bacterial infection-related diseases including diarrhoea., Antidiarrhoeal drug chloroxine (Endiaron, Endiaron, Sanofi-aventis, Prague, Czechia), a derivative of 8-hydroxyquinoline, can be mentioned as an example (Wiki web 2023). Nitroxoline, another synthetic derivative of 8-hydroxyquinoline, has been in clinical use in Europe for the treatment of UTIs (Sobke et al. 2012).

2.7.5 Combination effects of phytochemicals

Secondary metabolites occur in plants as mixtures (Wink 2008), and thus their synergistic and antagonistic effects can either enhance or reduce the activities . Examples of plants that contain mixtures of compounds producing stronger antimicrobial activity than individual compound are including *H. canadensis* (berberine, and hydrastine), *Allium sativum* (allicin, and ajoene), *Quercus rubra* (tannins, and quercetin), and *Piper betel* (catechols, eugenol) (Cowan 1999). Generally, plant extracts are complex mixtures of phytochemicals which may target multiple biological pathways simultaneously, providing broader therapeutic potential. Comparatively, pure compounds have a more

defined mechanism of action, allowing for targeted therapy with predictable outcomes. Also, plant extracts face more difficulties in clinical trials because it is harder to attribute effects to specific compounds and standardize doses whereas pure compounds with well-defined chemical structures and mechanisms of action, are easier to test in clinical trials. Since plant extracts contain mixtures of bioactive compounds, there is a chance of working in synergism to enhance therapeutic effects. Screening studies using plant derivatives with antimicrobial activity aiming to identify synergistic interactions, provide an important source of biologically active agents that could be exploited in combination therapies. Such compounds or active fractions may not necessarily have strong antibacterial activities themselves but may synergize with commercial antibiotics via known or novel modes of action (Rakholiya et al. 2013). For example, berberine has demonstrated inhibitory activity against efflux pumps of *E. coli* which increased intracellular accumulation of ciprofloxacin to restore susceptibility of the earlier mentioned bacteria (Li & Ge 2013). The investigation of Dwivedi et al. (2019) has revealed synergistic antibacterial activity of clavine alkaloid chanoclavine when combined with tetracycline against multidrug resistant clinical isolate of *E. coli*. It was further reported that chanoclavine inhibited the efflux of tetracycline from multidrug resistant *E. coli* and thus making the antibiotic available inside the cell for its action. Additional experiments have demonstrated that indole alkaloids (e.g., isoreserpiline, reserpiline, and serpentine) enhanced synergistic effect of nalidixic acid and tetracycline by inhibiting efflux pumps of diarrheic *E. coli* (Dwivedi et al. 2015). Furthermore, sanguinarine has been reported to synergistically enhance the activity of streptomycin against clinical isolates of diarrhoea-causing *E. coli* (Hamoud et al. 2015). It has been also observed that quercetin in combination with either chlortetracycline, doxycycline oxytetracycline, or tetracycline produced synergistic antibacterial effect against both standard and clinical isolates *E. coli in vitro* (Qu et al. 2019). It has been further demonstrated that eugenol-colistin combinations exhibited strong synergistic activity against clinical isolated *E. coli* strains (Wang et al. 2018). Additionally, synergistic antistaphylococcal effect of tannic acid when combined with fusidic acid, cefotaxime, minocycline, and rifampicin has been established (Kyaw et al. 2012). In another studies, berberine in combination with probiotics increased the clinical cure rate of diarrhoea in

children and adults in a randomized controlled trial (Yu et al. 2020). It is worth noting that in the complex environment of the intestinal tract, combination of antibiotics with natural substances bonded by a weak chemical bond could potentially lead to a decrease in biological activity of one or both components due to factors such as variations in pH, enzymatic activity, microbial metabolism, and drug-drug interactions in the gut.

2.7.6 Effect of plant compounds on health beneficial microbiota

The human microbiota constitutes a complex microbial ecology of interacting components that regulates important pathways in the host. This microbiota contributes to many critical physiological activities including digestion, and absorption which may determine the host's health (Santhiravel et al. 2022). Since diarrhoea is associated with disturbance in gut microbiota, especially the decline of microbial diversity, predominance of harmful bacteria, and alteration of bacterial metabolic pathways (Du et al. 2023), the use of alternative products based on phytochemicals, and its derivatives seems favourable to maintain a balanced and healthy gut microbiota. It has previously been reported that the intake of herbs and healthy dietary components can increase the number of beneficial bacteria and contribute to restoring the healthy microbiome and reversing dysbiosis (Lal et al. 2023). Also, phytochemicals influence the gut microbiota by exhibiting direct bactericidal or bacteriostatic effects to reduce pathogenic microbes, and the same time acting as substrates for beneficial ones for gut colonization and improvement of gut barrier integrity (Dingeo et al. 2020). It has been reported that fermentation of polyphenols in the intestinal lumen increases the abundance of beneficial microbiota such as *Bifidobacteria* and reduces the Bacillota and Bacteroidota ratio (Yang et al. 2020). It has earlier been mentioned that herbal preparations orally administered show poor bioavailability, however, the molecule easily escaped inside the small intestine (only 5–10% of polyphenols can be absorbed in small intestine) and accumulates in the colon, where it modulates gut microbiota composition (Feng et al. 2018). Natural alkaloid products with pharmacological effect such as berberine, coptisine, and epiberberine have been reported to alleviate gut dysbiosis by restoring beneficial gut microbes including *Akkermansia* and *Sporobacter* and reducing harmful bacteria (e.g., *Desulfovibrio*, and *E*.

coli). In addition, allicin (diallyl-dithiosulfinate), a constituent of raw *A. sativum*, has been cited to increase gut microbial diversity and inhibits pathogenic bacteria, such as *Proteus penneri*, and *E. fergusonii* (Mikaili et al. 2013; Lal et al. 2023).

Although *in vitro* selective growth-inhibitory activity of various plant-derived compounds and their synthetic analogues against diarrhoea-causing and beneficial bacteria has previously been described, the selective combinatory effect of natural compounds and their synthetic derivatives with commercial antibiotics has not previously been reported.

2.7.7 Nitroxoline

Synonym: 8-Hydroxy-5-nitroquinoline, 5-nitroquinolin-8-ol, 5-Nitro-8-hydroxyquinoline, 5-Nitro-8-quinolinol, Nibiol, Nitroxolin, Noxibio, etc

Nitroxoline ($C_9H_6N_2O_3$), is a yellowish crystalline, odourless powder, and a heterocyclic aromatic compound which was derived from 8-hydroxyquinoline molecule detected previously in *Centaurea diffusa* (Voronin et al. 1976; Vivanco et al. 2004; Repac et al. 2022). In the area of pharmacology, nitroxoline has been used since sixties for the treatment and prophylaxis of acute and recurrent UTIs caused by *E. coli* in children and adults (Wijma et al. 2018; Cho et al. 2019). It has also been approved by the FDA and is widely used as an anti-neurodegenerative drug to treat Alzheimer's disease and cancer in humans (Cherdtrakulkiat et al. 2016). Nitroxoline has been identified as a potent reversible inhibitor of cathepsin B (Mitrović et al. 2019), a typical cysteine lysosomal protease involved in a variety of physiologic and pathological processes (Ni et al. 2022). The complexes of nitroxoline have been also reported to show certain luminescence properties in the solid state at room temperature. Therefore, its nonpharmacological application for various purposes, including fluorescent indicators, fluorescent paint, and light-emitting instruments, has been mentioned (El-Wakiel & El-Ghamry 2017). This quinoline-based compound (Van Hau et al. 2019), has been widely studied in the clinical microbiology because of its broad-spectrum antimicrobial activities. It has been described to be effective against both Gram-positive and Gram-negative bacteria and also against

fungal pathogens (e.g., *Candida* spp.) (Zhang et al. 2014). For example, anti-uropathogenic activity of nitroxoline with sulphamethizole, marketed in equal combination as Nicene (Debat Laboratories, France), commonly used to treat urinary infections (e.g., pyelonephritis, cystitis, urethritis, prostatitis) in South Africa has been reported (Jacobs et al. 1978). Nitroxoline has also demonstrated excellent *in vitro* inhibition of clinical isolates of carbapenemase-producing Enterobacterales, including *E.* coli (Fuchs & Hamprecht 2019). In a randomized, single-blind clinical studies, nitroxoline 250 mg drug administered caused eradication of bacteriuria in more than 90% of the patients (Naber et al. 2014). This agent showed a strong growth-inhibitory activity against diarrheic bacteria, including *B. cereus*, clostridial species, *E. coli*, and *S. flexneri* assessed *in vitro*, at MICs values ranging from 2 to 4 µg/mL (Kudera at al. 2020). Antifungal activity of previously mentioned agent has been observed against UTI *Candida* isolates *in vitro* (Fuchs et al. 2022). Nitroxoline antibacterial action mechanism has been established on its ability to chelate divalent cations, such as magnesium ion (Mg^{2+}), manganese ion (Mn^{2+}), and zinc ion (Zn^{2+}), but not calcium ion (Ca^{2+}), which are essential for bacterial growth (Pelletier et al. 1995; Sobke et al. 2012). This drug is rapidly absorbed by the gastrointestinal tract and is excreted unchanged by kidneys (Zhang et al. 2014; Jakhar et al. 2019).

2.7.8 Sanguinarine

Sanguinarine ($C_{20}H_{14}NO_4$) [13-methyl(1,3)benzodioxolo(5,6-c)-1,3-dioxolo(4,5)phenanthridinium] is an isoquinoline derivative and a benzophenanthridine alkaloid, which was detected previously in many plant species of the families *Papaveraceae, Fumariaceae, Ranunculaceae,* and *Rutaceae* (Han et al. 2013; Zhang et al. 2020). This compound has been extracted from many plants, such as *Sanguinaria canadensis* L., *Chelidonium majus* L., *Argemone mexicana* L. and *Macleaya cordata* (Hu et al. 2000; 314 Wang et al. 2021). Sanguinarine occurs either as chloride or sulphated crystalline salts, where both are orange-red coloured, and it is sparingly soluble in aqueous conditions but highly soluble in many organic solvents (Basu & Kumar 2016). Furthermore, it exists in either iminium form (at pH less than 6) which actively binds to

nucleic acids or alkanolamine form (at pH greater than 7) that is more lipophilic and has greater penetration and cellular availability (Croaker et al. 2016). Sanguinarine has been reported to demonstrate wide array of biological activities, including antifungal, antimicrobial, antiinflammatory, antioxidant, anticancer, and immunomodulatory activities (Lopus & Panda 2006; Hallock et al. 2007; Zhang et al. 2013; Zhang et al. 2020). Also, this compound has been used in oral health care products, such as toothpastes and mouthwashes as antiplaque and antigingivitis agents (Munro et al. 1999; Malikova et al. 2006). In a previous experiment, sanguinarine demonstrated *in vitro* antibacterial activities against Gram-positive and Gram-negative bacteria, such as *S. aureus* and *E. coli*, respectively (Miao et al. 2011). Again, selective antibacterial effect of sanguinarine on some diarrhoea-causing (e.g., *V. parahaemolyticus*) and beneficial bacteria (e.g., *L. casei*) has been documented in literature (Kudera et al. 2020). Other studies have illustrated sanguinarine effectiveness against biofilm formation by *Providencia rettgeri*, which is considered to cause gastroenteritis, bacteraemia and travelers' diarrhoea (Zhang et al. 2020). Similarly, sanguinarine has demonstrated synergistic interaction with streptomycin when combined against the standard strains and clinical isolates of *E. coli* in *in vitro* studies (Hamoud et al. 2015). Sangrovit (Phytobiotics, Germany), a feed additive containing extract of *M. cordata* with standardized content of sanguinarine, improved growth performance, intestinal morphology and microflora populations in early weaned piglets as well as increased amounts of *Lacticaseibacillus* and SCFAs in the contents of their intestinal lumina whereas the level of *E. coli* and *Salmonella* spp. was reduced (Chen et al. 2018). In another experiment, feeding of sangrovit to broiler chickens, had a significant effect on caecal microflora activity and increased the concentration of total SCFAs in the caecal digesta (Juskiewicz et al. 2011).

Several action mechanisms of this agent have been described. For example, it has been reported that the iminium form of sanguinarine binds to DNA by intercalation with a higher specificity binding to GC-rich DNA, while the alkanolamine form does not bind to DNA (Bai et al. 2006). Other studies have suggested that this agent can compromise the bacterial cytoplasmic membrane, which may result in cell death (Obiang-Obounou et al. 2011). Furthermore, it has been illustrated that sanguinarine has the ability to block

cytokinesis in bacteria by inhibiting Z-ring formation (Beuria et al. 2005), and also can cause damage to bacterial, and fungal cell membrane (Zhao et al. 2019; Zhang et al. 2020). Some adverse health effects associated with the use of sanguinarine have previously been reported. For instance, it has been reported to cause outbreaks of human poisoning called epidemic dropsy (a condition occurs from ingesting sanguinarine), which is characterized by oedema of the legs, congestive heart failure, hepatomegaly, ataxia, and glaucoma (Dalvi 1985). Additionally, sanguinarine has been found to kill cells and destroy tissue of the skin (producing bleeding wounds and massive scab) when applied to the body surface. As such, this isoquinoline alkaloid is termed an escharotic agent (Cienki & Zaret 2010). The acute oral median lethal dose (LD_{50}) in rats is extremely high (1658 mg/kg) with death resulting from respiratory paralysis (Becci et al. 1987). Since this compound has been used as feed additives in livestock production, it is considered as safe for consumption. This is supported by public health agencies such as EFSA, and FDA.

2.7.9 Zinc pyrithione

Synonym: zinc omadine

Zinc pyrithione is a coordination complex of zinc ion (Jung et al. 2019), and a pyrithione (1-hydroxy-2-pyridinethione), which is synthesised from the antimicrobial metabolite aspergillic acid of *Aspergillus flavus* (Park et al. 2018) or found in Chinese medicinal plant called *Polyalthia nemoralis* (Han G et al. 1981). It is a white crystalline material in the dry state (Schwartz 2016), and it is insoluble in water, organic solvents, or surfactants (Guthery et al. 2005). Because of its broad spectrum of antimicrobial activity, zinc pyrithione has been commonly used as an algaecide, bactericide, and fungicide (Min et al. 2019). This agent is widely used as an active ingredient in most of daily hair care products in the market, such as shampoos, conditioner, and hair-rinses (Shih et al. 2004), to treat dandruff and seborrheic dermatitis (Schwartz 2016), caused by *Malassezia* spp. (Park et al. 2018; Tucker & Masood 2023). In accordance with The Scientific Committee on Consumer Safety (SCCS) opinion, zinc pyrithione is presently regulated as a preservative in rinse-off and leave-on hair products (with the exemption of oral hygiene

products) in a concentration up to 0.5% and 0.1% respectively, and up to 1.0 % in hair products. The committee further considers the agent as safe when used as an anti-dandruff in rinse-off hair products up to a maximum concentration of 1% (SCCS 2020). Likewise, it is a biocide widely used as preservatives in various industrial applications, including fuel lines, fuel tanks, antifouling paints (Cooney 1969; Yebra et al. 2004; Soon et al. 2019), and antimicrobial finishing agents in textile production (Jain & Tesema 2017). The *in vitro* growth-inhibitory activity of zinc pyrithione has been demonstrated against some pathogenic bacteria, including *B. cereus*, *E. coli*, and *S. aureus* using tube dilution assay (Khattar et al. 1988). Again, it has proven to possess *in vitro* selective antibacterial properties against diarrhoeagenic bacteria as reported in the work of Kudera et al. (2020). In another study conducted by Mala et al. (2022), it was observed that zinc pyrithione demonstrated significant synergistic antistreptococcal and antistaphylococcal activity when combined with gentamicin. Previous literature has reported that zinc pyrithione is membrane active (Guthery et al. 2005), thus, it acts on bacterial cell membrane by chelation or salt formation with the divalent metal cations which are present in the bacterial envelope (i.e. Mg^{2+}, Mn^{+2}, Ca^{2+}, etc.) and subsequent passive diffusion of the pyrithione-metal chelates into the cytosol (Dinning et al. 1998). Furthermore, an increase in cellular zinc levels, inhibition of mitochondrial functions, and a decrease in lipase expression has been associated with zinc pyrithione inhibitory mechanisms against *Malassezia* spp (Park et al. 2018). This agent has been classified as a moderately toxic agent with LD_{50} values ranging from 92 to 266 mg/kg and from 160 to 1000 mg/kg when administered orally to rats and mice, respectively (SCCS 2020).

3. Research questions

1. Which classes of antibiotics will produce combinatory and/or selective effects with antidiarrhoeal/anti-infective phytochemicals and alkaloid-related compounds against diarrhoea-causing and gut beneficial bacteria?
2. Which antibiotic will produce the selective synergistic effect with phytochemicals and their structural analogues against intestinal diarrhoeagenic and gut beneficial bacteria?
3. Which antibiotic will produce the synergistic effect with phytochemicals and their structural analogues against intestinal diarrhoeagenic bacteria?

4. Hypotheses

1. Various combinations of representatives of different classes of antibiotics with antidiarrhoeal/anti-infective phytochemicals and alkaloid-related compounds will produce broad spectrum of combinatory and/or selective effects against diarrhoea-causing and gut beneficial bacteria.
2. The combination of antidiarrhoeal/anti-infective phytochemicals and their structural analogues with antibiotics used for treatment of infectious diarrhoea such as ciprofloxacin will produce the selective synergistic effect against intestinal diarrhoeagenic and gut beneficial bacteria.
3. The combination of antidiarrhoeal/anti-infective phytochemicals and their structural analogues with other antibiotics will produce the synergistic effect against intestinal diarrhoeagenic bacteria.

5. Objectives

The aim of this study was to evaluate the *in vitro* selective and/or combinatory effects of representatives of different classes of antibiotics with phytochemicals and their synthetic analogues against diarrhoea-causing and gut beneficial bacteria.

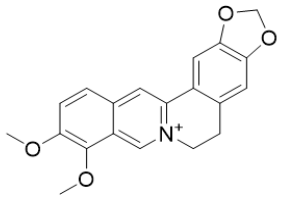
The specific objectives of the study are as follows:

1. Screening of combinations of phytochemicals and alkaloid-related compounds with representatives of different classes of antibiotics for their combinatory and selective effects against diarrhoea-causing and gut beneficial bacteria.
2. Determination of selective combinatory effect of ciprofloxacin with either nitroxoline, sanguinarine, and zinc pyrithione against intestinal diarrhoeagenic and gut beneficial bacteria.
3. Determination of combinatory effects of tetracycline with either nitroxoline, sanguinarine, and zinc pyrithione against diarrhoea-causing bacteria.

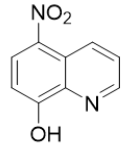
6. Materials and methods

6.1 Chemicals

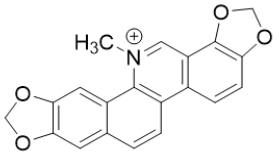
Phytochemicals (berberine chloride, sanguinarine chloride, and tannic acid), synthetic analogues of plant compounds (nitroxoline, and zinc pyrithione) and conventional antibiotics (ceftriaxone, ciprofloxacin, chloramphenicol, tetracycline and vancomycin) used in this study were purchased from Sigma-Aldrich (Prague, Czech Republic). Compounds were selected based on their antidiarrhoeal and/or anti-infective action described in literature (Kudera et al. 2020) as well as historical or ethnopharmacological use with regards to long-standing application in treating various bacterial infections in traditional medicine. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Prague, Czech Republic) was used to prepare the stock solutions of all tested compounds, excluding ciprofloxacin, and vancomycin which were prepared by using distilled water. Tannic acid, and tetracycline were also prepared by using 96% ethanol (Penta, Prague, Czech Republic). Tannic acid when diluted with all the antibiotics formed sediments in the solution which exhibited low solubility. The chemical structures of individual compounds tested are shown in Figure 2.



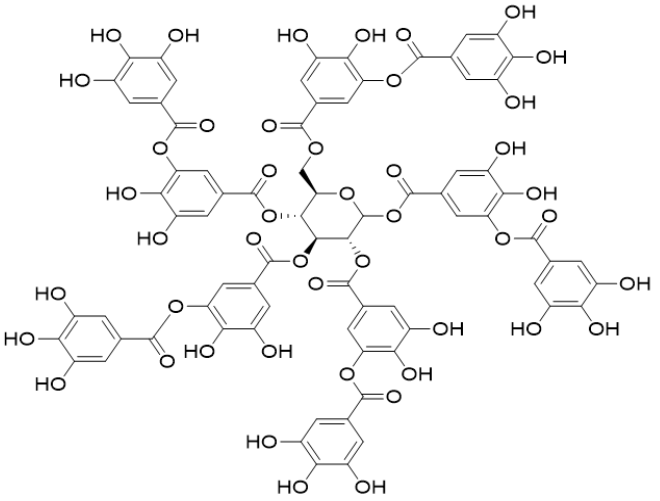
1 Berberine



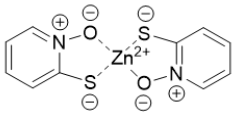
2 Nitroxoline



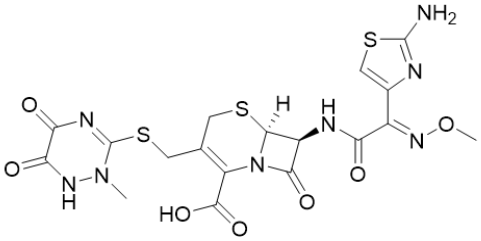
3 Sanguinarine



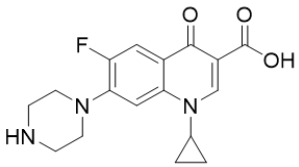
4 Tannic acid



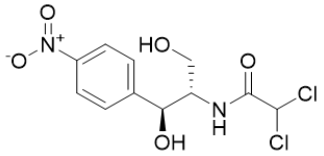
5 Zinc pyrithione



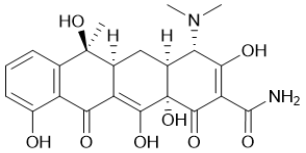
6 Ceftriaxone



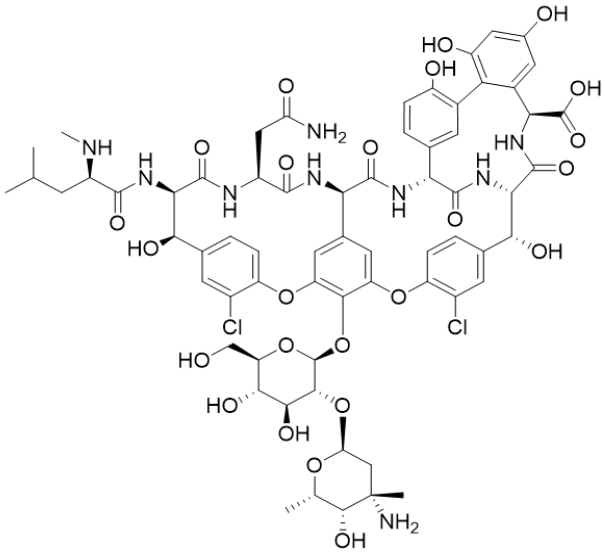
7 Ciprofloxacin



8 Chloramphenicol



9 Tetracycline



10 Vancomycin

Figure 2. The chemical structures of individual compounds tested**.**

6.2 Bacterial strains and growth media

The standard bacterial strains used were obtained from the American Type Culture Collection (ATCC, Rockville, USA), Czech Collection of Microorganisms (CCM; Brno, Czech Republic), German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany), and the National Collection of Type Cultures (NCTC, London, UK). Ten different diarrhoea-causing pathogens were tested in this study, which were selected as representatives of Gram-negative bacteria [*E. coli* O175:H7-VT (N) NCTC 1290, *E. coli* ATCC 25922, *E. coli* ATCC 35218, *Salmonella Typhimurium* ATCC 14028, *S. flexneri* ATCC 12022, *V. parahaemolyticus* ATCC 17802, and *Y. enterocolitica* ATCC 9610] and Gram-positive bacteria (*B. cereus* ATCC 14579, *E. faecalis* ATCC 29212, and *L. monocytogenes* ATCC 7644). According to ATCC Genome Portal (2024), all the above-mentioned pathogens are recommended for pharmaceutical and food testing as well as for infectious and enteric disease research. They are normally associated with either foodborne, waterborne, or nosocomial infections. In addition, five beneficial bacterial strains (*B. adolescentis* DSMZ 20087, *B. animalis* subsp. *lactis* DSMZ 10140, *B. breve* ATCC 15700, *L. casei* DSMZ 20011, and *L. rhamnosus* CCM 7091), which are among the three predominant bacterial phyla in the human gut were used in this study. Although *B. animalis* subsp. *lactis*, *L. casei*, and *L. rhamnosus* do not occur naturally in the human intestinal tract, it is evidenced that they inhabit the human gut since they are consumed in substantial quantities in various food products (e.g. in probiotic preparations), and for this reason, they are considered as secondary invaders of the gut. (Ahrné et al. 1998; Oozeer et al. 2006; Tsukimi et al. 2020; Kim et al. 2022; Guo et al. 2023). Mueller-Hinton broth (MHB) (Oxoid, Basingstoke, UK) was used as the growth medium for bacteria that grow aerobically. This was supplemented with 3 % NaCl (Sigma-Aldrich, Prague, Czech Republic) for the culture of *V. parahaemolyticus*. The anaerobic bacteria (bifidobacteria), including the facultative species (*Lacticaseibacillus*) were cultured in Wilkins-Chalgren broth (WCH) (Oxoid) supplemented with 5 g/L soya peptone and 0.5 g/L cysteine. All bacterial strains were grown in MHB (aerobic bacteria) and WCB (anaerobic bacteria) for 24 h at 37°C prior to testing.

6.3 Chequerboard assay

In this study, a chequerboard microdilution assay was used to assess the MIC values of antibiotics and alkaloid-related agents individually and in combination, simultaneously within the same 96-well plate, following the Clinical and Laboratory Standards Institute CLSI (2015) guidelines and the Clinical Microbiology Procedures Handbook (Lebar 2016). For the combinations of antibiotics with each of the alkaloid-related agents, eight two-fold serial dilutions of antibiotics in the horizontal rows of the 96-well microtiter plate were cross-diluted vertically, by eight two-fold serial dilutions of the test agents, using a Freedom EVO 100 automated pipetting platform (Tecan, Mannedorf, Switzerland). The final MHB and WCB volume per well was 100µL. The initial concentration for antibiotics was 16 µg/mL whereas that of the alkaloid-related agents was 512 µg/mL. The inoculum was adjusted to a final bacterial concentration of 1.5×10^8 CFU/mL in the MHB and WCB according to the 0.5 McFarland standard scale, using a Densi-La-Meter II (Lachema, Brno, Czech Republic). The microtiter plates were inoculated with the bacteria (5 µL/well). The plates for the aerobic bacteria were incubated for 24 h whilst those plates with obligate anaerobes and *Lacticaseibacillus* were incubated (using the Whitley A35 anaerobic workstation (Don Whitley Scientific, Bingley, UK) for 48 h, both at 37°C. The anaerobic conditions were created by the supply of standard anaerobic gas mixture of 10% H_2, 10% CO_2, and 80% N_2 (Linde Gas, Prague, Czech Republic). Afterwards, the optical density of the bacterial cultures was determined at a wavelength of 405 nm to assess growth inhibition using a Cytation 3 Imaging Reader (BioTek, Winooski, USA) (Cos et al. 2006). The MICs were expressed as the lowest concentration that inhibited bacterial growth by ≥ 80% compared with that of the agent-free growth control (Jorgensen et al. 1999). With exception of the results of screening test focused on identification of the most promising combinations of antibacterial agents which was assayed in a single experiment, the obtained data is presented as the average values of three independent experiments, each performed in triplicate (Okoliegbe et al. 2021). The antibiotics (tested in the same row of the microplate used for MIC determination) were employed as a positive control for the verification of susceptibility of the bacterial strains in the broth medium. A drug-free bacterial culture served as the

negative control. There was no change in turbidity (no contamination) in the negative control wells. The highest concentration of DMSO and ethanol (both at 1%) present in the microtiter plates did not inhibit bacterial growth of any strain tested.

6.4 Evaluation of combination effects

The FICI (= ∑FIC), a measure recommended by the European Committee on Antimicrobial Susceptibility Testing, has been used for the assessment of combinatory effect of antibacterial agents (EUCAST 2000). The combined effects of antibiotics (A) and alkaloid-related agents (B) were calculated using the following equation: $\sum FIC = FIC_A + FIC_B$ where, $FIC_A = MIC_{A\ combination\ B}/MIC_{A\ alone}$ and $FIC_B = MIC_{B\ combination\ A}/MIC_{B\ alone}$ (Rakholiya et al. 2013). With the aim of avoiding reproducibility errors in MIC chequerboard interpretation, the effects were evaluated according to strict criteria proposed by Odds (2003), using average values of the FICIs. The results were interpreted as follows: synergy if $\sum FIC \leq 0.5$; no interaction if $\sum FIC > 0.5 - 4$, and antagonism if $\sum FIC > 4$. Based on calculated FICIs, two antimicrobial agents producing the synergistic inhibition of diarrhoea-causing pathogens and, simultaneously, antagonistic action on gut beneficial microbiota were identified as a combination producing a selective combinatory effect. In order to describe the synergistic and antagonistic interactions of antimicrobial agents, the minimum and maximum FICI values were used, respectively. For evaluation of results obtained from initial screening test, the averages of FICI values were calculated and compared using Microsoft Excel 365 (Microsoft, Redmond, WA, USA). For the purpose of the calculation, the combination of antimicrobial agents showing no inhibition of bacterial culture was evaluated as non-active and value 1 (representing no interaction) was used for average calculation. To aid in the interpretation of results in Tables 3-8, graphical representations of the FICI data obtained for the most-sensitive bacteria are illustrated in the form of isobolograms (Fig. 3-5). Following the description, a synergy effect is shown by an upward concave isobole, no interaction is shown by a straight line on the x and y-axis (linear isobole), and an antagonistic effect is represented by a convex isobole (Williamson 2001). The border of synergy was calculated according to the following equation: $0.5 - (MIC_{A\ combination\ B}/MIC_{A\ alone}) \times MIC_{B\ alone}$, which is based on the

conservative interpretation of results eliminating reproducibility errors in MIC values determined by the chequerboard methodology (Odds 2003).

7. Results

7.1 Screening of combinatory and/or selective effects of phytochemicals and synthetic analogues with antibiotics

In the first step of the study, broad spectrum of combinations of the phytochemicals and synthetic analogues with antibiotics were tested for their *in vitro* combinatory and/or selective activities against 10 diarrhoeagenic and 6 gut beneficial bacteria. Combinations of antibacterial agents produced synergistic effects with FICI values ranging from 0.071 to 0.5. Ciprofloxacin-zinc pyrithione (FICI = 0.071) and ciprofloxacin-tannic acid (FICI = 0.078) combinations showed the best synergistic effect against *S. flexneri*, followed by combination of ciprofloxacin with sanguinarine against *Y. enterocolitica* (FICI = 0.125). The FICI values observed for ceftriaxone in combination with either nitroxoline, sanguinarine, tannic acid or zinc pyrithione against *B. cereus, E. faecalis,* and *L. monocytogenes* were ranging from 0.188 - 0.375. Similarly, chloramphenicol produced synergistic effect when it was combined with either nitroxoline or sanguinarine against *E. faecalis, S. flexneri,* and *Y. enterocolitica* at FICI values ranging from 0.141 - 0.266. The greatest synergistic effect was observed with chloramphenicol *vs* sanguinarine combinations against *S. flexneri* at FICI value of 0.141. Tetracycline exhibited synergistic interactions with either nitroxoline, sanguinarine, tannic acid or zinc pyrithione against different bacterial strains tested, namely, *E. coli, E. faecalis, L. monocytogenes, S. flexneri, S. typhimurium, V. parahaemolyticus,* and *Y. enterocolitica* with FICI values ranging from 0.086 - 0.313. Tetracycline/nitroxoline combinations produced the strongest synergistic effect in *S. flexneri* (FICI = 0.086). Furthermore, vancomycin in the presence of either of the plant-derived compounds against *E. faecalis, S. flexneri,* or *Y. enterocolitica* produced synergistic activity at FICI values ranging from 0.188 - 0.500. Berberine chloride did not produce any synergistic effect with phytochemicals and their synthetic analogues. Interestingly, most of the combinations exhibited antagonistic effects against all the *Bifidobacterium* strains with FICIs values ranging from 4.023 to 8.023. The greatest antagonism was observed towards *B. breve* when ciprofloxacin was combined with nitroxoline with FICI value of 8.023, followed by ciprofloxacin-sanguinarine against *B.*

adolescentis and tetracycline-nitroxoline against *B. animalis* spp. *lactis* with FICIs = 8.016 for both combinations. On the other hand, all the tested *Lacticaseibacillus* strains demonstrated no interaction effects to the antimicrobial combinations with FICIs values ranging from 1.019 to 2.063. Tables 1 and 2 summarise the FICI values produced by combinations of phytochemicals and synthetic analogues with antibiotics against the diarrhoea-causing and gut beneficial bacteria. According to calculated FICI averages, the most efficient antimicrobial combinations were as follows: nitroxoline with tetracycline (0.380) and ciprofloxacin (0.497), zinc pyrithione with tetracycline (0.447) and ciprofloxacin (0.519), sanguinarine with ciprofloxacin (0.610) and tetracycline (0.631). The combinations of berberine with all antibiotics (1), tannic acid with chloramphenicol (0.910), and with vancomycin (0.902) produced the weakest antimicrobial effect. Based on above mentioned data, combinations that produced the most promising results as well as the combinations which have not been previously studied were chosen for testing in further steps of the research.

Table 1. *In vitro* susceptibility of diarrhoea-causing bacteria to phytochemicals and synthetic analogues in combination with antibiotics

Antibiotic	Bacterium/ Compound/FICI											
	Bacillus cereus	*Enterococcus faecalis*	*Escherichia coli* ATCC 25922	*E. coli* ATCC 35218	*E. coli* O175:H7-VT (N) NCTC 12900	*Listeria monocytogenes*	*Shigella flexneri*	*Salmonella Typhimurium*	*Vibrio parahaemolyticus*	*Yersinia enterocolitica*	**Average FICI**	**Total average FICI**
Berberine chloride												
Ciprofloxacin	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	1	1
Ceftriaxone	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	1	
Chloramphenicol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	1	
Tetracycline	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	1	
Vancomycin	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	1	
Nitroxoline												
Ciprofloxacin	0.281	0.266	1.008	0.504	0.516	0.094	0.281	n.a.	0.516	0.5	**0.497**	**0.609**
Ceftriaxone	0.188	n.a.	n.a.	0.508	1.063	0.188	0.531	0.625	0.508	n.a.	0.661	
Chloramphenicol	n.a.	0.266	n.a.	n.a.	n.a.	0.508	0.531	n.a.	0.516	n.a.	0.782	
Tetracycline	0.516	0.313	n.a.	0.516	0.156	0.172	0.086	0.508	0.282	0.254	**0.380**	
Vancomycin	0.75	0.188	n.a.	0.75	n.a.	0.516	1.031	n.a.	0.5	0.5	0.724	
Sanguinarine												
Ciprofloxacin	0.094	0.313	0.563	1.016	0.531	0.616	0.266	2.016	0.562	0.125	**0.610**	**0.680**
Ceftriaxone	0.188	0.258	n.a.	0.508	0.531	0.375	n.a.	n.a.	0.516	n.a.	0.638	
Chloramphenicol	0.531	0.266	n.a.	n.a.	n.a.	0.625	0.141	n.a.	1.008	0.25	0.682	
Tetracycline	0.531	0.313	1.016	1.008	0.504	0.288	1.004	0.563	0.563	0.516	**0.631**	

Vancomycin	1.031	0.563	n.a.	0.516	1.25	1.016	0.281	n.a.	n.a.	0.75	0.841	
Tannic acid												
Ciprofloxacin	n.a.	n.a.	n.a.	n.a.	0.506	0.531	0.078	n.a.	0.281	n.a.	0.740	0.827
Ceftriaxone	n.a.	n.a.	n.a.	n.a.	n.a.	0.266	0.531	n.a.	0.508	n.a.	0.831	
Chloramphenicol	n.a.	n.a.	n.a.	n.a.	n.a.	0.563	0.516	n.a.	1.016	n.a.	0.910	
Tetracycline	n.a.	n.a.	n.a.	n.a.	0.187	0.313	0.516	n.a.	0.508	n.a.	0.752	
Vancomycin	n.a.	n.a.	n.a.	n.a.	n.a.	0.75	0.266	n.a.	n.a.	n.a.	0.902	
Zinc pyrithione												
Ciprofloxacin	0.188	0.258	0.508	0.516	0.504	0.13	0.071	n.a.	1.008	1.008	**0.519**	**0.629**
Ceftriaxone	0.188	n.a.	n.a.	0.531	n.a.	0.375	0.516	0.516	0.563	n.a.	0.669	
Chloramphenicol	0.625	n.a.	n.a.	n.a.	n.a.	0.563	0.516	n.a.	1.063	0.508	0.828	
Tetracycline	0.531	0.75	n.a.	0.516	0.254	0.254	0.25	0.516	0.141	0.258	**0.447**	
Vancomycin	0.508	0.516	n.a.	0.508	n.a.	1.016	0.266	n.a.	0.516	0.508	0.684	

FICI, Fractional inhibitory concentration index. n.a. (non-active), the combination of antimicrobial agents showing no inhibition of bacterial culture (for average FICI calculation n.a. was replaced by 1 that is value representing no interaction).

Table 2. *In vitro* susceptibility of gut beneficial bacteria to phytochemicals and synthetic analogues in combination with antibiotics.

Antibiotic	Bacterium/ Compounds /FICI					
	Bifidobacterium adolescentis	*Bifidobacterium animalis* spp. *lactis*	*Bifidobacterium breve*	*Lacticaseibacillus casei*	*Limosilactobacillus reuteri*	*Lacticaseibacillus rhamnosus*
Nitroxoline						
Ciprofloxacin	4.016	8.012	8.023	2.047	1.063	2.031
Tetracycline	8.011	8.016	4.250	1.031	1.008	2.031
Sanguinarine						
Ciprofloxacin	8.016	2.416	4.023	2.031	1.125	1.019
Tetracycline	8.004	2.016	2.250	2.031	1.008	1.063
Zinc pyrithione						
Ciprofloxacin	0.516	1.425	4.832	2.063	1.125	2.038
Tetracycline	8.001	4.250	0.750	1.031	1.250	1.063

FICI, Fractional inhibitory concentration index.

7.2 Selective combinatory effect of ciprofloxacin with nitroxoline, sanguinarine, and zinc pyrithione against diarrhoea-causing and gut beneficial bacteria

In correspondence with results obtained from the initial screening test, it was observed that combinations of ciprofloxacin with either nitroxoline, sanguinarine, or zinc pyrithione revealed synergistic activity against diarrhoea-causing bacteria, and at the same time, their antagonistic action decreases potential harmful effect of these agents on gut beneficial bacteria. The MIC values (alone) obtained for nitroxoline, sanguinarine, and zinc pyrithione against the diarrhoea-causing bacteria tested ranged from 2 to 16, from 8 to 64, and from 4 to 16 µg/mL, respectively, whereas those for beneficial bacteria ranged from 4 to 16 µg/mL (nitroxoline), from 4 to 32 µg/mL (sanguinarine), and from 13.333 to 64 µg/mL (zinc pyrithione). Among all antimicrobial agents tested, combination of ciprofloxacin with zinc pyrithione produced the greatest synergistic action (FICI = 0.071) against the diarrhoea-causing pathogens, whereas the strongest antagonism on gut beneficial bacteria was demonstrated by nitroxoline when combined with ciprofloxacin (FICI = 8.023). Based on FIC indices, *S. flexneri* was the most susceptible bacterium to combination of ciprofloxacin with zinc pyrithione, whilst growth of *B. breve* was least affected by active concentrations of nitroxoline and ciprofloxacin combination.

For combination of ciprofloxacin with nitroxoline, several synergistic interactions were observed for all the five standard diarrhoeagenic bacteria tested with FICI values ranging from 0.094 to 0.477. The strongest synergy (FICI of 0.094) was obtained against *L. monocytogenes* at a nitroxoline concentration of 0.25 µg/mL, when a 13-fold ciprofloxacin MIC decrease was achieved (from 1.333 to 0.104 µg/mL). Furthermore, the highest ciprofloxacin MIC reduction was observed for *V. parahaemolyticus*, where nitroxoline concentration of 1 µg/mL caused a 16-fold reduction of ciprofloxacin MIC from 0.125 to 0.008 µg/mL with FICI of 0.313. In the case of the gut beneficial bacteria, the mixtures of these antimicrobial agents revealed a clear antagonistic effect on three of five species tested with FICI values ranging from 4.012 to 8.023. The significant antagonistic action (FICI = 8.023) was observed towards *B. breve* at a ciprofloxacin concentration of 0.125 µg/mL, when 8-fold of nitroxoline MIC increase was achieved (from 4 to 32 µg/mL). Also, *B.*

animalis subsp. *lactis* showed high resistance to this combination where ciprofloxacin MIC of 10.667 µg/mL caused 8-fold rise in inhibitory concentration of nitroxoline (4 to 32 µg/mL) to produce FICI value of 8.012. No interaction relationship was observed when these agents were combined against *L. casei* and *L. rhamnosus*. The results summarized in Table 3 show the complete data on growth-inhibitory activities of tested ciprofloxacin with nitroxoline against diarrhoeagenic and beneficial bacteria, including calculated FICI values.

The combination of ciprofloxacin/sanguinarine exerted growth-inhibitory interaction against the five diarrhoeal causing pathogens tested, with synergistic effect ranging between FICI values of 0.134 and 0.5. It also exhibited antagonism towards two out of five beneficial bacteria tested in this study, with FICI ranging from 4.012 to 8.016. Moreover, no interaction relationship was observed when these agents were combined against *L. casei* and *L. rhamnosus*. The most significant synergistic effect with FICI value of 0.134 was documented against *E. faecalis* at a sanguinarine concentration of 0.25 µg/mL, when an 8-fold ciprofloxacin MIC decrease was achieved from 0.5 to 0.063 µg/mL. *B. cereus* was another diarrhoea-causing bacterium significantly affected by combination of these agents. In this case, ciprofloxacin at concentration of 0.25 µg/mL caused a decline in the sanguinarine MIC by 32-fold (from 64 to 2 µg/mL) resulting in FICI value of 0.198. Additionally, the highest ciprofloxacin MIC reduction was achieved for *L. monocytogenes*, where sanguinarine concentration of 2 µg/mL produced 104-fold reduction of ciprofloxacin MIC from 1.667 to 0.016 µg/mL with FICI of 0.259. Based on FICI evaluation, the above-mentioned agents demonstrated strongest antagonistic effect towards *B. adolescentis*, at a ciprofloxacin concentration of 4 µg/mL, when 8-fold MIC increase of sanguinarine was achieved (from 4 to 32 µg/mL) with FICI value of 8.016. This was followed by *B. breve* which showed resistance to the combination with FICI of 4.023, where ciprofloxacin MIC of 5.333 µg/mL caused 4-fold rise in active concentration of sanguinarine (from 8 to 32 µg/mL). The individual MICs of ciprofloxacin and sanguinarine against diarrhoea-causing and beneficial bacteria as well as the MICs of its combinations with corresponding FICI values are summarized in Table 4.

In reference to the data summarized in Table 5, the combination of ciprofloxacin with zinc pyrithione produced synergistic antimicrobial effect (FICI ranging from 0.071 to 0.5)

against all the pathogenic bacteria. The strongest synergistic activity with FICI value of 0.071 was achieved against *S. flexneri* at a zinc pyrithione concentration of 0.125 µg/mL, when a 17-fold ciprofloxacin MIC decrease was attained (from 0.083 to 0.005 µg/mL). In addition, the highest ciprofloxacin MIC reduction was observed for *L. monocytogenes*, where zinc pyrithione concentration of 2 µg/mL caused a 63-fold decrease of ciprofloxacin MIC from 1 to 0.016 µg/mL with FICI of 0.316. Considering the gut beneficial microbes assessed in this work, the combinations of ciprofloxacin and zinc pyrithione showed antagonistic action towards *B. breve* at a ciprofloxacin concentration of 0.125 µg/mL, when 5-fold of zinc pyrithione MIC increase was achieved from 13.333 to 64 µg/mL with FICI value of 4.832. The mixture of both agents exhibited no interaction on *L. casei* and *L. rhamnosus*.

The combination profiles of the most sensitive and resistant bacteria are presented graphically in form of isobologram curves (Figure 3 and 4), which represents the results of the checkerboard assay and the FICI values, whereas the axes of each isobolograms are the dose-axes of the individual agents. The resulting isobolograms confirmed the synergistic effect of ciprofloxacin in combination with nitroxoline, sanguinarine and zinc pyrithione against *B. cereus*, *E. faecalis* and *S. flexneri*, where synergy was observed for three ratios in the isobolograms of each strain. Also, antagonistic effect of ciprofloxacin/zinc pyrithione combination was observed towards *B. breve* for one ratio whereas ciprofloxacin in combination with nitroxoline and sanguinarine showed two ratios towards *B. adolescentis*, *B. breve* and *B. animalis* subsp. *lactis*. In this regard, an upward concave curve (light green, orange, and purple solid lines) represents the confirmation of antimicrobial synergy observed against the diarrhoea causing bacteria whereas a downward concave curve (deep green, red, and blue dashed lines) distinctively suggests antagonism achieved towards bifidobacteria.

Table 3. *In vitro* susceptibilities of diarrhoea-causing and gut beneficial bacteria to ciprofloxacin and nitroxoline alone and in combination

Bacterium[a]	MIC[b] alone		MIC of NTX (values in bold)/MIC of CIP with FICI[c] of corresponding NTX-CIP combination															
			32		**16**		**8**		**4**		**2**		**1**		**0.5**		**0.25**	
	CIP	NTX	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FIC	MIC	FICI	MIC	FICI
Beneficial bacteria																		
B. a.	4	8	0.063	4.016	0.063	2.016	0.063	1.016	4	1.5	4	1.25	4	1.125	4	1.063	4	1.031
B. a. l.	10.667	4	0.125	8.012	0.125	4.012	0.125	2.012	9.333	1.875	10.667	1.5	10.67	1.25	10.667	1.125	10.667	1.063
B. b.	5.333	4	0.125	8.023	0.125	4.023	0.125	2.023	0.75	1.141	5.333	1.5	5.333	1.25	5.333	1.125	5.333	1.063
L. c.	1.333	16	0.063	2.047	0.063	1.047	1.333	1.5	1.333	1.25	2	1.625	2	1.563	2	1.532	2	1.516
L. r.	2	16	0.063	2.031	0.063	1.031	2	1.5	3.333	1.917	3.333	1.792	3.333	1.729	3.333	1.698	3.333	1.682
Diarrhoeal bacteria																		
E. f.	0.5	16	0.016	2.031	0.016	1.031	0.016	0.531	0.135	0.521	0.125	0.375	0.125	0.313	0.125	0.281	0.125	0.266
L. m.	1.333	16	0.016	2.012	0.016	1.012	0.016	0.512	0.5	0.625	0.416	0.437	0.833	0.687	0.5	0.406	0.104	0.094
			8		**4**		**2**		**1**		**0.5**		**0.25**		**0.125**		**0.063**	
B. c.	0.5	6.667	0.016	1.231	0.057	0.715	0.089	0.477	0.094	0.338	0.094	0.263	0.125	0.287	0.25	0.519	0.25	0.509
S. f.	0.016	2	0.002	4.125	0.002	2.125	0.002	1.125	0.008	1	0.008	0.75	0.008	0.625	0.008	0.563	0.004	0.281
V. p.	0.125	4	0.008	2.063	0.008	1.063	0.008	0.563	0.008	0.313	0.063	0.625	0.063	0.563	0.063	0.531	0.063	0.516

[a]*B. a.*, *Bifidobacterium adolescentis*; *B. a. l.*, *Bifidobacterium animalis subsp. lactis*; *B. b.*, *Bifidobacterium breve*; *L. c.*, *Lacticaseibacillus casei*; *L. r.*, *Lacticaseibacillus rhamnosus*; *E. f.*, *Enterococcus faecalis*; *L. m.*, *Listeria monocytogenes*; *B. c.*, *Bacillus cereus*; *S. f.*, *Shigella flexneri*; *V. p.*, *Vibrio parahaemolyticus*.
[b]MIC, minimum inhibitory concentration of ciprofloxacin (CIP) and nitroxoline (NTX) expressed as an average from three independent experiments each performed in triplicate. All MICs units are in µg/mL.
[c]FICI, fractional inhibitory concentration index; FICI values (≤ 0.5) indicate synergistic effects; FICI values (> 0.5 – 4) indicate no interaction effect; FICI values (> 4) indicate antagonistic effect.

Table 4. *In vitro* susceptibilities of diarrhoea-causing and gut beneficial bacteria to ciprofloxacin and sanguinarine alone and in combination

Bacterium[a]	MIC[b] alone		MIC of SNG (values in bold) / MIC of CIP with FICI[c] of corresponding SNG-CIP combination															
			32		**16**		**8**		**4**		**2**		**1**		**0.5**		**0.25**	
	CIP	SNG	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI
Beneficial bacteria																		
B. a.	4	4	0.063	8.016	0.063	4.016	0.063	2.016	0.063	1.016	4	1.5	4	1.25	4	1.125	4	1.063
B. a. l.	8	13.333	0.125	2.416	0.125	1.216	8.042	1.605	16	2.300	16	2.15	16	2.075	16	2.038	10.67	1.352
B. b.	5.333	8	0.125	4.023	0.125	2.023	1.417	1.266	1.417	0.766	5.333	1.25	5.333	1.125	5.333	1.063	5.333	1.031
L. c.	2	16	0.063	2.031	0.063	1.031	2.333	1.667	4	2.25	4	2.125	4	2.063	4	2.031	4	2.016
L. r.	3.333	32	0.063	1.019	2	1.100	2.667	1.050	2.667	0.925	2.667	0.863	2.667	0.831	2.667	0.816	3.333	1.008
Diarrhoeal bacteria																		
E. f.	0.5	26.667	0.016	1.231	0.016	0.631	0.016	0.331	0.016	0.181	0.094	0.263	0.125	0.287	0.125	0.269	0.063	0.134
S. f.	0.016	16	0.002	2.125	0.002	1.125	0.002	0.625	0.008	0.750	0.008	0.625	0.008	0.563	0.008	0.531	0.004	0.266
V. p.	0.125	32	0.004	1.032	0.004	0.532	0.007	0.306	0.042	0.461	0.063	0.567	0.083	0.695	0.104	0.848	0.125	1.008
			16		**8**		**4**		**2**		**1**		**0.5**		**0.25**		**0.125**	
L. m.	1.667	8	0.016	2.009	0.016	1.009	0.016	0.509	0.016	0.259	0.339	0.328	0.833	0.562	0.833	0.531	1	0.616
			256		**128**		**64**		**32**		**16**		**8**		**4**		**2**	
B. c.	0.25	64	0.016	4.063	0.016	2.063	0.016	1.063	0.063	0.75	0.063	0.5	0.063	0.375	0.063	0.313	0.042	0.198

[a]*B. a.*, *Bifidobacterium adolescentis*; *B. a. l.*, *Bifidobacterium animalis subsp. lactis*; *B. b.*, *Bifidobacterium breve*; *L. c.*, *Lacticaseibacillus casei*; *L. r.*, *Lacticaseibacillus rhamnosus*; *E. f.*, *Enterococcus faecalis*; *S. f.*, *Shigella flexneri*; *V. p.*, *Vibrio parahaemolyticus*; *L. m.*, *Listeria monocytogenes*; *B. c.*, *Bacillus cereus*.

[b]MIC, minimum inhibitory concentration of ciprofloxacin (CIP) and sanguinarine (SNG) expressed as an average from three independent experiments each performed in triplicate. All MICs units are in µg/mL.

[c]FICI, fractional inhibitory concentration index; FICI values (≤ 0.5) indicate synergistic effects; FICI values (> 0.5 – 4) indicate no interaction effect; FICI values (> 4) indicate antagonistic effect.

Table 5. *In vitro* susceptibilities of diarrhoea-causing and gut beneficial bacteria to ciprofloxacin and zinc pyrithione alone and in combination

Bacterium[a]	MIC[b] alone		MIC of ZPT (values in bold) / MIC of CIP with FICI[c] of corresponding ZPT-TET combination															
			32		**16**		**8**		**4**		**2**		**1**		**0.5**		**0.25**	
	CIP	ZPT	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI
Beneficial bacteria																		
B. a.	4	64	0.063	0.516	4	1.25	4	1.125	4	1.063	4	1.031	4	1.016	4	1.008	4	1.004
B. a. l.	9.333	22.667	0.125	1.425	5.375	1.282	5.375	0.929	5.375	0.752	6.667	0.803	8	0.901	8	0.879	8	0.868
			64		**32**		**16**		**8**		**4**		**2**		**1**		**0.5**	
B. b.	4	13.333	0.125	4.832	0.125	2.432	2.333	1.784	3.333	1.433	3.333	1.133	4	1.15	4	1.075	4	1.038
L. c.	1	32	0.063	2.063	0.063	1.063	0.354	0.854	0.5	0.75	0.833	0.958	0.833	0.896	0.833	0.864	0.833	0.849
L. r.	1.666	32	0.063	2.038	0.063	1.038	0.125	0.575	1	0.85	2	1.325	2	1.263	2	1.232	2	1.216
Diarrhoeal bacteria																		
			4		**2**		**1**		**0.5**		**0.25**		**0.125**		**0.063**		**0.031**	
L. m.	1	6.667	0.016	0.616	0.016	0.316	0.25	0.4	0.25	0.325	0.333	0.370	0.417	0.436	0.375	0.384	0.125	0.130
			8		**4**		**2**		**1**		**0.5**		**0.25**		**0.125**		**0.063**	
B. c.	0.417	4	0.016	2.037	0.016	1.037	0.037	0.588	0.037	0.338	0.073	0.300	0.094	0.287	0.167	0.431	0.104	0.266
E. f.	0.5	8	0.016	1.031	0.016	0.531	0.125	0.5	0.25	0.625	0.125	0.313	0.125	0.281	0.125	0.266	0.125	0.258
			16		**8**		**4**		**2**		**1**		**0.5**		**0.25**		**0.125**	
S. f.	0.083	16	0.002	1.024	0.002	0.524	0.006	0.321	0.007	0.203	0.007	0.141	0.007	0.110	0.026	0.329	0.005	0.071
V. p.	0.063	8	0.008	2.124	0.008	1.124	0.008	0.624	0.016	0.498	0.063	1.117	0.063	1.055	0.104	1.685	0.063	1.008

[a]*B. a.*, *Bifidobacterium adolescentis*; *B. a. l.*, *Bifidobacterium animalis subsp. lactis*; *B. b.*, *Bifidobacterium breve*; *L. c.*, *Lacticaseibacillus casei*; *L. r.*, *Lacticaseibacillus rhamnosus*; *E. f.*, *Enterococcus faecalis*; *S. f.*, *Shigella flexneri*; *V. p.*, *Vibrio parahaemolyticus*; *L. m.*, *Listeria monocytogenes*; *B. c.*, *Bacillus cereus*.
[b]MIC, minimum inhibitory concentration of ciprofloxacin (CIP) and sanguinarine (ZPT) expressed as an average from three independent experiments each performed in triplicate. All MICs units are in µg/mL.
[c]FICI, fractional inhibitory concentration index; FICI values (≤ 0.5) indicate synergistic effects; FICI values (> 0.5 – 4) indicate no interaction effect; FICI values (> 4) indicate antagonistic effect.

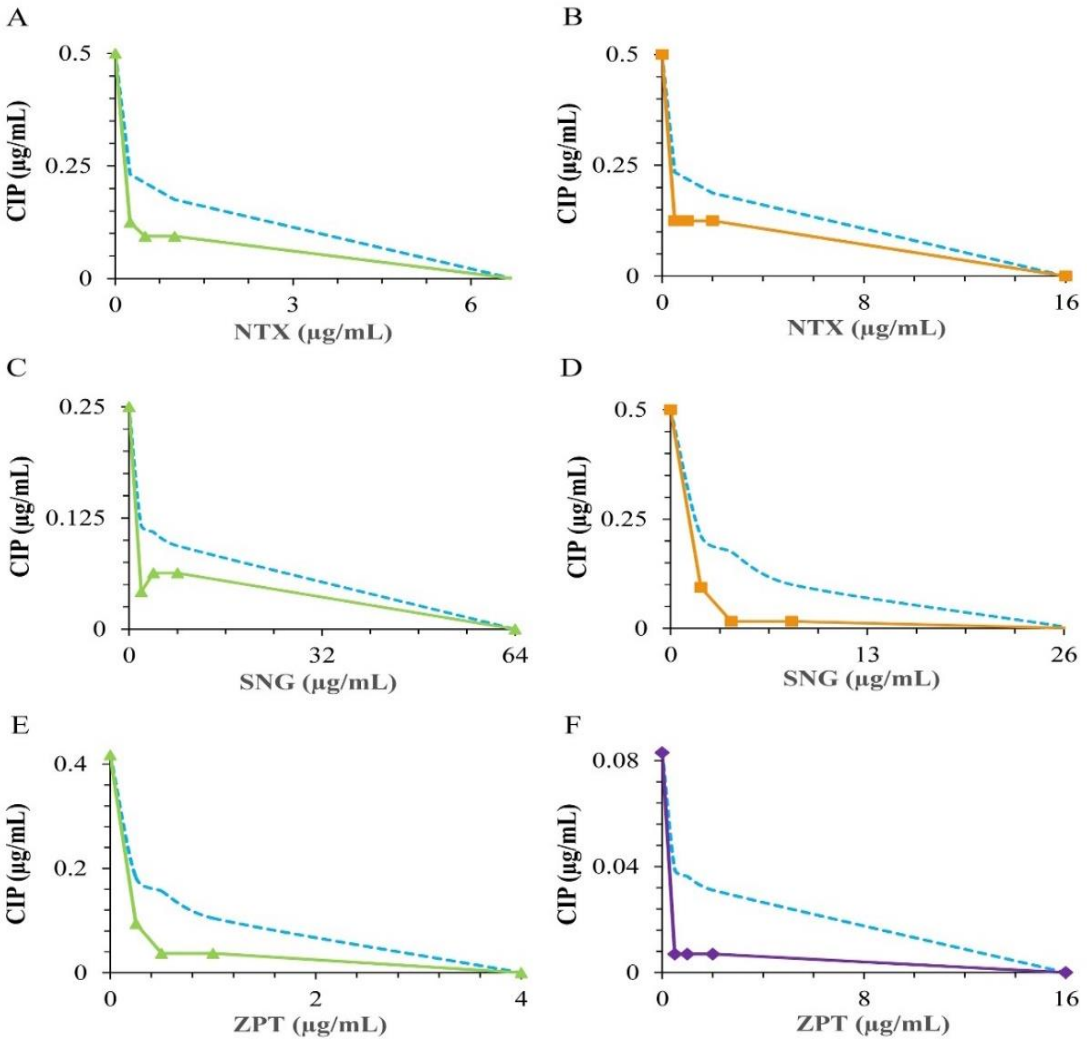


Figure legend

Figure 3. Isobolograms of the synergistic interactions for ciprofloxacin (CIP) with: nitroxoline (NTX) against *Bacillus cereus* (A) and *Enterococcus faecalis* (B); sanguinarine (SNG) against *B. cereus* (C) and *E. faecalis* (D); and zinc pyrithione (ZPT) against *B. cereus* (E) and *Shigella flexneri* (F).
B. cereus (▲), *E. faecalis* (■) and *S. flexneri* (♦); border for synergy (---).

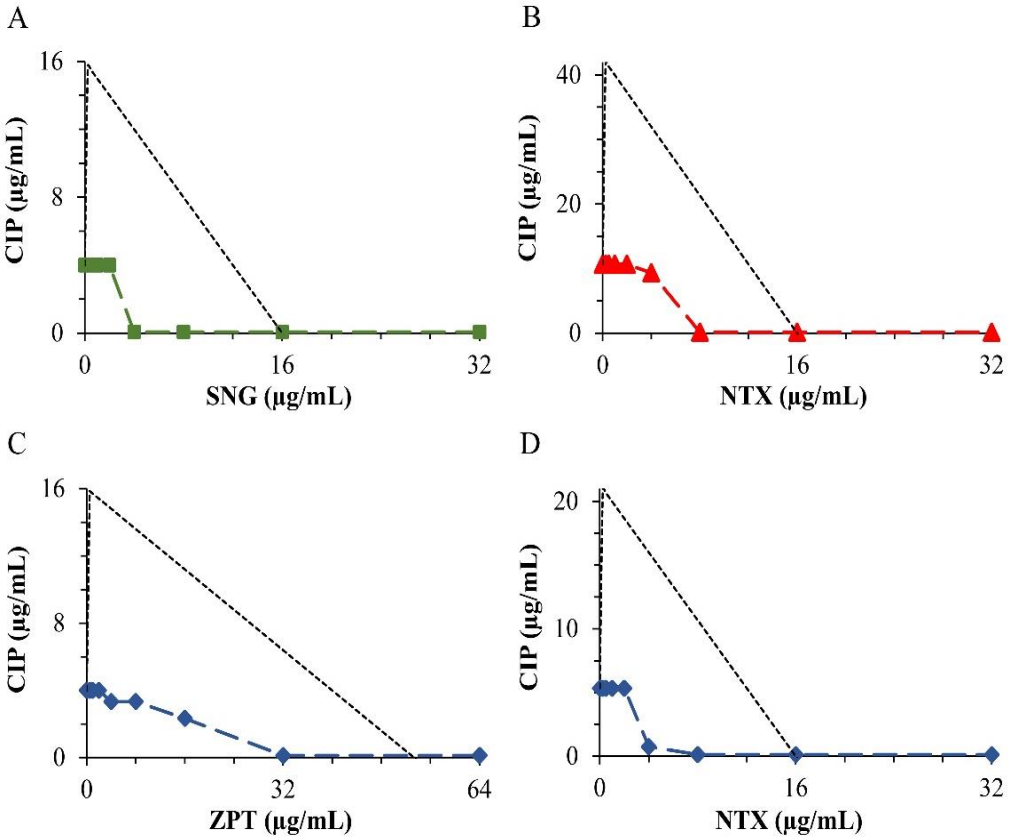


Figure 4. Isobolograms of the antagonistic interactions for ciprofloxacin (CIP) with: sanguinarine (SNG) against *Bifidobacterium adolescentis* (A); nitroxoline (NTX) against *B. animalis* subsp. *lactis* (B) and *B. breve* (D); and zinc pyrithione (ZPT) against *B. breve* (C).

B. adolescentis (■), *B. animalis* subsp. *lactis* (▲) and *B. breve* (♦); border for antagonism (---). Since the very low concentrations of ciprofloxacin in the combination with high concentrations of the alkaloid-related agents produced antagonistic effect, the convex isobole representing antagonistic interaction is not visible under measure used for drawing of graphs shown in the figures. A magnified resolution of the graphs presented in the manuscript would help to show it in more obvious way.

7.3 Combinatory effect of tetracycline with nitroxoline, sanguinarine, and zinc pyrithione against diarrhoea-causing bacteria

Besides the selective combinatory effect of certain antibiotics with phytochemicals and their synthetic analogues against diarrhoea-causing and gut beneficial bacteria described in previous section, several tested combinations produced only synergistic effect against diarrhoea-causing bacteria during the initial screening, which was, nevertheless, worth of further investigation. This was obvious especially in the case of combinations of tetracycline with either nitroxoline, sanguinarine, or zinc pyrithione, when synergistic activity was observed against several diarrhoea-causing bacteria.

According to the FICIs values, and the number of bacterial strains susceptible to the combination of agents tested, tetracycline produced the strongest interaction when combined with nitroxoline, followed by zinc pyrithione, and sanguinarine. The values of MIC alone attained for nitroxoline, sanguinarine, and zinc pyrithione against the different strains of bacteria tested ranged from 2 to 16, from 3.33 to 128, and from 8 to 16 µg/mL, respectively. Table 6 shows the individual and combined MICs of tetracycline and nitroxoline against the selected diarrhoea-causing bacteria, together with their corresponding fractional inhibitory concentration indices (FICIs). The tetracycline-nitroxoline combination produced various synergistic interactions against all bacterial strains, and the sum of the FICIs ranged from 0.086 to 0.5. The greatest synergistic effect was observed against *S. flexneri* (FICI: 0.086) at 0.063 µg/mL nitroxoline, resulting in a 16-fold decrease in tetracycline MIC (from 4 to 0.25 µg/mL). Additionally, the greatest MIC reduction (from 0.667 to 0.083 µg/mL) for tetracycline was observed against *L. monocytogenes*, at 0.5 µg/mL nitroxoline (FICI: 0.156).

The combination of tetracycline and zinc pyrithione produced synergistic antibacterial effects against five out of the six diarrhoea-causing pathogens (FICI ranging from 0.109 to 0.479; Table 7). The greatest synergistic effect was documented against *S. flexneri* (FICI value of 0.109), resulting in an 11-fold decrease in tetracycline MIC (from 5.333 to 0.5 µg/mL) at a zinc pyrithione concentration of 0.125 µg/mL. Also, the greatest tetracycline MIC reduction was reported against *V. parahaemolyticus*, at a zinc pyrithione concentration of 1 µg/mL, which resulted in a 129-fold decrease of the MIC of tetracycline (from 4 to 0.031 µg/mL) and a FICI of 0.133.

The tetracycline-sanguinarine combination demonstrated synergistic interactions against two out of the six diarrhoeal bacteria evaluated, with FICI values ranging from 0.288 to 0.5 (Table 8). The greatest synergistic effect was achieved against *L. monocytogenes* (FICI value of 0.288) at a sanguinarine concentration of 0.125 µg/mL, which caused a four-fold tetracycline MIC decrease (from 0.5 to 0.125 µg/mL). Furthermore, the greatest tetracycline MIC reduction was also observed for this bacterium (FICI of 0.428), where 1 µg/mL of sanguinarine caused an eight-fold reduction of tetracycline MIC (from 0.5 to 0.063 µg/mL).

Isobologram curves established from the results of the chequerboard assays and the calculated FICI values for the most-susceptible bacteria are presented in Figure 5. The axes of each isobologram represent the dose-axes of the individual agents. The isobolograms confirmed the synergistic antimicrobial effects of tetracycline when combined with either nitroxoline, sanguinarine, or zinc pyrithione against *E. faecalis*, *E. coli*, *L. monocytogenes*, *S. flexneri*, *V. parahaemolyticus*, and *Y. enterocolitica*. Synergy was observed for three ratios in the isobolograms of each bacterial pathogen. The concave isobole (represented by the round dotted lines) indicates the confirmation of antimicrobial synergy observed against the tested diarrhoea-causing bacteria.

Table 6. *In vitro* susceptibility of diarrhoeagenic bacteria to tetracycline and nitroxoline alone and in combination.

Bacterium[a]	**MIC**[b] **alone**		**MIC of NTX (values in bold) / MIC of TET with FICI**[c] **of corresponding NTX-TET combination**															
			32		**16**		**8**		**4**		**2**		**1**		**0.5**		**0.25**	
	TET	NTX	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI
E. faecalis	1	16	0.031	2.031	0.031	1.031	0.031	0.531	0.25	0.5	0.25	0.375	0.25	0.313	1	1.031	1	1.016
L. monocytogenes	0.667	16	0.016	2.024	0.016	1.024	0.052	0.578	0.109	0.413	0.083	0.249	0.083	0.187	0.083	0.156	0.104	0.172
			8		**4**		**2**		**1**		**0.5**		**0.25**		**0.125**		**0.063**	
E. coli O175:H7	4	2	0.063	4.016	0.063	2.016	0.063	1.016	1	0.75	4	1.25	1	0.375	1	0.313	0.5	0.157
S. flexneri	4	2.667	0.063	3.015	0.063	1.516	0.063	0.766	2.333	0.958	3	0.937	1	0.344	1	0.297	0.25	0.086
V. parahaemolyticus	2	2	0.016	4.008	0.016	2.008	0.016	1.008	0.016	0.508	0.5	0.50	0.5	0.375	0.5	0.313	0.5	0.282
			4		**2**		**1**		**0.5**		**0.25**		**0.125**		**0.063**		**0.031**	
Y. enterocolitica	8	6.667	0.031	0.604	1	0.425	2	0.400	2	0.325	2	0.287	2	0.269	2	0.259	2	0.255

[a:] *Enterococcus faecalis*; *Escherichia coli* O175:H7; *Listeria monocytogenes*; *Shigella flexneri*; *Vibrio parahaemolyticus*; *Yersinia enterocolitica*.
[b] MIC, minimum inhibitory concentration of TET and NTX expressed as an average of three independent experiments, each performed in triplicate. All MICs units are in µg/mL.
[c] FICI, fractional inhibitory concentration index; FICI values (≤ 0.5) indicate synergistic effects; FICI values (> 0.5 – 4) indicate no interaction effect; FICI values (> 4) indicate antagonistic effect.

Table 7. *In vitro* susceptibility of diarrhoeagenic bacteria to tetracycline and zinc pyrithione alone and in combination.

Bacterium[a]	MIC[b] alone		MIC of ZPT (values in bold) / MIC of TET with FICI[c] of corresponding ZPT-TET combination															
			16		**8**		**4**		**2**		**1**		**0.5**		**0.25**		**0.125**	
	TET	ZPT	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI
S. flexneri	5.333	8	0.063	2.012	0.063	1.012	1	0.688	1	0.438	1	0.313	1	0.250	1	0.219	0.5	0.109
V. parahaemolyticus	4	8	0.016	2.004	0.016	1.004	0.016	0.504	0.031	0.258	0.031	0.133	0.5	0.188	0.5	0.156	0.5	0.141
			8		**4**		**2**		**1**		**0.5**		**0.25**		**0.125**		**0.063**	
E. coli O175:H7	4	16	0.063	0.516	**0.25**	0.313	1	0.375	1	0.313	1	0.281	2	0.516	2	0.508	1	0.254
E. faecalis	1	8	0.031	1.031	0.031	0.531	0.5	0.75	1	1.125	1	1.063	1	1.031	1	1.016	1	1.008
Y. enterocolitica	8	8	0.031	1.004	0.25	0.531	1	0.375	1	0.25	2	0.313	2	0.281	2	0.266	2	0.258
			4		**2**		**1**		**0.5**		**0.25**		**0.125**		**0.063**		**0.031**	
L. monocytogenes	0.5	8	0.016	0.532	0.016	0.282	0.125	0.375	0.208	0.479	0.208	0.447	0.125	0.266	0.125	0.258	0.125	0.254

[a:] *Enterococcus faecalis*; *Escherichia coli* O175:H7; *Listeria monocytogenes*; *Shigella flexneri*; *Vibrio parahaemolyticus*; *Yersinia enterocolitica*.
[b] MIC, minimum inhibitory concentration of TET and ZPT expressed as an average of three independent experiments, each performed in triplicate. All MICs units are in µg/mL.
[c] FICI, fractional inhibitory concentration index; FICI values (≤ 0.5) indicate synergistic effects; FICI values (> 0.5 – 4) indicate no interaction effect; FICI values (> 4) indicate antagonistic effect.

Table 8. *In vitro* susceptibility of diarrhoeagenic bacteria to tetracycline and sanguinarine alone and in combination.

Bacterium[a]	MIC[b] alone		MIC of SGN (values in bold) / MIC of TET with FICI[c] of corresponding SGN-TET combination															
			64		**32**		**16**		**8**		**4**		**2**		**1**		**0.5**	
	TET	SGN	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI
E. coli O175:H7	4	128	0.25	0.563	4	1.25	4	1.125	4	1.063	4	1.031	4	1.016	2	0.508	2	0.504
			32		**16**		**8**		**4**		**2**		**1**		**0.5**		**0.25**	
E. faecalis	1	16	0.031	2.031	0.031	1.031	0.031	0.531	0.25	0.5	0.25	0.375	0.25	0.313	1	1.031	1	1.016
Y. enterocolitica	2	64	0.031	0.516	2	1.25	2	1.125	2	1.063	2	1.031	2	1.016	2	1.008	2	1.004
			16		**8**		**4**		**2**		**1**		**0.5**		**0.25**		**0.125**	
L. monocytogenes	0.5	3.333	0.016	4.832	0.016	2.432	0.016	1.232	0.031	0.662	0.063	0.426	0.104	0.358	0.125	0.325	0.125	0.288
			8		**4**		**2**		**1**		**0.5**		**0.25**		**0.125**		**0.063**	
S. flexneri	1	16	1	1.5	1	1.25	1	1.125	1	1.063	0.5	0.531	0.5	0.516	1	1.008	1	1.004
V. parahaemolyticus	1	16	0.25	0.75	0.5	0.75	0.5	0.625	0.5	0.563	1	1.031	1	1.016	1	1.008	1	1.004

[a:] *Enterococcus faecalis*; *Escherichia coli* O175:H7; *Listeria monocytogenes*; *Shigella flexneri*; *Vibrio parahaemolyticus*; *Yersinia enterocolitica*.
[b] MIC, minimum inhibitory concentration of TET and SGN expressed as an average of three independent experiments, each performed in triplicate. All MICs units are in µg/mL.
[c] FICI, fractional inhibitory concentration index; FICI values (≤ 0.5) indicate synergistic effects; FICI values (> 0.5 – 4) indicate no interaction effect; FICI values (> 4) indicate antagonistic effect.

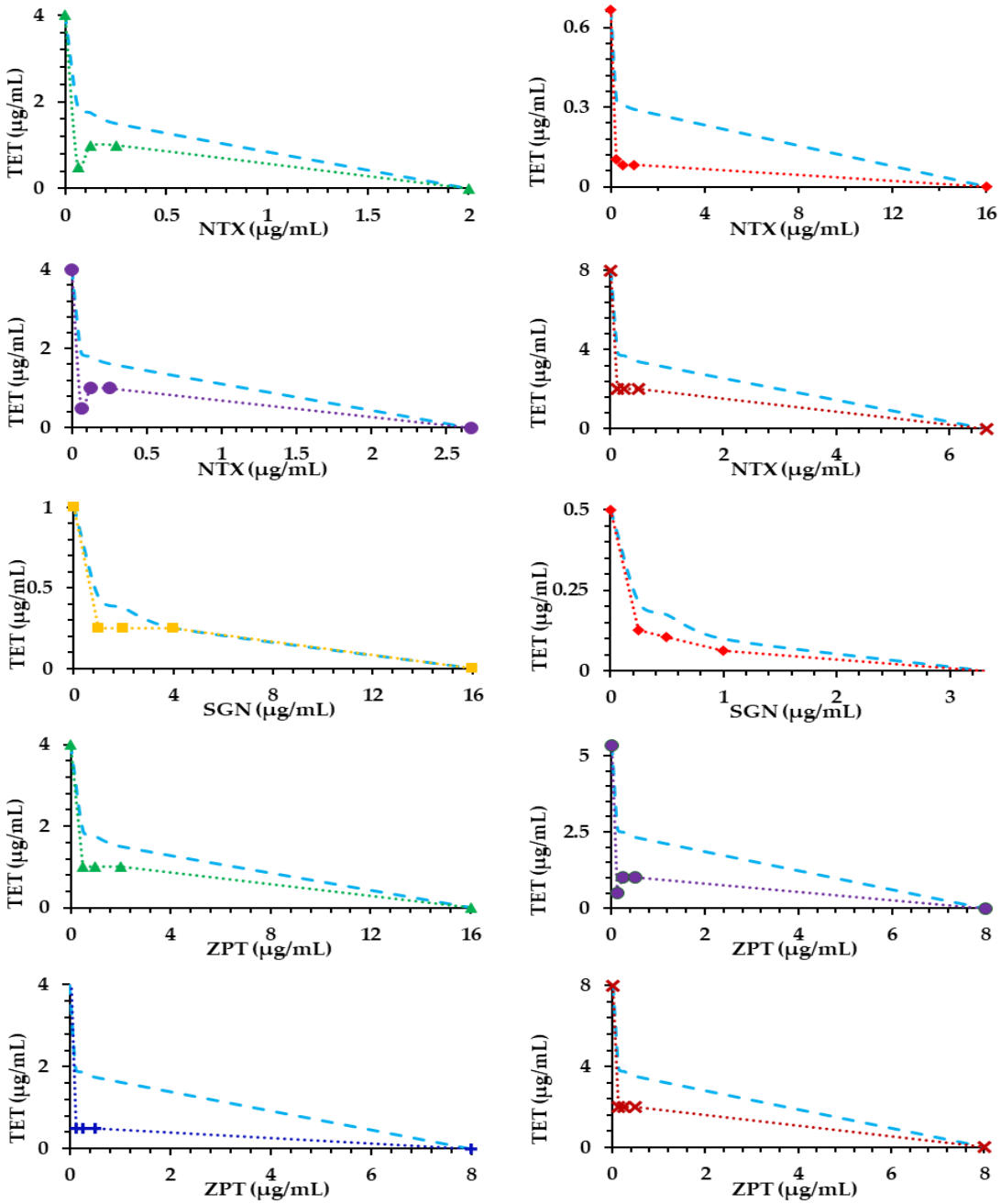


Figure 5. Isobolograms of the synergistic interactions for combination of tetracycline (TET) with: nitroxoline (NTX) against *Escherichia coli* (A), *Listeria monocytogenes* (B), *Shigella flexneri* (C) and *Yersinia enterocolitica* (D); sanguinarine (SGN) against *Enterococcus faecalis* (E) and *L. monocytogenes* (F); and for combination of TET with zinc pyrithione (ZPT) against *E. coli* (G), *S. flexneri* (H), *Vibrio parahaemolyticus* (I) and *Y. enterocolitica* (J). *E. coli* (▲), *E. faecalis* (■), *L. monocytogenes* (♦), *S. flexneri* (●), *V. parahaemolyticus* (+) and *Y. enterocolitica* (x); border for synergy (---) calculated for $\sum FIC \leq 0.5$.

8. Discussion

In recent times, combination treatments have been utilised in many cases to offer more efficacy and improved therapeutic options for patients in comparison with monotherapies. Combinations of plant-derived agents with conventional antibiotics have attracted the attention of many researchers. In the present study, the results of preliminary screening test indicated that combinations of alkaloid-related compounds with certain antibiotics such as ciprofloxacin and tetracycline are producing strong synergistic interactions. In addition, the results also suggested that certain alkaloid-related compounds can enhance antagonistic antibacterial activity of antibiotics against gut beneficial bacteria. In correspondence with published standards (Odds 2003), the FICIs range showing synergy and antagonism (≤ 05 - ≥ 4) were observed against some diarrhoeagenic and beneficial bacteria in case of several antimicrobial combinations. Selective antibacterial activities of the tested agents against the pathogenic and the gut beneficial bacteria have been previously documented (Kudera et al. 2020). In contrast, this is the first report on selective combinatory effect of antimicrobial agents. The antimicrobial synergistic activities of berberine in combination with conventional antibiotics have been described in many studies. For example, was demonstrated that berberine exhibited synergistic effect with vancomycin toward clinical isolates of *C. difficile* strains with FICI values ranging from 0.063-0.5 (Wultanska et al. 2020). Similarly, significant synergistic effect was observed when the aforementioned agent was combined with meropenem (FICI = 0.266) and sulbactam (FICI = 0.313) against strains of multidrug resistant *A. baumannii* (Li et al. 2021). In addition, antistaphylococcal synergistic effect of berberine-oxacillin combination has been observed with FICI value of 0.500 (Yu et al. 2005). In the previous study, berberine has been reported to demonstrate synergistic activities with ciprofloxacin against diarrhoeagenic bacteria *E. coli* and *S. enteritidis* (Ebrahimi et al. 2016). However, our results showed that the combination involving this compound and antibiotics was not active against the microorganisms. In this regard, the discrepancy with our study findings may be explained that the earlier study used berberine extracts (from the stem of *Berberis vulgaris*) in combination with ciprofloxacin as compared with standard berberine hydrochloride that we employed. Although the number of reports on synergistic activity of tannic acid with antibiotics against the tested diarrhoea-causing bacteria are limited, synergistic antistaphylococcal activity of the aforementioned phytocompound with fusidic

acid and rifampicin has been documented at FICI values ranging from 0.375-05 (Kyaw et al. 2011). Additionally, the work of Kırmusaoğlu (2019) observed that tannic acid enhanced synergistic efficacy of ampicillin (FICI: ranged from 0.295 to 0.398), oxacillin (FICI: ranged from 0.173 to 0.477), and penicillin (FICI: ranged from 0.174 to 0.223) against Methicillin-resistant *S. aureus* clinical isolates. These are in consonance with the results we observed for the susceptible diarrhoeal pathogens (FICI values ranging from 0.078 to 0.5). The antibacterial activity of tannic acid may be attributed to its astringent property, ability to penetrate bacterial cell membrane and interfere with cell metabolism, as well as its strong tendency to complex with metal ions (Dusane et al. 2015; Kaczmarek 2020). In a previously published report, mixtures of sanguinarine and streptomycin produced synergistic effect against diarrhoeagenic *E. coli* with FICI value of 0.375 (Hamoud et al. 2015). It has also been observed that sanguinarine synergistically enhanced growth inhibition of polymyxin B against pathogenic strains of *E. coli* at FICI values ranging from 0.375-0.5 (Qiao et al. 2024). As in another studies, significant results were obtained when sanguinarine was combined with either ciprofloxacin or vancomycin against strains of *S. aureus* with FICI values ranging from 0.06 - 0.5 (Obiang-Obounou et al. 2011; Andima et al. 2024). Comparatively, these results are within the range of our findings. In a recently published study, zinc pyrithione exhibited enhancement of both antistaphylococcal and antistreptococcal activities for gentamicin with FICI values in the range from 0.20-0.43 (Mala et al. 2022). This suggests a strong antibacterial synergistic effect of this agent in the presence of antibiotics as it is in agreement with our results. Although published data describing synergistic effect of ceftriaxone and chloramphenicol with the tested plant-derived agents is limited, the above-mentioned antibiotics have shown interactions with other bioactive plant compounds against diarrhoea-causing pathogens. For instance, synergism effect of ceftriaxone and quercetin was achieved in the range of 0.40-0.48 against clinical isolate of *E. coli* (Alnour et al. 2022). Conversely, ceftriaxone in combination with the various tested plant-based antimicrobials against *E. coli* strains determined did not exhibit synergism. The discrepancy may be that the previous study utilised clinical isolates of *E. coli* whilst standard strains were analysed in our study. In another research, chloramphenicol *vs Cinnamomum verum* essential oil in combination demonstrated synergistic interaction against diarrhoeic *E. coli* at FICI = 0.5 (El Atki et al. 2019). This did not agree with the results we observed because, the phytocompound agent used in the mixture by the previous workers was different from our own. Subsequently, antimicrobial

combinations that showed promising were further investigated and remarkable results were observed as in the case of ciprofloxacin as well as tetracycline in combination with either nitroxoline, sanguinarine or zinc pyrithione against the tested microorganisms.

In accordance with the standardized antimicrobial susceptibility testing breakpoint data (Murray et al. 1999), the MIC range of ciprofloxacin (0.016 - 1 μg/mL) observed in this study for pathogenic bacteria indicates their susceptibility to this antibiotic. Its previously reported MIC values against *B. cereus*, *E. faecalis*, *L. monocytogenes*, *S. flexneri* and *V. parahaemolyticus* were in respective ranges of 0.03 - 1, 0.25 - 1, 1 - 4, 0.008 - 0.03 and 0.20 - 0.39 μg/ml (Rolston et al. 2004; Cohen et al. 1991; Cherubin & Stratton 1995; Felmingham et al. 1997; Inagaki et al. 1989), which is well corresponding with the results of our experiments. In the case of beneficial bacteria, Masco et al. (2006) has reported ciprofloxacin MIC values ranging from 1 to 16 μg/ml towards the standard strains of bifidobacteria which is similar data recorded in this study. Moreover, consistent with our findings, Rozman et al. (2020) reported MIC values of 1 and 2 μg/ml towards *L. casei* and *L. rhamnosus*, respectively. In another study conducted by Chang et al. (2012), ciprofloxacin produced MIC values ranging from 4-64 μg/ml against *L. casei* isolated from faecal samples of children. Compared with our report, this discrepancy may be caused by different susceptibility of standard bacterial strain used in this study. According to the recently published studies, alkaloid-related agents (nitroxoline, sanguinarine and zinc pyrithione) have demonstrated growth-inhibitory effect against the diarrhoea-causing bacteria tested, at MIC values ranging from 1-128 μg/mL. In addition, the study further reported MIC values of the above-mentioned agents towards the standard gut beneficial bacteria ranging from 16-512 μg/mL (Kudera et al. 2020). The MIC values recorded in our study belong to this range. It is therefore suggested that our result describes bacteriostatic effect (bacterial growth inhibition) of the antibacterial agents tested rather than its bactericidal effect (kill bacterial growth) (Pankey & Sabath 2004), which may provide valuable information in the area of pharmacological research. However, further research on the nature of bacterial growth inhibitory effects of these antimicrobial agents identified in this study is required. The synergistic effect of ciprofloxacin with other antibiotics (e.g., gentamicin and trimethoprim) has previously been reported against clinical isolates of bacterial pathogens causing diarrhoea (Mandal et al. 2003; Huovinen et al. 1992). Furthermore, another study has shown that combination of this antibiotic with isoquinoline

alkaloid berberine produced synergistic action against strain species of diarrhoeagenic bacteria (Shi et al. 2018). Nevertheless, experimental data on combined effect of ciprofloxacin with nitroxoline, sanguinarine and zinc pyrithione are completely missing. Recently, the paper on selective antibacterial action of the above-mentioned agents against diarrheic and beneficial gut bacteria appeared in the literature (Kudera et al. 2020). In our investigation, ciprofloxacin in combination with nitroxoline, sanguinarine and zinc pyrithione at various inhibitory concentrations produced significant synergism against diarrhoeal pathogens as well as antagonism on bifidobacteria. To the best of our knowledge, this is the first report demonstrating selective combinatory effect of antimicrobial agents against diarrhoea-causing and gut beneficial bacteria.

From the results presented in this study, it is not possible to conclude what action mechanisms are responsible for synergistic and antagonistic interactions between ciprofloxacin and tested agents. However, based on literature data, it can be hypothesized that different mechanisms are responsible for the ability of nitroxoline, sanguinarine and zinc pyrithione to reduce MIC of ciprofloxacin against the diarrhoeagenic bacteria as well as for the capacity of ciprofloxacin to increase the MICs of the earlier mentioned agents towards the beneficial bacteria. It has been documented that ciprofloxacin targets topoisomerase enzymes essential for bacterial DNA synthesis, namely DNA gyrase and topoisomerase IV (Drlica & Zhao 1997). Metal ion cofactors (e.g., magnesium ion) are known to affect substantially the biological properties of topoisomerases (Sissi & Palumbo 2009). It has previously been described that activity of the quinolones is reduced in the presence of divalent cations, such as Mg^{2+} (Marshall and Piddock. 1994). Since nitroxoline and zinc pyrithione have been observed to chelate metal ions (Pelletier et al. 1995; Dinning et al. 1998), it is possible to assume that Mg^{2+} bonded to these compounds is not available as a cofactor necessary for proper functions of topoisomerases, and, at the same time, it does not affect activity of ciprofloxacin. The ability of both agents to form complexes with Mg^{2+} may therefore significantly contribute to their synergistic antibacterial action with ciprofloxacin observed in this study. Regarding the antagonistic activity observed in this study, we assume that the ability of bifidobacteria sequester and accumulate metal ions from their environment (Kot & Bezkorovainy 1991), can contribute to its increased resistance to nitroxoline and zinc pyrithione in presence of ciprofloxacin. It can also be hypothesized that ciprofloxacin, which increases bacterial membrane permeability by

the release of bound Ca^{2+} into the cytosol (Zawadzka et al. 2019), can enhance penetration of sanguinarine, which blocks cytokinesis in bacteria by inhibiting Z-ring formation (Beuria et al. 2005), into the bacterial cells. On the other hand, sanguinarine may also enhance intracellular penetration of ciprofloxacin because it has been observed that permeability of bacterial cell membrane increases in the presence of this alkaloid (Zhang et al. 2020, Obiang-Obounou et al. 2011). This may be suggested as a probable mechanism underlying the synergistic antibacterial interaction between these two antimicrobial agents against the diarrhoeal-causing bacteria examined in our studies. Although the presence of sanguinarine in the feed administered to experimental animals has previously been observed to increase the levels of bifidobacteria in the contents of their intestinal lumina (Chen et al. 2018), the mechanism responsible for increased resistance of this bacteria to sanguinarine in the presence of ciprofloxacin is unclear. Interestingly, ciprofloxacin MICs were greatly reduced at high concentrations of the second compound, whereas those concentrations were in many cases above the MIC of the second agent. A similar phenomenon was observed in study of Lalouckova et al (2021), who researched antagonistic effect of oxacillin in combination with palm seed crude oils and lauric acid against *Staphylococcus aureus*.

According to the CLSI (2020) antimicrobial susceptibility testing breakpoint data interpretation, the MIC range of tetracycline observed in this study (0.5 to 8 µg/mL) proves sensitivity of most of the diarrhoeagenic bacteria to this antibiotic. Previously reported MIC values for tetracycline against *E. faecalis*, *E. coli*, *V. parahaemolyticus* and *Y. enterocolitica* were in the respective ranges of 0.5 to 32, 0.5 to 64, 0.06 to 2, and 2 to 4 µg/mL (Sirichoat et al. 2020; Pohl et al. 2018; Han et al. 2007; Stock & Wiedemann 1999), which correspond with the findings of the current study. In contrast to the MICs determined in the current study for *L. monocytogenes* and *S. flexneri*, other researchers have reported higher MICs values (≥ 256 µg/mL) (Li et al. 2007; Madiyarov et al. 2010). The variations in susceptibility of both bacteria to tetracycline (observed in the current study *vs.* the previously published data) can be explained by the use of different strains. Despite the fact that the antibacterial activities of nitroxoline, sanguinarine, and zinc pyrithione have been demonstrated in various studies, data on their *in vitro* growth-inhibitory effects against the diarrheic bacteria tested in the present study are limited. The MIC values observed for the tested antimicrobial compounds in the current study are well corresponding with the results of recently published data from the same laboratory, demonstrating their effectiveness against most of the tested diarrhoeal pathogens

at MICs ranging from 2 to 512 µg/mL (Kudera et al. 2020). Synergistic effects of tetracycline and other antibiotics (e.g., augmentin and ciprofloxacin) have previously been documented for some selected diarrhoea-causing bacteria isolated from poultry droppings (Omoya 2016). According to our best knowledge, the combined growth-inhibitory activity of nitroxoline, sanguinarine, and zinc pyrithione with tetracycline against diarrhoea-causing bacteria is reported for first time in this study. Tetracycline inhibits bacterial protein synthesis by preventing the association of aminoacyl-transfer ribonucleic acid (tRNA) with the 30S subunit of the bacterial ribosome (Chopra & Roberts 2001). In addition, tetracycline forms a complex with magnesium ions (Mg^{2+}) and binds to the A-site of ribosomes (Grossman 2016; White & Cantor 1971). Although the details of its mechanism of action are still not elucidated, it is known that the antibacterial activity of nitroxoline is due to its indirect ability to chelate cations essential for bacterial growth, especially Mg^{2+} (Repac et al. 2022; Wang et al. 2019). Similarly, zinc pyrithione chelates metal ions, including Mg^{2+} (Dinning et al. 1998). In order to diffuse through the bacterial cell membrane, tetracycline has to be fully protonated because the Mg-chelate cannot enter the cell (Repac et al. 2022). Therefore, it is possible to assume that nitroxoline and zinc pyrithione scavenge Mg^{2+} from the environment, which creates favourable conditions for tetracycline to enter the bacterial cell membrane. Inside of the cell, tetracycline forms a complex with Mg^{2+}, which is the only form which is able to inhibit bacterial growth by binding to the bacterial 30S ribosomal subunit. The chelation properties of all three agents may therefore contribute to their synergistic antibacterial activity. In the present study, the combination of sanguinarine with tetracycline produced a synergistic growth-inhibitory effect against Gram-positive bacteria. Since it has previously been suggested that the anti-staphylococcal action of sanguinarine is based on its ability to compromise the cytoplasmic membrane (Obiang-Obounou et al. 2011), it can be hypothesized that this compound can help tetracycline to enter the bacteria by disturbing the cell membrane. Subsequently, tetracycline can effectively inhibit bacterial protein synthesis inside the cell. Both antibacterial agents can therefore act together against *E. faecalis* and *L. monocytogenes* (as examined in the present study) through their synergistic activity.

Since both ciprofloxacin and nitroxoline are drugs commonly used in clinical practice for treatment of bacterial infections (Louie 1994; Naber et al. 2014) the use of their combination could be a potential treatment strategy against several fluoroquinolone-resistant infections of gastrointestinal tract, such as ciprofloxacin-resistant shigellosis which has been

a recurrent challenge in many parts of developing world (Taneja 2007). Also, because tetracycline and nitroxoline are therapeutic drugs for bacteria-related diseases, their combined use could improve the efficacy of tetracycline against gastrointestinal diseases caused by diarrhoeagenic bacteria, because nitroxoline would chelate cations (e.g., calcium, Mg^{2+}) which are reported to lower the absorption of this antibiotic in the gut (Shutter & Akhondi 2022; Poiger & Schlatter 1979). In addition, as the above-mentioned antibacterial agents are used for the management of urinary tract infections (UTIs) (Rosenstock et al. 1985; Naber et al. 2014), their combination could also be a potential treatment strategy against increased acquired resistance to orally administered antibiotics against *E. coli* caused UTIs (Kresken & Körber-Irrgang 2014), which has been a growing healthcare concern worldwide. The standard daily dosage of nitroxoline is 250 mg administered every 8 hours which may be associated with side effects, including discoloured urine, headache, nausea, and stomach pains. Since sanguinarine has been observed to be a slightly toxic substance when administered orally to rats [median lethal dose (LD_{50}) =1658 mg/kg] (Becci et al. 1987), its pharmacological use seems to be limited. According to the Scientific SCCS (2020), zinc pyrithione is classified as a moderately toxic agent, with LD_{50} values ranging from 92 to 266 mg/kg and from 160 to 1000 mg/kg when administered orally to rats and mice, respectively. Although zinc pyrithione is an anti-fungal ingredient well-known in cosmetic and shampoo products, its use in form of orally administered agent therefore seems to be even less realistic than the use of sanguinarine. Nevertheless, based on the FICI values achieved in this study, it is possible to suppose that the significantly lowered active concentrations of sanguinarine and zinc pyrithione resulting from their antibacterial combination with the antibiotics can produce lower toxicological response in target organisms. Furthermore, as reported in the study of Kudera et al. (2019), sanguinarine and zinc pyrithione revealed increased toxicity to normal intestinal cells. However, the lower concentrations/doses used in the combination are expected to reduce the toxic effects of the agents. Also, there is a chance that the toxicity would be modulated by commensal microorganisms and would not particularly affect the host as such ability of gut microbiota has been previously described in several studies (O'Keefe 2016; Alexander et al. 2017). Also, further toxicological studies are needed to examine the therapeutic safety of either ciprofloxacin or tetracycline in combinations with the above-mentioned alkaloid-related agents before their possible pharmacological usage.

9 Conclusions

In summary, the present study has demonstrated that representatives of several classes of antibiotics produce synergistic effect with antidiarrhoeal/anti-infective phytochemicals and synthetic analogues against diarrhoea-causing, and, in certain cases, antagonistic action on beneficial intestinal bacteria. More specifically, combinations of ciprofloxacin with either nitroxoline, sanguinarine or zinc pyrithione produced an antibacterial synergistic effect against diarrhoea-causing bacteria, namely *B. cereus*, *E. faecalis*, *L. monocytogenes*, *S. flexneri* and *V. parahaemolyticus*, and, simultaneously, has shown antagonistic action towards gut beneficial strains of bifidobacteria such as *B. adolescentis*, *B. animalis* subsp. *Lactis* and *B. breve*. Besides the selective combinatory effect observed for above mentioned agents, several tested combinations produced only synergistic effect against diarrhoea-causing bacteria. Particularly, tetracycline in combination with either nitroxoline, sanguinarine, or zinc pyrithione produced antibacterial synergistic interactions against most of the diarrhoea-causing bacteria tested. *S. flexneri* was the most susceptible bacterium to the combination of tetracycline and nitroxoline. For the first time, this study demonstrates selective combinatory effect of ciprofloxacin together with either nitroxoline, sanguinarine, or zinc pyrithione against diarrhoea-causing and gut beneficial bacteria. The observed antagonism of tested agents towards gut microbiota can be considered as positive effect contributing to the safety of the therapeutic agents whereas their synergism against diarrhoeal bacteria significantly potentiates total antimicrobial efficacy. Again, to the best of our knowledge, this is the first report of the synergistic interactions between tetracycline and the above-mentioned alkaloid-related compounds against most of the selected diarrhoeagenic bacteria strains tested. Based on the FICI values obtained in this study, the synergistic actions suggested that the combination of the antimicrobials was more active against the diarrhoeal microorganisms than the activity of the single agents alone. The results suggest that certain combinations of agents tested in this study can be used for development of antidiarrhoeal therapeutic agents with reduced harmful action on the gastrointestinal microbiome. If these combinations will be introduced to the clinical practice, they would allow to use lower concentrations of antibiotics in the treatment of diarrhoeal disease of bacterial origin. Since synergistic interactions produce lower concentrations/doses of the combined agents, certain combinations are potential to reduce the toxicity effects in human. Lastly, the combinatory effect based on the compounds

with different action mechanisms and broad-spectrum antibacterial effect can overcome or slow down development of resistance of bacterial strains.

9.1 Recommendations for future work

The results of this study suggest combinations of antibiotics with antidiarrhoeal/anti-infective phytochemicals and synthetic analogues as promising approach for development of drugs for treatment of diarrhoea safer to beneficial bacteria. However, further studies focused on their *in vivo* anti-diarrhoeal activity and safety followed by human clinical trials will be needed prior their consideration for the use in clinical practice. Furthermore, investigations concerning the exact mechanism of their selective combinatory actions should be conducted. Moreover, additional *in vitro* studies, such as time-kill assay, which can help with understanding of the interactions between microbial strains and combinations of antimicrobial agents, especially to their time-dependent effect, can also be recommended for the future. In addition, further investigation of the *in vitro* combinatory and/or selective effect of tannic acid with antibiotics against diarrheic and gut beneficial bacteria can bring interesting new results. Lastly, systematic evaluation of *in vitro* synergistic interaction of ceftriaxone with various plant-derived agents against diarrhoeal pathogens is also recommended for future studies since it produced positive results in the initial screening test.

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11. Appendices

11.1 *Curriculum vitae*

Personal details

Name	**Ing. Hayford Osei-Owusu**
Address	Gregorova 2087/1, Praha 4
	Czech Republic
Mobile phone number	+420 774890346
E-mail	osei-owusu@ftz.czu.cz
Date of birth	30 June 1980
Nationality	Ghanaian

Education

2018 – present	**Doctoral Study**
	Czech University of Life Sciences Prague
	Faculty of Tropical AgriSciences
	Study Programme: Tropical Agrobiology and Bioresource Management
	Thesis: Selective combinatory effect of antibiotics with phytochemicals and synthetic analogues of plant-derived compounds against diarrhoea-causing and beneficial intestinal bacteria.
2015 – 2017	**Master's degree**
	Czech University of Life Sciences Prague
	Faculty of Tropical AgriSciences

	Study Programme: Tropical Crop Management and Ecology Thesis: Evaluation of *in vitro* anti-clostridial effect of 8-hydroxyquinoline and its derivatives.
2010	Institute of Commercial Management (ICM); Bournemouth, England. Diploma Certification in Business Management and Administration.
2002 – 2006	**Bachelor's degree** University of Cape Coast. Ghana Faculty of Agriculture Study Programme: Agricultural science

Professional experience

2014 – 2015	Banking Operations Manager, and SMEs and Project Credit Manager. Institution: Oval Microfinance Ltd. Accra, Ghana.
2009 – 2014	Credit Officer Institution: Akim Bosome Rural Bank Ltd**.** Akim Swedru, Ghana.
2006 – 2007	National service Institution: Ministry of Food and Agriculture. Ghana.

Pedagogic activities

Department of Crop Sciences and Agroforestry, Faculty of Tropical AgriSciences, Czech University of Life Sciences Prague.

Subjects:	ICI008E Special crops seminar
	ICI003E Economic botany seminar

Award

Rector’s prize award for the academic year 2021 - 2022 for PhD students with outstanding research and publication results. Czech University of Life Sciences Prague.

Project participation

2018- 2022	Internal grant agency (IGA).
	Czech University of Life Sciences Prague
	Faculty of Tropical AgriSciences
	Role: Co-investigator.

Language skills

	Akan (Twi): Native tongue
	English: Proficient user

11.2. List of author's publications

a). Publications in scientific journals:

Osei-Owusu, H., Rondevaldova, J., Houdkova, M., Kudera, T., Needham, T., Mascellani, A., Kokoska, L. 2024. Evaluation of *in vitro* synergistic effects of tetracycline with alkaloid-related compounds against diarrhoeic bacteria. International Journal of Molecular Sciences, **25**(11), 6038. (IF 5.6).

Osei-Owusu, H., Kudera, T., Strakova, M., Rondevaldova, J., Skrivanova E., Novy, P., Kokoska, L., 2022, *In vitro* selective combinatory effect of ciprofloxacin with nitroxoline, sanguinarine, and zinc pyrithione against diarrhea-causing and gut beneficial bacteria, Microbiology Spectrum, **10**(5), e0106322. (IF 3.7).

Okpala, O.E., Rondevaldova, J., **Osei-Owusu, H.,** Kudera, T., Kokoskova, T., Kokoska, L. 2024. Susceptibility of *Staphylococcus aureus* to anti-inflammatory drugs with a focus on the combinatory effect of celecoxib with oxacillin in vitro. Molecules **29** (15), 3665. (IF 4.2

b). Oral communications:

Okpala, O.E., **Osei-Owusu, H**., Rondevaldova, J., Kokoska, L. Celecoxib potentiates the *in vitro* anti-staphylococcal effect of oxacillin. Workshop: Recent progress in Pharmacognosy and Phytochemistry 2022. Hradec Kralove, Czech Republic. (poster).

Okpala, O.E., **Osei-Owusu, H.**, Rondevaldova, J., Kokoska, L. Non-steroidal anti-inflammatory drug celecoxib potentiates the *in vitro* anti-staphylococcal effect of oxacillin. Federation of European Microbiological Societies (FEMS) 2022. Belgrade, Serbia. (poster).

Osei-Owusu, H., Kudera, T., Kokoska, L. *In vitro* synergistic interactions of phytochemicals and their synthetic analogs with tetracycline against diarrhoeal causing bacteria. 69th International congress and annual meetings of the society of medicinal plants and natural product research (GA). Planta Medica 2021, **87**(15): 1264-1265. Bonn, Germany (poster).

Osei-Owusu, H., Kudera, T., Strakova, M., Rondevaldova, J., Skrivanova E., Kokoska, L. Combinations of ciprofloxacin with alkaloid related compounds are producing selective synergistic and antagonistic effects against diarrheagenic and probiotic bacteria *in vitro*. World Microbe Forum conference in collaboration with American Society for Microbiology (ASM) and Federation of European Microbiological Society (FEMS) 2021. Online conference. (poster).

Osei-Owusu, H., Kudera, K., Subrtova, SH., Kokoska, L. Selective growth-inhibitory effect of combinations of plant compounds and their derivatives with conventional antibiotics on diarrhoea causing and gut beneficial bacteria. Federation of European Microbiological Society conference on Microbiology in association with the Serbian Society of Microbiology 2020. Book of abstract page 267. Belgrade, Serbia. (poster).

Osei-Owusu, H., Kudera, T., Strakova, M., Rondevaldova, J., Kokoska, L. Combinatory effect of plant compounds and their derivatives with conventional antibiotics on diarrhoea-causing bacteria. 67th International Congress and Annual Meetings of the Society for Medicinal Plant and Natural Product Research (GA) in cooperation with the French Society of Pharmacognosy AFERP. Planta Medica 2019, **85**(18):1523 . Innsbruck, Austria. (poster).