Studies on the manipulation of gastrointestinal tract bacteria

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Studies on the Manipulation of Gastrointestinal Tract Bacteria

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ABSTRACT

Increasing awareness that the human intestinal flora is a major factor in health and disease has led to different strategies to manipulate the flora to promote health. These approaches include changes to the diet by inclusion of prebiotics and probiotics. Prebiotics are non-digestible polysaccharide food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the gastrointestinal tract (GIT). Probiotics on the other hand are viable culture of bacteria, which applied to animals or humans, beneficially affect the host by improving the properties of the indigenous microflora. One mechanism of action of probiotics is the production of antimicrobial substances called bacteriocins, such as colicins, that inhibit the growth of their competitors. The experiments described in this thesis examined the potential use of prebiotics and probiotics to manipulate GIT bacteria.

A crude polysaccharide extract (HW) from the medicinal mushroom *Ganoderma lucidium* was prepared by extracting the fruiting body with boiling water. The extract was then purified by ethanol precipitation resulting in the hot water-ethanol (HWE) extract. Groups of mice were fed these extracts over a period of three weeks at a concentration of 150 μg/ml in sterile drinking water and the mice then euthanised after three weeks. Changes in population dynamics of lumen bacteria were determined in the duodenum, ileum, colon and faeces by rigorous washing of excised segments while adherent bacteria were released with the non-ionic detergent Triton X100, which does not affect the viability of the bacteria. The prevalence of haemolytic colonies was assessed by plating washouts onto blood agar. Total colony forming units were enumerated on bacteriological media selective for *Enterobacteriaceae, Streptococci, Enterococci* and Lactic acid Bacteria (LAB). Results showed that there was little change in population dynamics elicited by extract feeding. The exception was a significant reduction in haemolytic lumen bacteria and increase in LAB lumen bacteria recovered from the colon of HWE treated mice.

A multiplex PCR was optimized and applied to survey the prevalence of eight common colicin genes (Colicins A, D, E1, E2, E6, E7, Ia and V) in *Escherichia coli* (*E. coli)* isolates. The study focused on 39 clinical isolates from

humans and 68 isolates from pigs with post-weaning diarrhoea. In addition, 152 porcine commensal *E. coli* isolates obtained from different compartments of the GIT (duodenum, ileum, colon and faeces) were included in the PCR analysis. Six individual colicins (E1, E2, E6, E7, Ia and V) and four dual colicin combinations (E1/E2, E1/E7, E2/E7, & E2/Ia) were detected. Approximately 28.2 % of the human pathogenic isolates had at least one colicin gene with colicins D, E1, E7 and V occurring at frequencies of 5.1 % each. Colicins E6, Ia and the dual colicin, E2/Ia, were less frequent and were found in about 2.6 % of clones. Only 4 % of the porcine pathogenic isolates possessed a colicin gene and these were exclusively E1 and V. In contrast, there was a significantly higher carriage (36.2%) of colicin genes in commensal porcine *E.* coli. Of these, E1, E7 and Ia accounted for 87 % of all colicin genes detected. Six of the commensal strains possessed multiple types of colicins with the most common being the E2/E7 combination. Furthermore, there appeared to be differences in the type of colicins found in commensal *E. coli* isolates recovered from different intestinal compartments.

Seven porcine commensal *E. coli* strains producing standard colicins were evaluated for inhibitory activity against five pathogenic *E. coli* of human and porcine origin. The experiment utilized a kinetic inhibitory microtitre assay (KIMA) to assess inhibition using non-induced supernatants and supernatants induced with 0.2 μg/ml of mitomycin C to stimulate colicin production. The level of inhibition was found to be variable with most of the commensal porcine *E. coli* strains showing little or no inhibitory action against the five pathogenic strains. However, two commensal strains, ILC33 and CC89 were found to highly inhibitory to three porcine pathogenic *E. coli* strains of serotypes O141:K85, 0141:K88 and 0149:K88.

The findings of this thesis suggest that purified polysaccharide extracts (HWE) from *Ganoderma lucidium* have the potential to be used in further studies as prebiotics in view of their positive effects on beneficial LAB. In addition, the use of colicin-bearing strains as probiotic bacteria is justifiable because of the low incidence of colicin genes in pathogenic *E. coli* compared to commensals. Finally, these findings indicate that potential probiotic bacterial strains have to be scrutinised for their inhibitory activity against individual pathogenic strains prior to being subjected to further assessments.

CERTIFICATION

I, Peter Njuguna, declare that this thesis, submitted in partial fulfilment of the requirements for the award of the degree of Master of Science (Research), in the School of Biological Sciences, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualification at any other academic institution.

Peter Njuguna

PUBLICATIONS AND PRESENTATIONS

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TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

LIST OF TABLES

Chapter 1

Chapter 3

LIST OF ABBREVIATIONS

BA	**Blood agar**
BP	**Base pairs**
CATC	**Citric Azide Tween Carbonate agar**
CC	**Colon commensal**
CFU	**Colony forming units**
DC	**Duodenum commensal**
E. coli	**Escherichia coli**
EHEC	**Enterohemorrhagic *E. coli***
ETEC	**Enterotoxigenic *E. coli***
FC	**Faecal commensal**
GIT	**Gastrointestinal tract**
HW	**Hot water extract**
HWE	**Hot water-ethanol extract**
ILC	**Ileum commensal**
KDa	**Kilodaltons**
KEA	**Kanamycin Esculin Azide agar**
LA	**Luria agar**
LAB	**Lactic acid bacteria**
LB	**Luria agar broth**
MAC	**MacConkeys agar**
ML	**Milliliter**
MRS	**Deman, Rogosa and Sharpe agar**
NDGOS	**Non-digestible galacto-oligosaccharides**
O.D.	**Optical density**
PBS	**Phosphate Buffer Saline**
PCR	**Polymerase chain reaction**
PWD	**Post-weaning diarrhoea**
RPM	**Rotations per minute**
SCFA	**Short-chain fatty acids**
μg	**Micro-gram**
TX	**Triton-X 100**

Chapter 1

Review of Literature

1.1 The Human Gastrointestinal Tract

The gastrointestinal tract (GIT) of humans and animals is divided into different sections each that perform a specific function (Figure 1.1). The main function of the GIT is the digestion of food (stomach and the small intestine), absorption of nutrients and water (small and large intestine), and the storage of wastes (large intestine) (Schneeman, 2002).

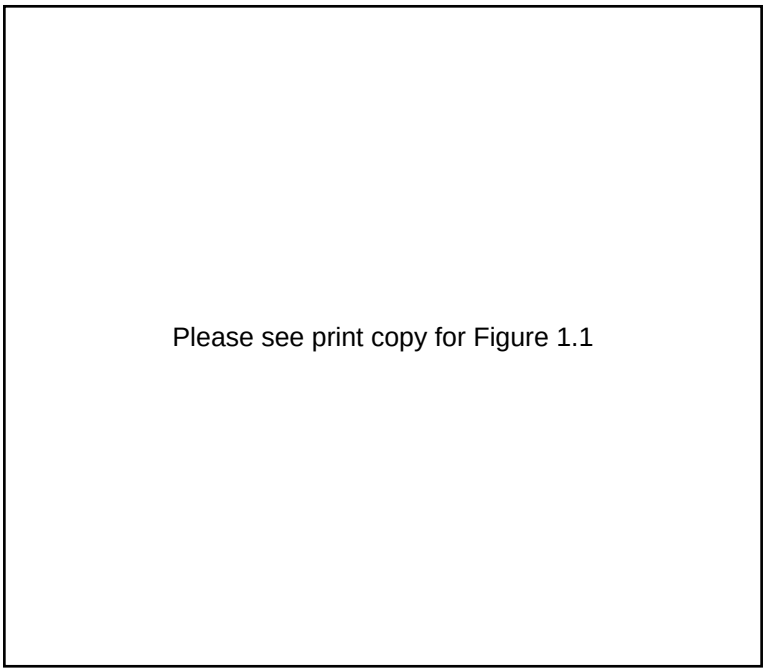


FIG 1.1 Diagrammatic representation of the human gastrointestinal tract showing the major regions. Taken from www.pharmanex.com

The microbes characteristically found at different sites of the GIT of healthy subjects, are referred to as the normal (commensal) microflora (Berg, 1996). They will colonise specific GIT regions and stay there until organisms better adapted to occupy the habitat displace them. In addition to these colonising microbes, various transient microbes can be observed. Since they just pass through the tract, unless some factor induces a change in the normal

microflora whereby the transient microbe can establish, their influence on the intestinal ecosystem is variable. In addition, colonisation of the intestine by the pathogens will be influenced by these factors.

The human gastrointestinal tract is made up of complex consortia of microorganisms, which colonise the length of the gut. The GIT microflora does not appear spontaneously in newborn humans or animals; instead, certain microbes colonise particular intestinal habitats at various times after birth that are characteristic of that particular habitat and animal host (Falk *et al.,* 1998). The GIT microflora are not just randomly distributed throughout the GIT but are found at characteristic population levels in particular regions of the tract.

The anatomy, morphology and physiology of the GIT exert a large influence on the normal microflora and possible enteropathogens that live in the intestine. The stomach and the upper two-thirds of the small intestine (duodenum and jejunum) contain only low numbers of microorganisms (10^3-10^4 bacteria ml^{-1} of gastric or intestinal contents) (Tannock, 1995). Microbial numbers are restricted in these areas because of the low pH of the stomach contents and peristalsis through the stomach and the small bowel (Berg, 1996). The principal microbial types found in these regions are usually those that can survive low pH as well as those that can adhere the stomach epithelium. Certain species of *Lactobacillus* and *Streptococcus* are the dominant bacterial species found in this region (Guarner and Malagelada, 2003).

The distal small intestine (ileum) is considered a 'transition zone' between the relatively sparse microflora of the upper bowel and the tremendous numbers of bacteria found in the large intestine (Tannock, 1995). Compared with the upper small intestine, the ileum with decreased peristalsis and lower

oxidation-reduction potentials, maintains a more diverse microflora and higher bacterial populations (10^8 bacteria ml^{-1} of intestinal contents) (Berg, 1996). Major constituents are *Lactobacillus*, *Streptococcus* and members of the *Enterobacteriaceae* family such as *Escherichia coli* (*E. coli*).

The large intestine (colon) is the primary site of microbial colonisation in humans and animals. This is probably because of the slow intestinal transit times of up to sixty hours encountered here and the very low oxidation-reduction potentials in this region (Tannock, 1995). Consequently, the colon harbours great numbers of bacteria (10^{10}-10^{11} bacteria g^{-1} of intestinal contents) comprising an estimated 400-500 species (Falk *et al.,* 1998). The major genera found in the colon are *Bifidobacterium, Eubacterium, Bacillus, Clostridium, Bifidobacterium,* and *Lactobacillus* (Berg, 1996).

1.1.1 Beneficial Effects of GIT Microflora

Use of animals bred under germ-free conditions has provided important information about the effect of the microbial community of the gut on host physiology and pathology (Falk *et al.,* 1998). Evidence obtained through such studies suggests that microflora have important and specific metabolic, trophic, and protective functions.

The microflora is dominated by strict anaerobes that ferment endogenous and exogenous substrates. Short-chain fatty acids (SCFA), especially acetate, propionate, butyrate and lactate, contribute towards energy metabolism of the large mucosa and colonic cell growth, and are also metabolized systematically by host tissues such as the liver, muscle and brain (Steer *et al.,* 2000). The fermentive capabilities of the GIT microflora also

contribute towards large bowel digestive function, acting on recalcitrant compounds of dietary origin and on host secretions, allowing recovery of nutrients otherwise lost to the host (Guarner and Malagelada, 2003). The colonic microflora is involved in bowel motility, enterohepatic cycling of primary bile acids and possibly the metabolism of cholesterol, resulting in the production of faecal neutral sterols (Steer *et al.,* 2000). The indigenous GIT microflora stimulate the development of the immune defense system so that the host can respond more rapidly to potential pathogens - often a major determining factor as to whether the host will survive or succumb to an infection (Edwards and Parrett, 2002). Finally, the stability of the microflora effectively limits the capacity of invading microorganisms, including pathogens, to colonise the gut, giving rise to what has been termed 'colonisation resistance' (Guarner and Malagelada, 2003).

1.1.2 Manipulation of GIT Microflora

An increase in stress and modern day living, which makes a consequential demand on the immune system, can disrupt the homeostasis of the GIT. Another contributory factor includes the consumption of pharmaceutical compounds, in particular antibiotics, which by design destroy bacteria, and can therefore have a detrimental effect on the balance of the GIT microbiota (Kyriakis *et al.*, 1999). These factors combine to shift the balance of the gut microflora away from potentially beneficial or health-promoting bacteria such as *lactobacilli* and *bifidobacteria*, towards an increase in harmful or pathogenic microorganisms like *Clostridia* (Fooks and Gibson, 2002). Therefore, it is of considerable benefit to the host to maintain a good community structure through

increased levels of beneficial bacteria preferably at the expense of the pathogenic ones.

1.1.3 Nutraceuticals

Nutraceuticals are considered to be a generic class of nutritional supplements. They improve the overall health of humans and animals when added as a dietary supplement to food. Apart from a possible direct effect on improving nutrient absorption and digestive physiology, they may also have a different mechanism aimed at promoting growth of friendly bacteria in the GIT by generating conditions that are unfavourable for the growth of pathogens. There are many different kinds of dietary supplements in use that are diverse in composition and probably differ in their mode of action. This thesis will discuss the prebiotics and probiotics.

1.1.4 Prebiotics

Prebiotics can be defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the GIT (Cummings *et al.,* 2002). Non-digestible oligosaccharides represent one of the most commonly used prebiotics in the animal industry and have also been used as a dietary supplement for humans (Roberfroid, 2001). Bouhnik *et al.* (1996) evaluated the benefits of non-digestible galacto-oligosaccharides (NDGOS) in the human diet. Their study found that NDGOS were selectively utilised by *bifidobacteria*, which fermented the carbohydrate to meet their energy requirements for growth and division. As *bifidobacteria* are considered to be friendly bacteria, their

proliferation in the GIT would not pose a threat to the health of the human host. In addition to this, they suggested that the products of fermentation of NDGOS in the GIT have a role in the improvement of the GIT environment by supplying metabolisable SCFA in the form of acetate and butyrate to the GIT epithelium. The observed beneficial potential of prebiotics may be ascribed to molecular structure; linear chains composed of mainly (1-2)-β linked fructose molecules (Steer *et al.,* 2000).

1.1.5 Probiotics

Probiotics can be defined as a viable mono- or mixed culture of microorganisms, which applied to animals or humans, beneficially affects the host by improving the properties of the indigenous microflora (Fooks and Gibson, 2002). Probiotic*s* are said to work by a number of mechanisms. *In vitro*, bacteria compete for attachment sites in the brush border of intestinal epithelial cells (Bernet *et al.,* 1994). Adherent non-pathogenic bacteria (probiotics) can prevent attachment and subsequent entry of pathogenic enteroinvasive bacteria into the epithelial cells (Guarner and Malagelada, 2003). Furthermore, bacteria compete for nutrient availability in ecological niches and maintain their collective habitat by administering and consuming all resources (Fooks and Gibson, 2002). The host actively provides a nutrient that the bacterium needs, and the bacterium actively indicates how much it needs to the host. Increasing the numbers of friendly bacteria by way of a probiotic may thereby decrease the substrate availability for the other bacterial populations, especially pathogenic ones. Finally, probiotics can inhibit the growth of their competitors by producing antimicrobial substances called bacteriocins (Jack *et al.,* 1995).

The literature on probiotics is extensive with most articles focussing on the use of probiotics in maintaining human health, as opposed to their use in animals. Reports of the health benefits obtained from the consumption of probiotics have been reported for the last three decades. The most common probiotics are Lactobacillus species such as *Lactobacillus* acidophilus and *Lactobacillus casei*. Whilst there is an ever-increasing amount of evidence to support the beneficial role of lactic acid bacteria (LAB), little work involving the probiotic applications of other bacterial species has been done. Pryde *et al.* (1999), in a study using the technique of terminal restriction length polymorphism to characterise the bacterial diversity presenting the different parts of the porcine GIT, found that some of the species detected had gone undetected by traditional cultural methods of identification. Further, they suggested that cultural methods have overestimated the contributions of certain groups, in particular *Lactobacillus* species, to the overall bacterial population of the GIT. Consequently, they suggested that existing isolates, notably *Lactobacillus* species, might not be the most appropriate bacteria to use for probiosis.

Thus, more extensive investigations involving a variety of other bacteria species (including gram-negative ones) is needed in order to not only determine their mode of action but also their efficacy.

1.2 Medical Mushrooms

1.2.1 General Information on Medicinal Mushrooms

Mushrooms have been used medicinally for centuries, particularly in traditional Chinese and Japanese medicine to maintain health and increase longevity. In many Asian cultures mushrooms are still used to promote good health and vitality and to increase the body's adaptive capabilities. In the Western world mushrooms are undoubtedly consumed much more for their texture and flavour, than for their nutritional and medicinal properties. The beneficial effects of several mushrooms are recorded in the first Chinese book on medicinal substances "Seng Nong Ben Cao Jing" a compendium of material medica, from 2000 years ago (Jong and Birmingham, 1992). It was not until this century, when antibiotics were obtained from *Penicillum*, that the medicinal value of mushrooms first gained worldwide attention. It is now well documented that beyond providing nutritional effect, mushrooms from the higher class of basidomycetes may also modulate various functions in the body that are relevant for health. It is estimated that that there are about 10,000 mushroom species worldwide, about 700 of these are edible, and 50 – 200 of them show medicinal effects (Borchers, *et al.,* 1999).

Edible mushrooms are considered to be healthy because they are low in calories and fat, while rich in vegetable proteins chitin, vitamins, mineral, and dietary fibre (Manzi *et al.,* 1999). The dietary fibre component is of a mixture of polysaccharides, lignin, and other plant cell wall constituents that are resistant to hydrolysis by human enzymes (Manzi and Pizzoferrato, 2000). Among the most interesting functional components in this mixture are the β-glucans. These compounds, homo- and hetero glucans with β-(1-3), β-(1-4) and β-(1-6)

glucosidic linkages are hypothesised to play a key role in the healthy properties of these mushrooms (Miyazaki and Nishijima, 1981).

1.2.2 Medicinal Use of Mushrooms

Although medicinal mushrooms have been consumed for thousands of years as part of traditional medicine, it was not until recently that clinical studies started to be carried out. It is now well established from *in vitro* and animal studies that polysaccharides isolated from higher basidiomycetes, by hot water extraction show anti-tumour activity (Bao *et al.,* 2001). It was in the late 1960s that Japanese researchers began to systematically investigate whether mushrooms contained substances able to inhibit tumour growth (Chihara *et al.,* 1970). Since then a wide variety of mushroom species have been investigated for their anti-tumour activity. So far, about 200 species of mushrooms have been found to markedly inhibit the growth of different tumours (Wasser and Weis 1999). Most research regarding biological activity for these polysaccharides is performed *in vitro* or in animal models. For all polysaccharides showing anti-tumour activity, the initial publications are reports on the fractionation and isolation of these substances and on their ability to inhibit the growth of tumours, most commonly sarcoma 180. However, these studies do not address the mechanism by which these compounds exert their anti-tumour effects. In the assessment of anti-tumour activity in humans, most studies have been small, and not placebo-controlled and double blinded. Most of them are poorly documented, with imprecise definition of the end products. Therefore it is difficult to evaluate the clinical effects of the isolated polysaccharides in humans. However, in Japan where most of the clinical

studies have been conducted, two of the most investigated polysaccharides, lentinan and schizophyllan, are approved for clinical use in humans (Borchers *et al.,* 1999).

Although most research regarding medicinal effects of mushrooms has been conducted on polysaccharides and their claimed anti-tumour activity, other components have been reported to show other biological effects. Wasser and Weis (1999) have reviewed the anti-viral, antibiotic, cardiovascular and hypercholesterolemia activity of lentinan and other higher mushrooms. Hikino *et al.* (1985) reported that a water extract of the dried fruiting bodies from *Ganoderma lucidum* decreased plasma sugar level in normal and alloxan induced hyperglycaemic mice. Lin *et al*. (1993) demonstrated the anti inflammatory and liver-protective effects of different higher basidomycetes. So far, most of the research regarding effects of the medicinal mushrooms has been conducted on isolated purified constituents, and few research groups have evaluated the effects of the entire mushroom. However, as the industrial interest in extractible nutraceuticals from mushrooms is increasing, so is the research regarding these compounds. These compounds are hypothesised to exhibit either medicinal and/or toxic qualities, and have immense potential as dietary supplements for use in the prevention and treatment of various human diseases (Chang and Buswell, 1999).

1.2.3 Genus *Ganoderma*

The Finnish mycologist Peter Karsten established the genus *Ganoderma* in 1881, with *Ganoderma lucidium* as the sole species (Gottlieb *et al.,* 2000). Since then, taxonomists have reported many more species belonging to the

genus, with more than 250 *Ganoderma* species described worldwide (Chang and Buswell, 1999). The most frequently cited species in relation to the pharmacology and medicinal effects of *Ganoderma* is *Ganoderma lucidium*. *Ganoderma lucidium* is also known by the common names Ling Zhi (Chinese), Reishi (Japanese) and Youngzhi (Korean) (Chang and Buswell, 1999). In reality, the *Ganoderma lucidium* complex encompasses several *Ganoderma* species including *Ganoderma tsugae*, *Ganoderma luteum*, *Ganoderma lucidium, Ganoderma applantum*, *Ganoderma australe* and several other taxa that are restricted to tropical areas (Hseu *et al.,* 1996). However, in therapeutic practices and literature citations, *Ganoderma* usually refers to the species *Ganoderma lucidium*.

1.2.4 Polysaccharides in Medicinal Mushrooms

The polysaccharides represent a structurally diverse class of macromolecules of relatively widespread occurrences. Unlike proteins and nucleic acids, they contain repetitive structural features that are polymers of monosaccharide residues joined to each other by glycosidic linkages. Among these macromolecules, polysaccharides have the greatest potential for structural variability because they can interconnect at several points to form a wide variety of branched or linear structures. Polysaccharides can also form secondary structures, depending on the conformation of sugar residues, the molecular mass, and the inter- and intra-chain hydrogen-bonding. This enormous potential variability in polysaccharide structure gives the necessary flexibility for the precise regulatory mechanisms of various cell-cell interactions in higher organisms (Ooi and Liu 2000). Both water-soluble and water-insoluble

polysaccharides have been isolated from medicinal mushrooms (Chen *et al.,* 1998). Most of the positive biological effects recorded are from studies on the water-soluble polysaccharides isolated by hot water extraction (Bao *et al.,* 2002). The amount and type of polysaccharides in different medicinal mushrooms varies, much because of different growth conditions, therefore different extractions procedures will reflect in the different amounts and types of polysaccharides in the extract. An ethanol precipitate obtained from the non-dialyzable fraction of a hot water extract of the dried fruiting bodies of *Ganoderma lucidum* contained 64.5 % neutral sugar (as glucose) (Miyazaki and Nishijima, 1981). Bao *et al.* (2001) obtained a yield of 3.8 % from the crude material from the dried bodies of *Ganoderma lucidum* by hot water extraction.

1.2.5 Polysaccharides with Immune Modulating Activity Isolated from Basidiomycetes

Studies of polysaccharides isolated from higher basidiomycetes showing immune modulating activity, have been carried out extensively since the first report by Ringer *et al.* (1957). The main source of these immune modulating polysaccharides is related to fungal cell walls, which consists of polysaccharides such as chitin, β-D-glucans, or polysaccharide-protein complexes such as galatomannan-protein glucuromannan-protein (Ooi and Liu, 2000). The most active polysaccharide is the branched (1-3)-β-D-glucans, sometimes referred to as (1-3)(1-6)-β-D- glucans (Mizuno, 1995).

Structural studies on immunologically active polysaccharides isolated from different basidiomycetes indicate that they all have a backbone of β-(1-3) linked D-glucopyranosyl residues with randomly dispersed β-D-glucopyranosyl

units substituted as the Carbon 6 glucosyl residues (Figure 1.2) (Miyazaki and Nishijima 1981, Bao *et al.,* 2001). The branches are usually composed of only one single glucose-residue although more than one unit may be present in some glucans (Sone *et al.,* 1985).

The biological and immunological properties of these polysaccharides are associated with the branching pattern, molecular weight and higher order structures (Bao *et al.,* 2002). Marchessault *et al.* (1967) demonstrated that biologically active (1-3)-β-D-glucans presented triple helical parallel strands with hydrogen bonding between Carbon 4 and Carbon 6 hydroxyl-groups present on the external surface of the triple helix. It is well established that most of the (1-3)-β-D-glucans contribute to tumour retarding effects based on the enhancement of the body's immune system, including activated microphages, natural killer cells and cytotoxic T cell cells and their secretory products, rather than a direct cytotoxic effect (Wang *et al.,* 1997). The underlying cellular and molecular mechanisms of these immune response modifications are yet to be fully understood. A specific β (1-3) D – glucan receptor on phagocytes and natural killer cells (Szabo *et al.,* 1995; Muller *et al.,* 1996) has been reported. A receptor binding process can be the first step in mediating the activating effects of β (1-3) D – glucan. However the intracellular events that occur after β (1-3) D – glucan has bound to the receptor are yet to be identified.

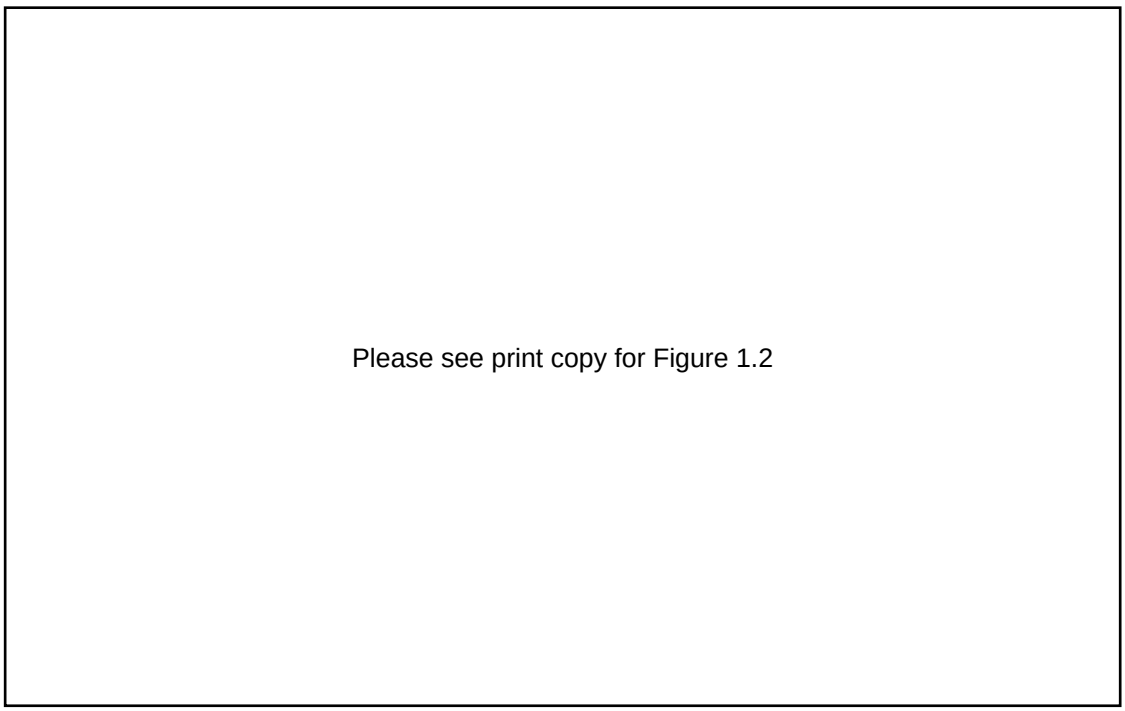


FIG. 1.2 Structure of branched (1-3)- β-D-glucans. The most biologically active polysaccharides isolated from higher basidomycetes appear to be branched (1-3)-β-D-glucans. All have a common structure, a main linear chain consisting of (1-3)-β-D-glucopyranosyl units along which are randomly dispersed single β-D-glucopyranosyl units attached by 1-6 linkages, giving a "comb-like" structure. Adapted from Mizuno (1995).

Not all β-D glucans isolated from higher basidiomycetes show immune modulating activity. The differences in activity can be correlated to the solubility in water, the molecular size, branching rate and conformation, which include the β (1-6) bonding system in the β (1-3) major chain (Wasser and Weis 1999). Modification of D-glucosyl groups of side chains to polyol groups has been reported to significantly enhance the immune modulating activity (Mizuno, 1995).

Some of the most known immune modulating polysaccharides isolated from higher basidiomycetes are schizophyllan, lentinan and PSK (Krestin). Schizophyllan (Komatsu *et al.,* 1969) is a pure high-molecular weight (1-3)-β-D-glucans prepared from *Schizophyllan commune* culture filtrates. Lentinan

(Chihara *et al.,* 1970) is a pure high-molecular weight (1-3)-β-D-glucans from *Lentinus edodes* fruiting bodies, whereas PSK (Mizuno, 1995) is a polysaccharide-protein complex extracted from *Coriolus versicolor* mycelia containing (1-4), (1-3) or (1-4), (1-6)-β-D-glucan. Polysaccharides isolated from *Ganoderma lucidum* have the same backbone as other (1-3)-β-D-glucans, and thus show immune modulating activity (Bao *et al.,* 2001; Wang *et al.,* 1997). However, in structural details these polysaccharides are different, both in degree of branching and in the length of the side chains.

So far there are no publications on non-digestible polysaccharide prebiotics isolated from medicinal mushrooms. Due to current knowledge on the structure of polysaccharides isolated from both the higher basidomycetes and prebiotics, it can be hypothesized that the polysaccharides from the mushrooms may perform similar effects on the gastrointestinal microflora as prebiotics have been documented to do.

1.3 Bacteriocins

Bacteria, in their competition for survival, frequently produce an extraordinary array of microbial defence mechanisms. Most anti-microbial products are synthesized during post-logarithmic growth when both food and space for bacterial multiplication are exhausted (Kleanthous and Walker, 2001). Further growth can only take place at the expense of bacterial competitors, and in the fight for survival, bacteria employ some exquisitely designed compounds that are toxic for specific microbial target cells. Their synthesis can be costly at a time of starvation and often requires the expression of several different genes.

These anti-microbial compounds include broad-spectrum classical antibiotics so critical to human health concerns, metabolic by-products such as the lactic acids produced by lactobacilli, lytic agents such as lysozymes found in many foods, numerous types of protein exotoxins, and bacteriocins (Riley and Wertz, 2002; Jakes, 1982).

Bacteriocins are extracellular proteins produced by certain bacteria, which exert their lethal effects on bacteria of the same or related groups (Jack *et al.,* 1995). Until relatively recently, most of the significant progress in bacteriocin research stemmed from investigations of a class of bacteriocins called colicins. Colicins are bacteriocins synthesised by, and active against, *E. coli* and closely related species such as *Citrobacter*, *Shigella* and *Salmonella* (Concepcion and Diaz, 2000). This has resulted in considerable in-depth knowledge of the genetic basis, domain structure, mode of formation, and killing action of these molecules. However, there now appears to be a remarkable resurgence of research activity centred upon the bacteriocin-like activities of gram-positive bacteria, particularly LAB, which are already widely used in the food industry to improve food preservation.

Bacteriocins differ from classical antibiotics in one critical way; they have a relatively narrow killing spectrum and are only toxic to bacteria closely related to the producing strain (Kleanthaus *et al.,* 1998). The bacteriocin family includes a diversity of proteins in terms of size, microbial targets, modes of action, and immunity mechanisms and are present in both gram-positive and gram-negative bacteria.

1.3.1 Colicins

Colicinogeny, the property of certain strains of coliform bacteria to produce cell-free substances that kill cells of other coliform strains, was first discovered by a Belgian microbiologist called Andre Gratia in 1925. He showed that *E. coli* strain V (virulent in experimental infections) produced in liquid media a heat-stable substance (later referred to as colicin V) that inhibited in high dilution the growth of *E. coli* ∅ (Reeves, 1972). The colicinogenic bacteria were specifically immune to killing by the colicin they made, but not to other colicins. In this way knowledge of the colicins advanced at a great rate and a large number of colicins have been identified, each characterised by the corresponding specific immunity.

Colicin-sensitive strains have receptors with which colicins combine. Receptors are defined by mutations that render the bacterial mutants resistant to one or more colicins and abolish the uptake of colicin (Alonso *et al.,* 2000). Some of these receptors have been identified with specific outer membrane proteins (Lazdunski *et al.,* 1998), which also participate in absorption of one or more bacteriophages or in transport of external substrates such as iron or vitamin B_{12} (Konisky, 1982). In addition, another class of bacterial mutants, called colicin tolerant, retain their colicin receptors but become insensitive to specific sets of colicins (Pugsley and Oudega, 1987).

1.3.2 Colicin Classification

Colicins have in the past been classified in various ways according to bacterial cross-resistance or cross-tolerance. However, these properties have proven to be too subject to genetic change and specify bacterial rather than

colicin properties. Consequently, based on studies of both colicins themselves and of their genetic determinants, colicins have been classified on their mechanism of action on sensitive bacteria (Riley *et al.,* 1994; Stroud *et al.,* 1998). To date, more than 20 different colicins have been identified (Riley, 1998).

1.3.3 Mechanism of Action

The biochemical effects of colicins on sensitive bacteria fall into two major classes: nuclease colicins and channel-forming colicins. Nuclease colicins kill sensitive cells through degradation of RNA thus inhibiting protein synthesis (RNAses), or by non-specific cleavage of chromosomal DNA (DNAses) (Jakes, 1982; James *et al.,* 2002). Channel-forming colicins, on the other hand, kill sensitive cells by interfering with energy metabolism through damage to the cytoplasmic membrane (Alonso *et al.,* 2000). Representatives of the most common colicins, depending on mode of action, are summarised in Table 1.1.

Experimental studies with colicins immobilised on solid substrates have supported the two major classifications. For example, colicins of the E2 and E3 groups, which must reach through the cytoplasmic membrane to affect their cytoplasmic targets, are inactive when fixed on solid particles whereas colicin E1, which damages the cytoplasmic membrane itself, is still lethal when immobilised on solid particles (Luria and Suit, 1987). To enter sensitive cells, colicins parasitises multi-protein systems used by sensitive cells for important biological functions. These proteins include outer membrane porins (OmpF, OmpA and OmpC), vitamin B_{12} (BtuB), sidephore and nucleoside (Tsx)

receptors, and the TolA and TolB proteins that cooperate with these proteins (Table 1.1). The most frequently used is BtuB, the vitamin B_{12} receptor, which defines the E type colicins (E1 to E9) (Kleanthous and Walker, 2001). Both colicins Ia and Ib share a common receptor, Cir that transports iron-chelated sidephores, including ferric dihydrobenzoate, and is also parasitised for the transport of catechol-substituted cephalosporin antibiotics (Stroud *et al.,* 1998). The mechanisms of colicin recognition and transport result in the very narrow target range of colicins in that they generally kill only *E. coli* and its close enteric relatives (Riley and Gordon, 1999).

1.3.3.1 Nuclease Colicins

Nuclease colicins cause single- and double-strand breaks in DNA or RNA of sensitive cells, the rate of breakdown depending on the colicin/cell ratio. Purified colicin E2, E7, E8 and E9 are non-specific DNAases that are cytotoxic by virtue of random cleavages of the chromosome that overwhelm the cellular DNA repair machinery (Kleanthous and Walker, 2001). Bacteria producing the nuclease colicins protect themselves by co-synthesising immunity proteins which bind their colicin with strong affinity and inhibit its enzymatic action (Kleanthous *et al.,* 1998). Tests with proteolytic digests of colicins indicate the C-terminal segment carries the nuclease activity and combines with the immunity protein.

Table 1.1 The mode of action, group, binding receptor, translocation system, and molecular weight for characterized colicins. KDa, kilodaltons; ND, not determined. Taken from Cursino *et al.* (2002).

Please see print copy for Table 1.1

1.3.3.2 Channel-Forming Colicins

Killing of bacterial cells by channel-forming colicins is normally accompanied by an arrest of macromolecular synthesis, a lowering of ATP levels, a loss of motility, and an arrest of active transport of β–galactosidases or amino acids (Luria and Suit, 1987). However, the block in macromolecular synthesis is not the primary cause of death as evidenced by experiments in *E. coli* mutants defective in the Ca^{2+} -Mg^{2+} - dependent ATPase. These mutant bacteria respond to colicin K by an increase in intracellular ATP levels and continue to synthesise macromolecules. The primary biochemical block has been attributed to a rapid loss of membrane potential and consequent decrease of the proton motive force across the cytoplasmic membrane (Stroud *et al.,* 1998). The main cause of cellular death is the reduction of the intracellular levels of K^+ and Mg^{2+}, and of small-phosphorylated metabolic intermediates for the affected bacteria (Alonso *et al.,* 2000). When cells of the ATPase-defective mutant attacked by colicin K are plated on a rich medium with high concentrations of K^+ and Mg^{2+}, most of them survive and give rise to colonies (Luria and Suit, 1987).

The mechanisms of colicin recognition and transport result in the very narrow target range of colicins in that they generally kill only *E. coli* and its close enteric relatives (Riley and Gordon, 1999).

1.3.4 Colicin Plasmid Genes and their Products

Plasmid DNA carries the determinants encoding production, release of and immunity to most colicins. A feature common to each colicin plasmid that has been well characterised is that there are three adjacent genes whose genes

determine colicin production, immunity, and release of colicin by lysis. The gene coding for the colicin is referred to as *cea*, the gene coding for the immunity protein as *imm*, and the gene coding for the lysis protein as *kil* (Konisky, 1982; Eraso *et al.,* 1996). Each of these genes has been sequenced for most of the characterised colicins and their transcriptional relations have been uncovered. For clarity, the genetic organisation of colicin genes can be divided onto two groups (Figure 1.3); the first group is typified by channel-forming colicins whereas the second organization is found in nuclease colicins.

In channel-forming colicins, the *cea* and *kil* genes are in an operon and are oriented from left to right (Figure 1.3-A) (Pilsl and Braun, 1995). The *imm* gene is located between these two genes and is oriented in the opposite direction. Expression of the operon in growing cells is controlled by the SOS system that regulates error-prone DNA repair in *E. coli* and involves the Lex A and Rec A proteins (Ebina *et al.,* 1983). The operon promoter (P1), generally very strong for channel-forming colicins, contains a *lex* operator sequence consisting of two overlapped SOS boxes to which Lex A protein binds, preventing synthesis of enzymes that repair damaged DNA (Ebina *et al.,* 1983). Lex A protein also prevents synthesis of colicin and lysis proteins. Therefore, under normal circumstances, colicins are not produced. Exposure to DNA-damaging agents or inhibitors of DNA replication such as UV irradiation and radiomimetic chemicals such as mitomycin C results in generation of an inducing signal responsible for reversible activation of the Rec A protein to its protease form (Craig and Roberts, 1980). The activated Rec A protease cleaves the Lex A repressor, resulting in depression of the SOS regulated genes, and subsequently production of the colicin and lysis proteins. Co-transcription of the

cea and *kil* genes insures that induction of colicin synthesis will lead to its release. Since the *imm* gene is oriented in opposition to the *cea* and *kil* genes, it is not under SOS control. Instead, it has its own weak promoter and is expressed constitutively (Pilsl and Braun, 1995). Constitutive expression of the immunity protein protects the producing cell from exogenous colicin.

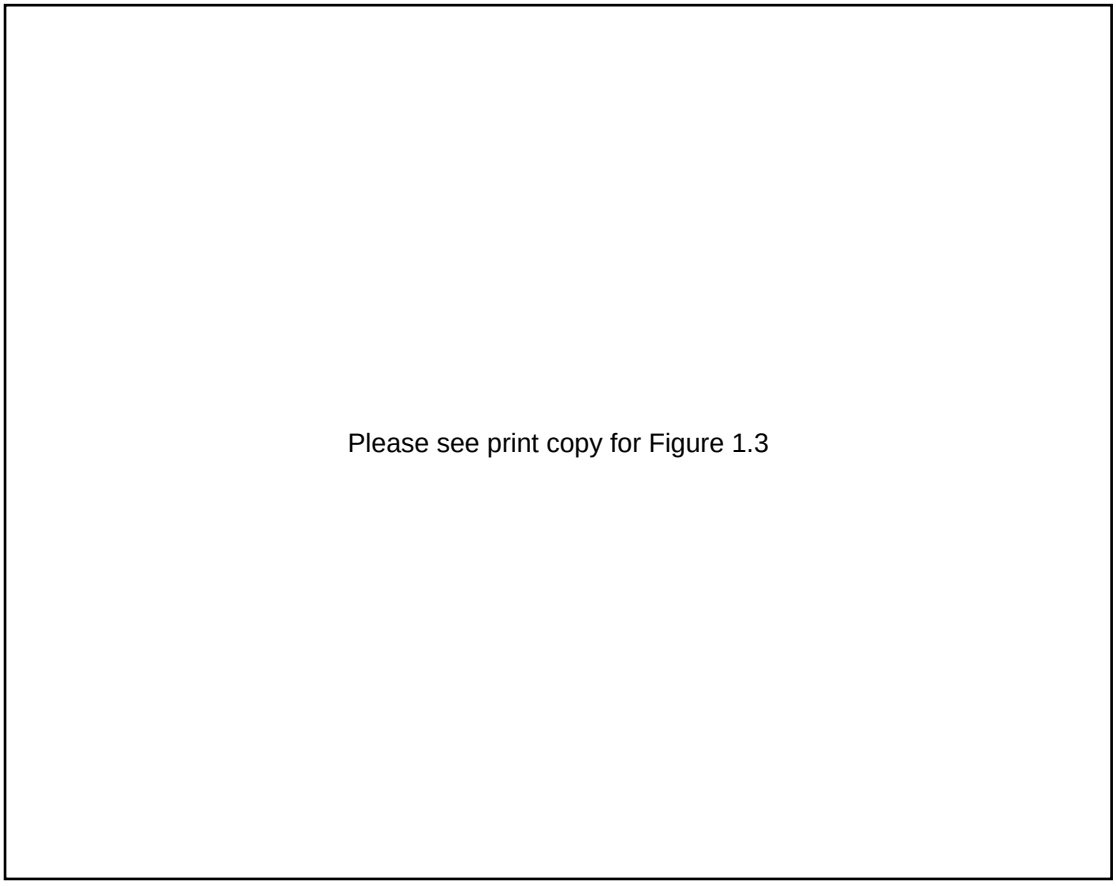


FIG. 1.3 Organization of the colicin-immunity-lysis gene operon of channel-forming (**A**) and nuclease colicins (**B**). Three flanking genes code for colicin (*cea*), immunity (*imm*), and lysis (*kil*). Two transcripts initiated at the SOS-inducible promoter PI terminate at T1 and T2 respectively. The longer transcript includes messenger from the *kil* gene. In nuclease colicins, the promoter for transcription of the *imm* gene in non-induced cells is probably located within the *cea* gene. In channel-forming colicins, the *imm* gene is transcribed in the opposite direction apparently from its own promoter. Not drawn to scale. Adapted from Luria and Suit (1987).

The genetic organisation for the second group is such that the *cea*, *imm* and *kil* genes are contained in a single operon (Figure 1.3-B) and are released from the cell in a stoichiometric complex (Kleanthous *et al.,* 1998). Like the operon for the channel-forming colicins, expression of this operon is SOS-inducible. In contrast, however, since the *imm* gene is part of the operon, the immunity protein is also produced by the SOS response (Pugsley and Oudega, 1987).

1.3.4.1 Colicin Gene

The colicin proteins responsible for killing sensitive cells are elongated, single polypeptide chains that are divided into three structural and functional domains that correspond to the steps of their mechanism of action (Figure 1.4). The central domain binds to a receptor that is specific for the colicin at the surface of target bacteria (Journet *et al.,* 2001). The N-terminal domain determines whether the TolA or TonB system will be used by the colicin in order to cross the envelope whereas the C-terminal domain carries the lethal activity (nuclease or channel-forming) (Bouveret *et al.,* 1998). Colicins which share the same receptor and the same translocation pathway, such as colicins Ia and Ib, display high levels of sequence homologies in their N-terminal and central domains (Alonso *et al.,* 2000).

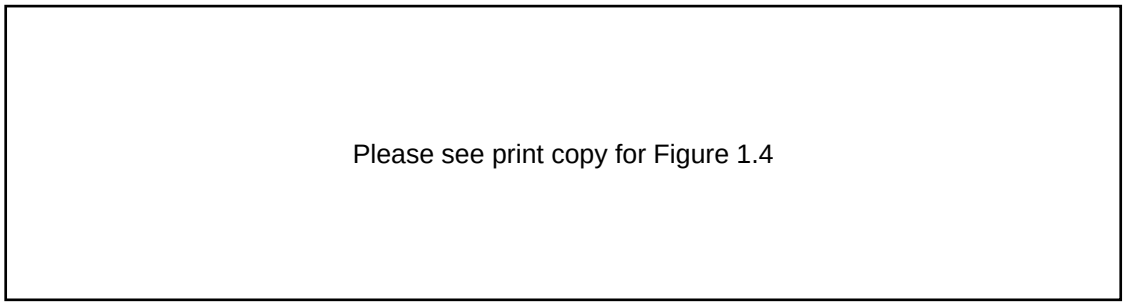


FIG. 1.4 Regions of the *cea* genes that correspond to the modular segments of the colicin molecule. The N-terminal domain (T) is involved in translocation of colicin from receptor to cytoplasmic membrane, the central domain (RR) functions in receptor recognition, and the C-terminal domain (C) is responsible for cell-killing activity. Not drawn to scale. Adapted from Alonso *et al.* (2000).

1.3.4.2 Immunity Gene

The lethal effect of a colicin is effectively neutralised by the immunity protein coded by the same plasmid. They confer protection against the colicin molecules absorbed by the target bacteria by binding to and inactivating the C-terminal domain (Pilsl and Braun, 1995).

Immunity proteins specific for channel-forming colicins reside in the inner membrane where the incoming toxin acts. In the colicin A system, the immunity protein (Cai) has been well characterised and provides an adequate model for other immunity proteins of the channel-forming colicins. The Cai protein is a minor component of the cell membrane and consists of four separate hydrophobic regions consistent with its crossing the cell membrane four times (Benedetti and Geli, 1996). Its N- and C-termini and a loop of hydrophilic amino acids designated L2 are located in the cellular cytoplasm while two hydrophilic loops termed L1 and L3 are located in the periplasm (Espesset *et al.,* 1996). The immunity protein acts by forming a tight intramembranous complex with the channel-forming region of the colicin (Espesset *et al.,* 1996).

Expressing a colicin with nuclease activity poses a survival problem to the host bacterium. To overcome this problem, immunity proteins are co-

synthesised with the colicin and bind the toxin in the producing cell and are released into the medium with the colicin itself as a heterodimeric complex (Kleanthous *et al.,* 1998). The heterodimeric complex is then secreted into the extracellular environment, and it is in this form that cell binding is initiated (Kleanthous and Walker, 2001). The immunity protein dissociates during translocation into the target cell, leaving the bound cell at the mercy of the active nuclease unless it is also colicinogenic and harbours the same immunity specificity. The precise mechanism by which the immunity protein dissociates itself is not yet known.

Neutralisation of a colicin by its immunity protein occurs through a highly specific interaction between the immunity protein and the C-terminal domain of the colicin (Pilsl and Braun, 1995). For example, colicins Ia and Ib are related both structurally and functionally and show 60 % homology at their C-terminal domain (Mankovich *et al.,* 1986). However, cells bearing colicin Ib plasmid show immunity against colicin Ib but are sensitive to colicin Ia (Isaacson and Konisky, 1972).

1.3.4.3 Lysis Gene

Colicins, as produced in the cell cytoplasm or as released by induced cells, have no leader sequence to guide them across the membrane (Cavard, 2002). Release upon induction of colicinogenic cells, for instance by mitomycin C, requires the function of a lytic protein, whose gene (*kil*) is part of the colicin operon, downstream from the colicin gene (Figure 1.3). The lysis proteins are lipoproteins and their mature forms have been found to be localized in both outer and inner membranes (Alonso *et al,* 2000) except for that of colicin N

which has only been found in the outer membrane (Benedetti and Geli, 1996). Lysis proteins are thought to promote a non-specific increase in the permeability of the cell envelope by exerting a direct effect on the inner membrane and an indirect effect (mediated by phospholipase A activation) on the outer membrane (Luria and Suit, 1987). The increase in permeability of the outer membrane induces a partial lysis of the colicinogenic cell, leading to its death. The partial lysis of the colicinogenic cell occurs not due to the action of intracellular colicin but as a result of colicin exportation. Colicin molecules are not able to insert themselves on the inner side of the membrane, presumably because the transmembrane potential polarity is contrary to that required for their correct folding (Alonso *et al*., 2000). Although colicin production is “suicidal” for the producing cell, it becomes a selective advantage for the remainder of the colicinogenic population, which is protected by the immunity protein.

Some colicins, such as colicin Ia, Ib and V, are not released via cell lysis (Riley *et al.,* 1994). These colicins are actively transported across the cell membrane into the external environment via mechanisms that are not well understood.

1.3.5 Mechanism of Protection Against Channel-Forming and Nuclease Colicins

To survive in natural environments where colicins and other toxin-like products are present, sensitive bacteria have developed mechanisms to protect themselves against these lethal products. This is especially important for non-colicinogenic bacteria that would be at a disadvantage when facing those bearing the colicinogenic determinant. To overcome this problem target bacteria

in addition to immunity, have developed other protection mechanisms. Two of these, resistance and tolerance are discussed below.

1.3.5.1 Resistance

Any alteration in the specificity of the colicin-receptor association prevents the recognition of the colicin, rendering the cell insensitive to the colicins lethal effect. As already mentioned, colicins recognize and adsorb to specific receptors on the surface of target cells. The inability of some cells to adsorb colicins is called resistance (Luria and Suit, 1987). Resistance has been studied by means of laboratory mutant strains that have lost their sensitivity to colicin action.

Colicin resistance caused by alterations in the receptor may or may not be related to deficiencies in the receptors natural substrate recognition and assimilation. The areas of the receptor that participate in both activities are highly related, thus associating the resistance phenotype with deficiencies in substrate incorporation (Riley and Gordon, 1999). However, there are reports of mutants of FepA protein (receptor for colicin B) where its receptor activity for enteroqueline, iron-chelating compound, is not affected suggesting that both mechanisms can be separated (Alonso *et al.,* 2000; Lazdunski *et al.,* 1998). Other mechanisms by which a sensitive cell becomes resistant include changes in the binding site, elimination of the binding site or production of a substance that competes with the colicin for the binding site (Alonso *et al.,* 2000).

Theoretically, it would be expected that by adsorbing to and utilising receptors that are vital to cell survival, the possibility of a resistant mutant being viable in nature is minimal. However, a study carried out by Gordon *et al*. (1998)

in 447 *E. coli* isolates from mice found that the level of resistance to colicins, especially E2, was as high as 70 %. However, it is possible that the high levels of resistance observed in the laboratory do not accurately reflect levels of resistance in nature due to high sensitivity of resistance assays (Riley, 1998).

1.3.5.2 Tolerance

A third mechanism by which target cells become insensitive to colicins is tolerance. Tolerance is produced by alterations in the envelope proteins, Tol and TonB, involved in the translocation of colicins into sensitive cells (Riley, 1998). Mutations in the Tol and TonB system proteins prevent the internalisation of the adsorbed colicin, producing a tolerance phenotype.

Two colicin-tolerant cell types have been identified; those that are mutated in the Tol system and are tolerant to group A colicins, and those mutated on the TonB system and are tolerant to group B colicins (Benedetti and Geli, 1996). The N-terminal domain of group B colicins have been shown to contain s consensus sequence of 5 to 6 amino acids called the TonB-box that is homologous to the TonB-box found in TonB-dependent receptors (Lazdunski *et al.,* 1998). It has been shown that colicins with mutations in the TonB-box are unable to be translocated (Bernadetti and Geli, 1996).

1.3.6 Ecological Role of Colicin Production

Since colicin production is encoded by plasmids, it has been seen as a dispensable and non-essential function for the survival of coliform bacteria (Luria and Suit, 1987). However, the high frequency of colicinogenic strains (at least 30 %) among *E.* coli isolates, and the highly conserved organisation of colicinogenic genes and amino acids sequences (Alonso *et al.,* 2000), argues that colicins could play a positive role in *E. coli* population dynamics. The evidence for a positive role of colicins in microbial communities comes from theoretical, *in vitro* and *in vivo* studies.

Using an *in vitro* model, Gordon and Riley (1999) demonstrated that colicin-producing strains E1, E2, E4, E6 and E8 were successful in displacing colicin sensitive cell populations. Furthermore, they found that the time required for the colicin sensitive cell population to be displaced declined as the initial frequency of the colicin-producing population increased and strains producing higher titres of colicin tended to displace the colicin sensitive strain more rapidly. Recently, Kirkup and Riley (2004) have demonstrated that colicins E1 and E2 are effective antagonistic agents within *E. coli* populations in the mouse colon. They concluded that colicins serve to promote microbial diversity in one of the dominant niches exploited by enteric bacteria, the mammalian colon. The prevalence of colicin producing strains in the GIT ecosystem could be explained if it were found to benefit the colonisation and defence of an ecological niche shared by non-colicinogenic sensitive micro-organisms with similar or shared nutritional requirements.

1.4 Aims of the Present Study

This review has highlighted the close relationship between prebiotics and probiotics in promoting the growth of beneficial bacteria in the GIT.

Polysaccharides, in particular β-(1-3)-D-glucans, isolated from higher basidiomycetes, have been shown to have beneficial effects on general health. While increasing evidence supports the beneficial role of these polysaccharides on enhanced immune response and their anti-tumour effects, little work involving their prebiotic actions has been undertaken. To evaluate the benefits of polysaccharides isolated from higher basidiomycetes, there is a need to formulate hypotheses that can be tested. The first experiment described in this thesis involves the use of a mouse model to investigate the prebiotic value of polysaccharides isolated from *Ganoderma lucidium*. Implicit in the experimental design was the testing of the following hypothesis in relation to dietary supplementation: This was

- That polysaccharides isolated from *Ganoderma lucidium* are able to alter the population dynamics of cultivable good and bad bacteria in the GIT.

One of the mechanisms by which probiotics inhibit the growth of pathogenic bacteria is by producing antimicrobial substances called bacteriocins (Jack *et al.,* 1995). The most common probiotics are Lactobacillus species such as *Lactobacillus* acidophilus and *Lactobacillus casei*. Whilst there is an ever-increasing amount of evidence to support the beneficial role of LAB, little work involving the probiotic applications of other bacterial species,

especially gram-negative ones, has been done. The main objectives of the second part of this thesis were:

- To develop and validate a multiplex PCR for colicin detection;
- To assemble a collection of commensal and pathogenic *E. coli* and apply the multiplex PCR to survey the presence of colicins in these *E. coli;* and
- To bioassay the inhibitory spectrum of colicin producing commensal *E. coli* strains that are able to inhibit the growth of pathogenic *E. coli*

Chapter 2

Effect of *Ganoderma lucidium* Fruiting Body Extracts on the Population Dynamics of Gastrointestinal Bacteria Grown on Selective Media

2.1 Introduction

Nutraceuticals are considered a generic class of nutritional supplements that improve the overall health of humans and animals when added as a dietary supplement to food. Medicinal mushrooms and mushroom products have been used in this way for thousand of years (Chang and Buswell, 1999). Previous research has demonstrated that many medicinal mushrooms have bioactive polysaccharides, which are able to confer their nutraceutical properties. *Ganoderma lucidium* is a medicinal mushroom with a long history of use in Asian medicine as a tonic and remedy for the treatment of various diseases such as hypertension, bronchitis, diabetes and cancer. The use of modern analytical techniques such as chromatography, spectrochemistry and bioassay systems has attributed these positive effects to polysaccharides found in the fruiting bodies of this mushroom (Wasser and Weis, 1999).

The nutraceutical effect of medicinal mushrooms on the population dynamics of bacteria in different parts of the intestine is something that has not been well studied. This will be the first study to look at the effects of *Ganoderma lucidium* polysaccharides on bacterial population dynamics in different parts of the intestine.

The aims of this chapter were:

- To extract and purify polysaccharide extracts from the fruiting body of *Ganoderma lucidium*; and
- To design a feeding trial in mice to compare the impact of polysaccharide extracts from the fruiting body of *Ganoderma lucidium* on changes in the population dynamics of cultivable lumen and adherent bacteria from different segments of mouse GIT.

2.2 Materials and Methods

2.2.1 Fruiting Body Extraction

Dried fruiting body from *Ganoderma* lucidium was obtained by Dr. Ren Zhang (University of Wollongong, Wollongong, Australia). Most bioactive polysaccharides are water soluble, and hence a hot water procedure was used to extract the polysaccharides from the ground fruiting body. Since a hot water extract contains more than polysaccharides, further purification of the extract is necessary. Polysaccharides are insoluble in alcoholic solutions, while other constituents are soluble.

The fruiting body was ground to a fine powder (2.5 g) using a blender and then extracted with 50 ml boiling sterile de-ionised water (dH_2O) for 8 hours. The solution was then filtered through a Whatman filter paper No. 1 (Crown Scientific, Moorebank, Australia) to remove insoluble matter.

The procedure used for the purification of the hot water (HW) extract was a modified method of Mizuno (1995). To purify the extract, the HW extract was mixed with two volumes of 95 % ethanol. The precipitate was isolated by

centrifugation at 10, 000 rpm in a J2-MC centrifuge (Beckman, Gladesville, Australia) for 10 min and then dissolved in sterile dH_2O. This process was then repeated for the solution by mixing it with 3 volumes of 95 % ethanol. Again, the precipitate was isolated by centrifugation (10, 000rpm, 10 min) in the same centrifuge, and dissolved in dH_2O. The resulting extract (HWE) was centrifuged further for 45 min at 20,000 rpm to remove small insoluble matter, and filtered through a non-pyrogenic sterile 0.22 μm filter (Sartorius, Waverley, Australia) under sterile conditions.

The extracts were assayed for polysaccharide content and aliquoted onto day amounts so that the final concentration was 150 μg/ml in drinking water. Aliquots were stored at -20 °C until used.

2.2.1.2 Polysaccharide Content of Extracts

The polysaccharide content of the extracts was assessed using the phenol-sulphuric acid test developed by Dubois *et al.* (1956). The method is based on the reaction between carbohydrates and phenol, which results in an orange/yellow colour of the solution. Sulphuric acid is added to the reaction to convert all non-reducing sugars into reducing sugars. The absorbance at 490 nm (O.D. 490) is proportional to the initial polysaccharide concentration in the sample.

To determine the total amount of polysaccharides present, a linearly fitted standard curve constructed for glucose was employed. The standards used were 0.04, 0.08, 0.12, 0.16 and 0.20 mg/ml glucose in dH_2O, and all samples were diluted to fall within the linear range of the standard curve. 400 μl of standards and samples were pipetted in triplicate into1.5 ml eppendorf tubes

(Sarstedt, Ingle farm, Australia) and 10 μl of 80 % phenol was added. One ml of concentrated sulphuric acid was rapidly added to the centre of the liquid surface to ensure thorough mixing of the solutions. The tubes were then stood for 10 min before being vortexed and placed in a water bath at 25 °C for 20 min. The O.D. 490 was measured using a UV spectrophotometer (Labsystems, model 1601, Chesire, UK).

2.2.1.3 Protein Content of Extracts

Proteins have aromatic side chains such as tryptophan, tyrosine and phenylalanine that absorb light at 280 nm. The HW and HWE extracts were subjected to a scan between the wavelengths 200-400 nm on a UV spectrophotometer (Labsystems, model 1601, Chesire, UK), to ensure that the HWE extract did not show a big absorbance at 280 nm.

2.2.2 Mice

2.2.2.1 Source

Female C57Bl mice were obtained from the John Curtin School of Medical Research, Australian National University, Canberra. All experimental work performed complied with the Australian code of conduct for the care and maintenance of animals for scientific purposes. The mice were acclimatised for a period of 14 days before being used in experiments.

2.2.2.2 Holding Conditions

Mice were confined to a maximum of 6 animals in autoclavable polypropylene cages (30 cm x 50 cm x 20cm). A deep litter system was used with a 1:1 mix of autoclaved sawdust / woodchips. This was applied in 3-5 cm layers every other day and completely changed at least twice a week.

2.2.2.3 Feed

Mice were fed *ad libitum* Barastoc rat and mouse cubes (Ridley Agriproducts, Pakenham, Australia) containing – wheat, barley, sorghum, cottonseed meal, canola meal, soybean meal, sunflower meal, full fat soybean meal, millrun, meat meal, molasses, bentonite, limestone, salt, lysine, vitamins and trace minerals. Water was administered in 50 ml centrifuge tubes (Sarstedt, Ingle farm, Australia) though a 5-10 cm sipper tube. The volume of water supplied per cage per day was 45 ml.

2.2.2.4 Care

The mice were monitored daily for general well being by close scrutiny for morbidity (lack of movement or a sustained display of lethargy with no scatter in response to handling). Healthy mice would also consume their share of feed pellets and water whilst sick or ill mice would go off feed completely. Water was changed every second day. Environmental conditions were kept stable with an air-conditioner and the ambient temperature in the mouse room was maintained at 22 ^{o}C.

2.2.2.5 Weight Estimations

Mice were weighed before the start of the experiment and individual weights recorded. Mice were allocated to separate cages so that the average mean weight of all mice in each treatment group was within 5 % of the group mean represented by the mean weight of all mice used in each experiment. Mice were weighed individually each week during the course of the experiment. A top pan balance (A&D, model GX-400, California, USA) with a hold function that enables weight measurements of moving objects was used to determine body weights.

2.2.3 Tissue Sampling

2.2.3.1 Anaesthesia

To minimise the trauma of intraperitoneal euthanasia, each mouse was pre-anaesthetised with 150 μL of a mixture of ketamine (100 mg/mL) and xylazine (100 mg/mL) solubilized in sterile Phosphate Buffer Saline (PBS). Euthanasia was accomplished by intraperitoneal injection of Valabarb (100 μL – 300 mg/mL sodium pentobarbitone) (Jurox, Silverwater, Australia) prior to recovery of tissue.

2.2.3.2 Removal of Intestinal Segments

A longitudinal incision was made along the ventral face of the abdomen. The intestinal tract was displaced to the side of the mouse starting from the stomach to the rectum. To prevent leakage, two surgical knots were secured 1 cm below the stomach and 1 cm above the anus. Further ligations were carried out to isolate 5 cm stretches of the duodenum and ileum, and on both ends of

the caecum. Each designated segment was excised below the secured ligations and transferred to pre-weighed petri dishes containing 2.5 mL PBS in 20 % glycerol and placed on ice. The petri dish was reweighed and segment weight was obtained by differential subtraction.

2.2.3.3 Recovery of Lumenal Bacteria / Digesta

Intestinal segments from each animal were blotted dry and then transferred to new medium in small petri dishes. Each segment was then cut along the longitudinal axis. Lumen bacteria and digesta were released by orbital shaking for 5 min at 150 rpm. The wash was transferred into labelled tubes. A second wash was carried out with 2 mL PBS in 20 % glycerol (2 min at 150 rpm). Both washes were pooled.

2.2.3.4 Recovery of Adherent Bacteria

Washed intestinal segments were blotted dry on sterile paper to remove any excess PBS and digesta. Each segment was further cut into three pieces and transferred to pre-weighed petri dishes containing 2 mL 0.1 % Triton-X 100 (TX) (Hach, Philadelphia, USA) in sterile water. After reweighing, segment weights were again determined by differential subtraction. Adherent bacteria were then released by orbital shaking for 15 min at 150 rpm. The wash was then transferred into labelled tubes. A second wash was carried out using 1 mL of TX (10 min at 150 rpm). Both washes were pooled. Viability assays previously performed at Elizabeth McArthur Agricultural Institute (EMAI, Camden, Australia) have shown that incubation of bacteria in TX for up to 4 hours did not affect survival.

2.2.4 Faecal Sampling

2.2.4.1 Collection and Processing of Faeces

Faecal samples were obtained from individual mice transferred to escape-proof and well-ventilated sterile containers. On average, each mouse would produce 6 faecal pellets in 10 min. Faecal pellets were accurately weighed before immediate resuspension in ice-cold PBS in 20 % glycerol to attain a final concentration of 50 faeces mg/ml. Homogeneity of each sample was ensured by vigorous vortexing of each suspension in the presence of 4 – 5 sterile glass beads (1 mm) for a period of 1 – 2 min. The homogenised faecal suspensions were transferred to 1.5 ml eppendorf tubes (Sarstedt, Ingle farm, Australia) and stored at –80 °C until used.

2.2.5 Bacteriological Media

2.2.5.1 Blood Agar (BA)

Blood agar (Oxoid, CM271) is a non-selective general-purpose medium for the cultivation of the majority of microorganisms (Appendix 1). It is particularly useful for cultivating fastidious microorganisms that are difficult to culture on selective media. Haemolysis is identified by clearly recognisable haloes around individual colonies; a clear halo is observed for β-haemolysis (or total haemolysis), while a greenish halo is observed for α-haemolysis (or partial haemolysis). A halo is not observed for non-haemolytic colonies.

2.2.5.2 DeMan, Rogosa and Sharpe (MRS) Agar

MRS agar (Merck, 1.10660) is a selective medium used for the cultivation of LAB (Appendix 1). Polysorbate, acetate, magnesium and

manganese act as special growth factors to support the growth of *Lactobacilli*. As the medium exhibits a low degree of selectivity, other LAB such as *Pediococcus* may also grow. MRS agar was used fro the enumeration of lactic acid producing bacteria.

2.2.5.3 Kanamycin Esculin Azide (KEA) Agar

KEA agar (Oxoid, CM591B) is a selective medium used for the cultivation and differentiation of Streptococci (Appendix 1). Kanamycin and azide inhibit accompanying microorganisms. Streptococci hydrolyse the glucoside esculin and the released esculin complexes with iron (III) ions resulting in a black zone surrounding the individual colonies.

2.2.5.4 Citric Azide Tween Carbonate (CATC) Agar

CATC agar (Merck, 1.10279) is a selective medium used for the cultivation and differentiation of Enterococci (Appendix 1). Accompanying microorganisms are inhibited by the high concentrations of citrate and azide. Enterococci reduce the colourless 2,3,5-triphenyltetrazolium chloride to the red formazan, leading to colonies that are red in colour.

2.2.5.5 MacConkey's (MAC) Agar

MacConkey's agar (Merck, 1.05465) is a selective medium used for the cultivation of coliforms, as well as other enteric bacteria, such as *Shigella*, *Salmonella* and *E. coli* (Appendix 1). Bile salts and crystal violet largely inhibit the growth of gram-positive organisms, while lactose and the pH indicator

neutral red allow the differentiation of lactose fermenting (pink colonies) and non-lactose fermenting (white colonies) organisms.

2.2.6 Processing and Enumeration of Bacteria

Intestinal wash samples were aliquoted into wells in a sterile 96-well tissue culture plate. Fourfold serial dilutions were carried out with a multi-channel pipette. Triplicate 10 μL drops representing each dilution were plated onto pre-dried square plates (100 mm x 100 mm, Sarstedt, Ingle farm, Australia) containing MAC, KEA, CATC, MRS and BA agar media. Once dry, the plates were inverted and incubated for 24 hours at 37 °C for MAC, KEA and BA; 48 hours at 37 °C for CATC; 48 hours at 37 °C in 5 % CO_2 for MRS. Colony forming units (CFU) distributed over each drop zone were counted and averaged for triplicates with countable bacteria from at least 2 dilutions. The totals from each dilution were averaged to give the final bacterial CFU count.

2.2.7 Experimental Protocol

Mice were organised into three groups each containing 12 mice; two of which were HW and HWE fed and the other was a negative control. The polysaccharide extracts were administered in the drinking water at a final concentration of 150 μg/ml. Control mice were given sterile dH_2O without any extract. Feeding started on day 1 and continued for 22 days. No extracts were mixed in the drinking water over weekends. Mice were weighed once a week for the entire duration of the experiment. Mice in all groups were euthanised on day 22 for removal of intestinal segments (Chapter 2.2.3.1).

2.2.8 Statistical Analysis

All statistical analyses were carried with the aid of Dr Idris Barchia (Special biometrician, EMAI). Based on previous data from other experiments at EMAI, the error distribution and variance heterogeneity for the data set on percentage haemolysis was subjected to logit transformation. Bacterial CFU count data from selective media platings including BA, MAC, KEA, CATC and MRS was subjected to logarithmic transformation to stabilise the variances before analysis. All transformed data were then analysed using a split plot analysis because of the fact that Lumen bacteria and Adherent bacteria represented nested data because they are actually subset samplings from each intestinal segment of a single animal. The transformed data (**Y**) may be mathematically represented by the following model:

Y = Treatment + *Error(A)* + "LB/AB" + Interaction + *Error(B)*

Pair-wise treatment differences were tested using the Least Significant Difference (LSD) test at the 5% significance level.

2.3 Results

2.3.1 Polysaccharide and Protein Content of Extracts

Using the phenol-sulphuric acid test, the concentration of polysaccharides in the HW and HWE extracts was found to be 470 μg/ml and 496 μg/ml, respectively. When these extracts were scanned for protein content, the HW extract was found to have a higher peak at 280 nm compared to the HWE (Figure 2.1). This result suggests that the ethanol precipitation was able to remove most proteins and that it was relatively pure.

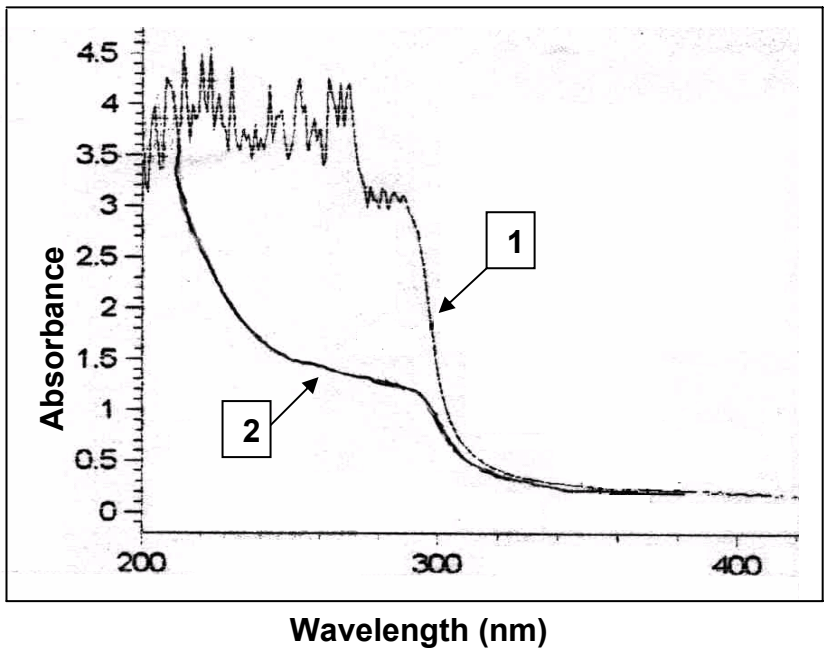


FIG 2.1 Scan of HW (1) and HWE (2) extracts from *Ganoderma lucidium* at wavelengths of 200 – 400 nm.

2.3.2 Weight Gain

The average weight of mice in each treatment group over the course of the 21 days feeding is shown in Figure 2.2. All groups showed comparable daily weight gain over this period. Although it appeared the HW and HWE treated mice were not gaining weight as fast as the control group, this trend did not continue by the end of the experiment. At the conclusion of the experiment, the control mice had a mean weight of 19.34 g. HW and HWE treated mice reached

a mean weight of 18.61 g and 18.58 g, respectively. Statistical significance was not reached between control groups.

2.3.3 Water Consumption

The average amount of water consumed by mice in each treatment group over the 21 days of the experiment is shown in Figure 2.3. There was no significant difference in the amount of water consumed between the treatment groups. The quantity of water consumed by the HW and HWE treated mice was 3.78 ml and 3.79 ml respectively while the unfed controls consumed 3.61 ml. In the course of the experiment HWE fed mice consumed on average 560 μg/ml polysaccharide per day.

2.3.4 Bacterial Morphology on Selective Media

Figure 3.4 shows the appearance of bacterial colonies cultured on each of the four selective media. Colonies on MAC gar appear as pink due to the fermentation of lactose (Figure 2.4-A). Colonies on CATC agar are red, indicating the reduction of the colourless 2,3,5-triphenyltetrazolium chloride to the red formozan (Figure 2.4-B). Colonies on KEA agar appear black in colour due to the hydrolysis of esculin (Figure 2.4-C). Colonies on MRS appear as white or cream due to phenotypic differences (Figure 2.4-D).

Figure 2.5 shows the appearance of haemolytic and non-haemolytic colonies on blood agar. α-haemolytic colonies appear with green halos around each colony representing α-haemolysis. β-haemolytic colonies appear with distinctive clear halos around each colony representing β-haemolysis. Non-haemolytic colonies appear as white colonies with no clear halo around the colony.

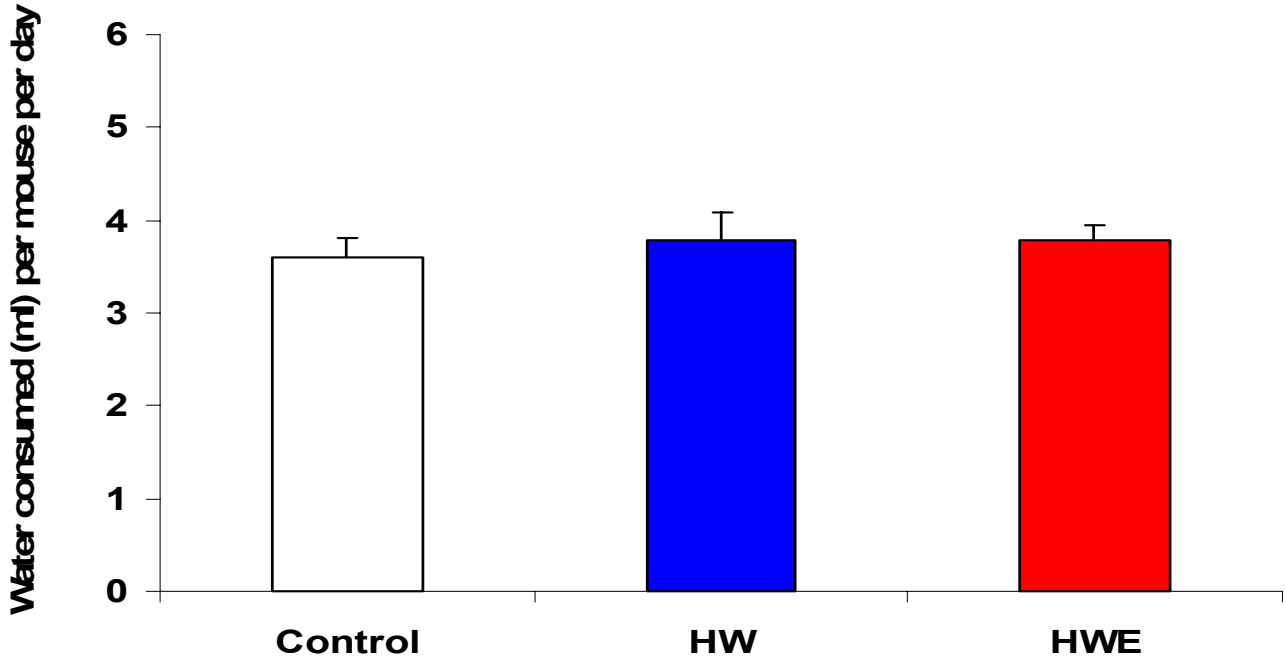


FIG 2.2 Changes in body weight over time due to mushroom extract feeding. Bars represent crude (HW) and purified (HWE) *Ganoderma* extracts. Control mice were fed drinking water without extracts. Data are means ± standard error for 12 mice per treatment group.

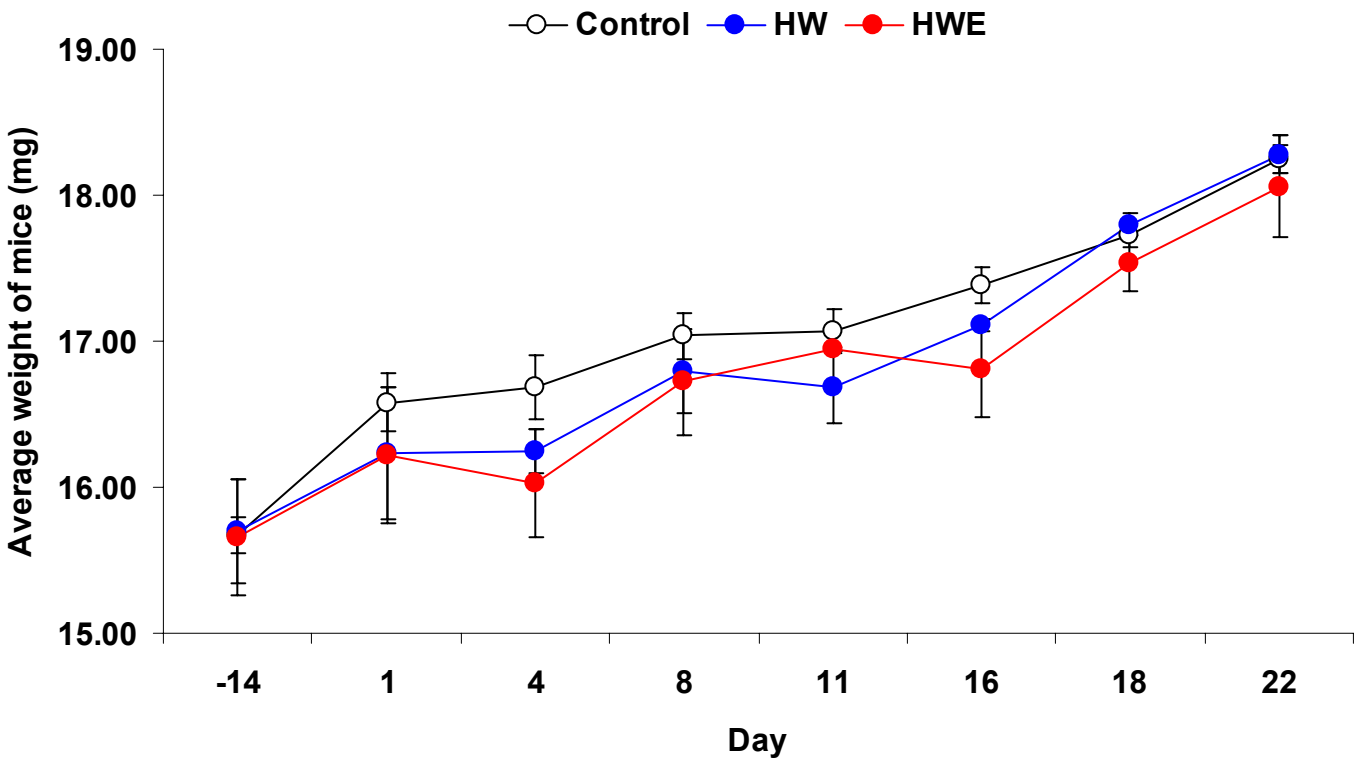


FIG 2.3 Average amount of water consumed per mouse per day for the *Ganoderma* fed and control groups over the course of the experiment. Crude (HW) and purified (HWE) *Ganoderma* extracts. Control mice were fed drinking water without extracts Data are ± standard error for 12 mice per treatment group.

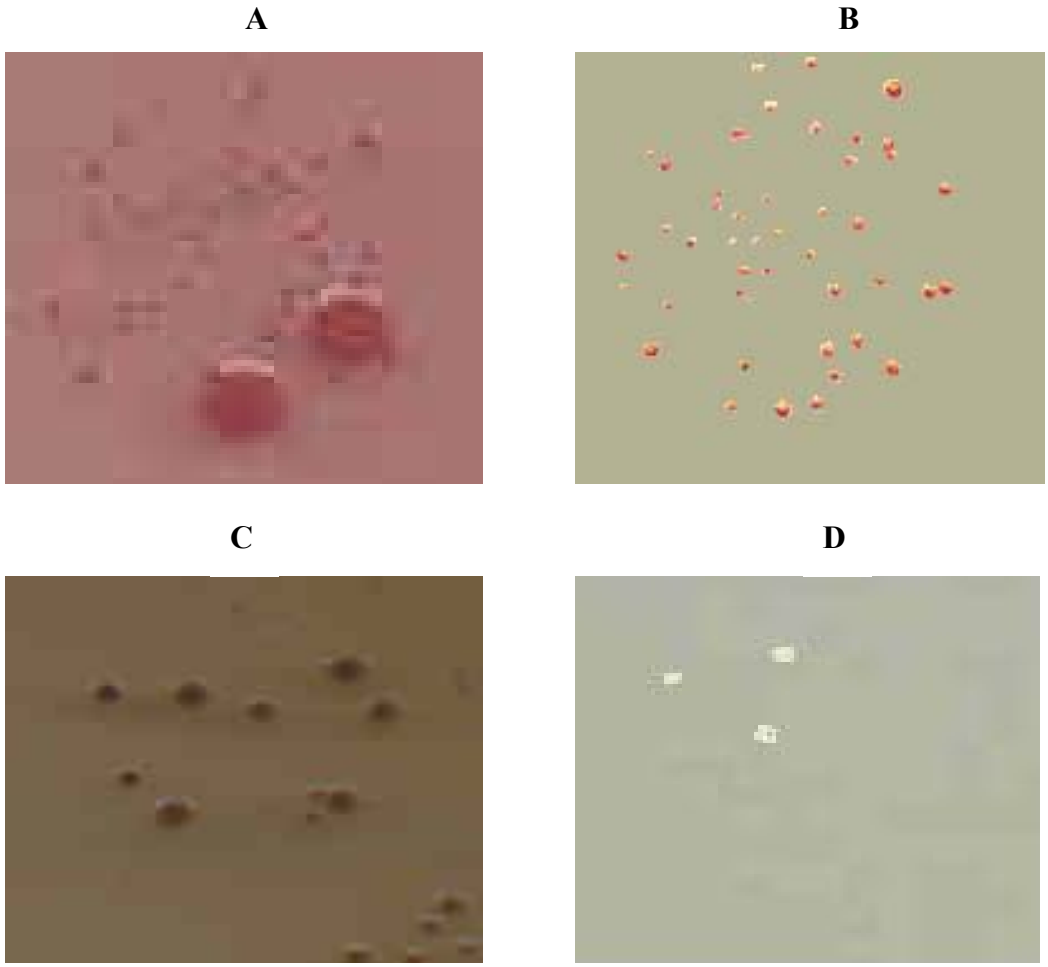


FIG 2.4 Morphology of bacterial populations on different selective media. **A** – *Enterobacteriaceae* on MAC agar appear as pink colonies due to the fermentation of lactose. **B** – *Enterococci* on CATC agar appear as red colonies due to the formation of a red formozan product. **C** – *Streptococci* on KEA agar appear as black colonies due to the hydrolysis of esculin. **D** – Lactic acid bacteria on MRS agar appear as white or cream colonies.

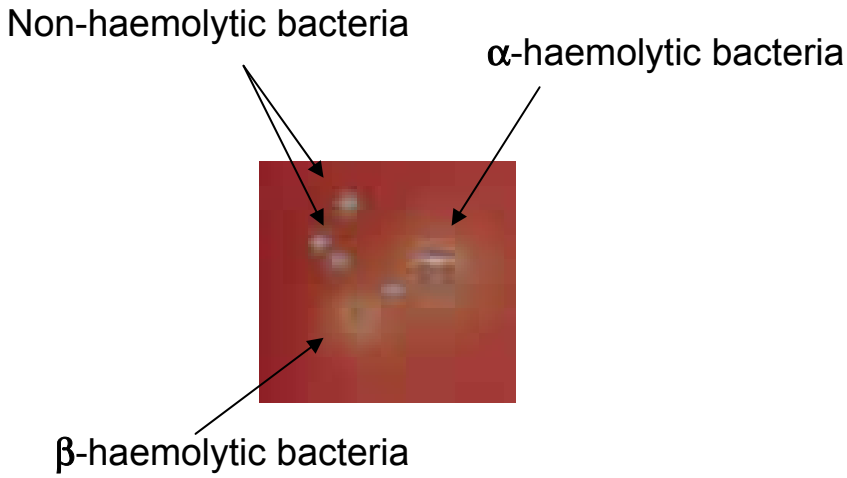


FIG 2.5 Bacterial morphology on blood agar. Non-haemolytic bacteria appear as white colonies with no halo. α-haemolytic bacteria have a green halo around them while β-haemolytic bacteria have a clear halo around them.

2.3.5 Haemolytic Colonies

The frequency of haemolytic colonies in the duodenum, the ileum and the colon segments was determined by plating washings on blood agar (Figure 2.6). An attempt was made to differentiate between populations that were in the lumen from adherent populations released by the non-ionic detergent Triton-X 100.

None of the viable luminal and adherent colonies on the blood agar isolated from duodenum in the control group and the two treatment groups (HW and HWE) showed haemolytic activity. Exception from this was a small amount of luminal bacteria isolated from the duodenum in HWE treated mice (1.79 %).

In the ileum, lumen bacteria of HW treated mice showed an average percentage haemolysis (36.9 %) that was comparable to the control group (37.4 %). HWE treated mice showed a positive trend in reduction of haemolytic bacteria (25.9 %). However, the reduction was not significant at a 5 % level. Adherent bacterial in HW treated mice (12.5 %) did not differ significantly from the controls (31.3 %), but there was a positive trend in reduction. HWE virtually eliminated all haemolytic colonies in the adherent population.

In the colon, a significant reduction was observed in the frequency of haemolytic colonies in the lumen population between HWE treated mice (22 %) and the controls (41.7 %). HW treated mice (37.2 %) did not show an average percentage haemolysis that was significantly different from the control group, however, a trend towards reduction was seen. Adherent bacteria in HW (42.5 %) and HWE (37.5 %) treated mice also showed a positive trend for reduction, but the differences were not significant at a 5 % level compared to controls (43.8 %).

No haemolytic colonies were present in the faeces in both the control and HW treatments. A small amount of adherent bacteria was isolated from the colon in HWE treated mice (1.79 %).

2.3.6 Total Bacterial Enumeration

Figure 2.7 shows the average total colony count of lumen and bacteria from the duodenum, the ileum and the colon as well as the faeces on non-specific blood agar after 21 days of feeding hot water and polysaccharide-enriched extracts to mice. Total lumen populations in the duodenum of HW (2.12×10^4) and HWE (5.32×10^4) treated mice were not significantly different from those of the controls (1.81×10^4). Adherent populations in the duodenum of HW (6.86×10^3) and HWE (3.66×10^2) treated mice as compared to controls (3.12×10^3) were also not significantly different.

Total lumen populations in the ileum of HW treated mice (2.00×10^6) were not significantly higher ($p = 0.005$) than that in the control group (1.22×10^4). HWE treated mice also showed a total luminal population (2.79×10^4) that was significantly higher than the control mice ($p = 0.004$). Total adherent bacterial populations in the ileum of HW (2.37×10^4) and HWE (8.10×10^3) treated mice were not significantly different from those of the controls (3.71×10^3).

In the colon, total lumen bacteria were significant higher in HW (6.93×10^6) treated mice compared to controls (8.27×10^5) ($p = 0.019$). No significant difference was observed between lumen populations in HWE treated mice (1.95×10^6) compared to the control group. Total adherent

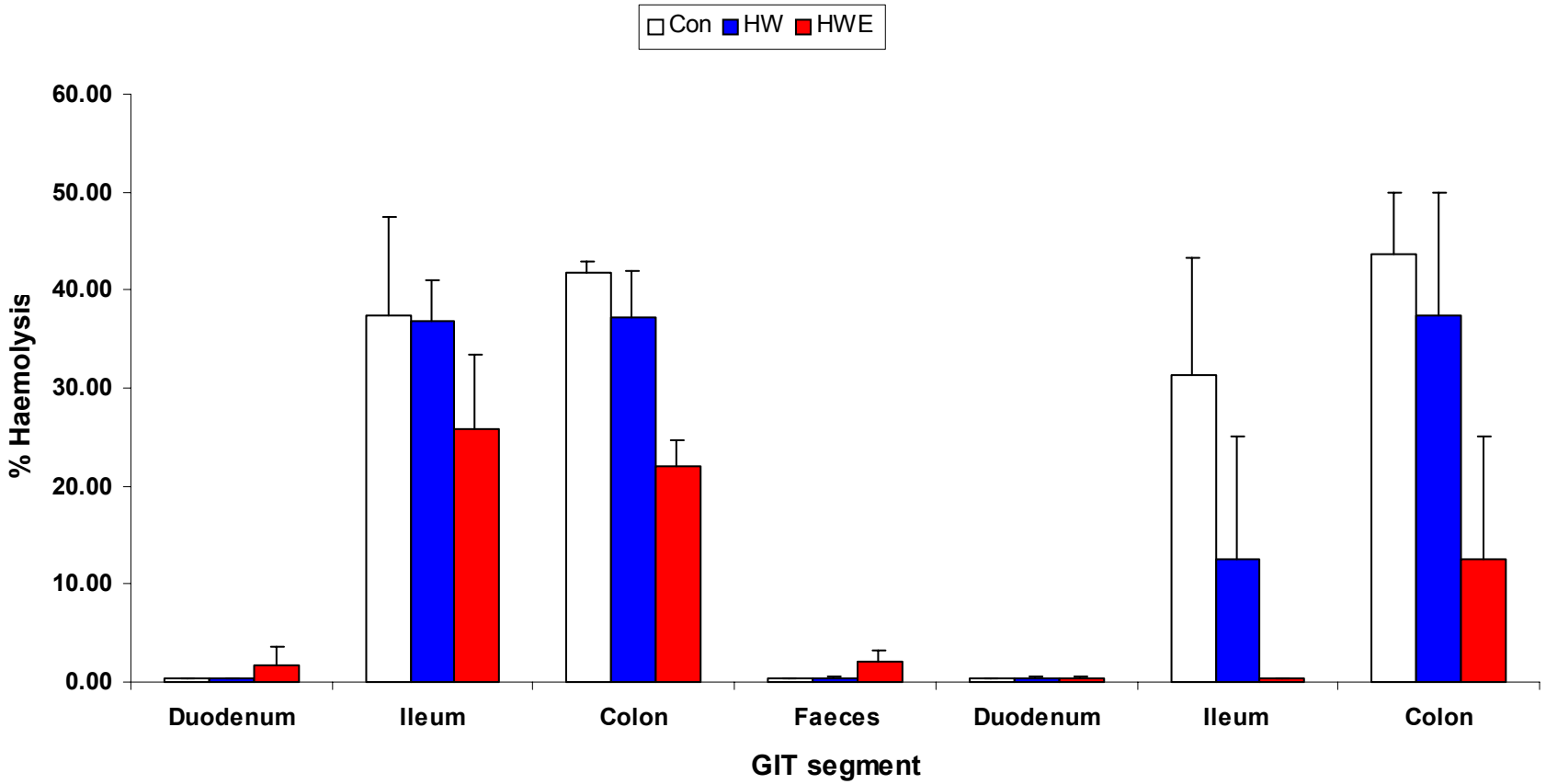


FIG 2.6 Frequency of haemolytic lumen and adherent bacterial populations recovered from intestinal segments and faeces of mice after 21 days of extract feeding from fruiting body of *Ganoderma lucidium*. Bars represent crude (HW) and purified (HWE) *Ganoderma* extracts. Control (Con) mice were fed drinking water without extracts. Values are means ± standard error of 12 mice in each treatment group.

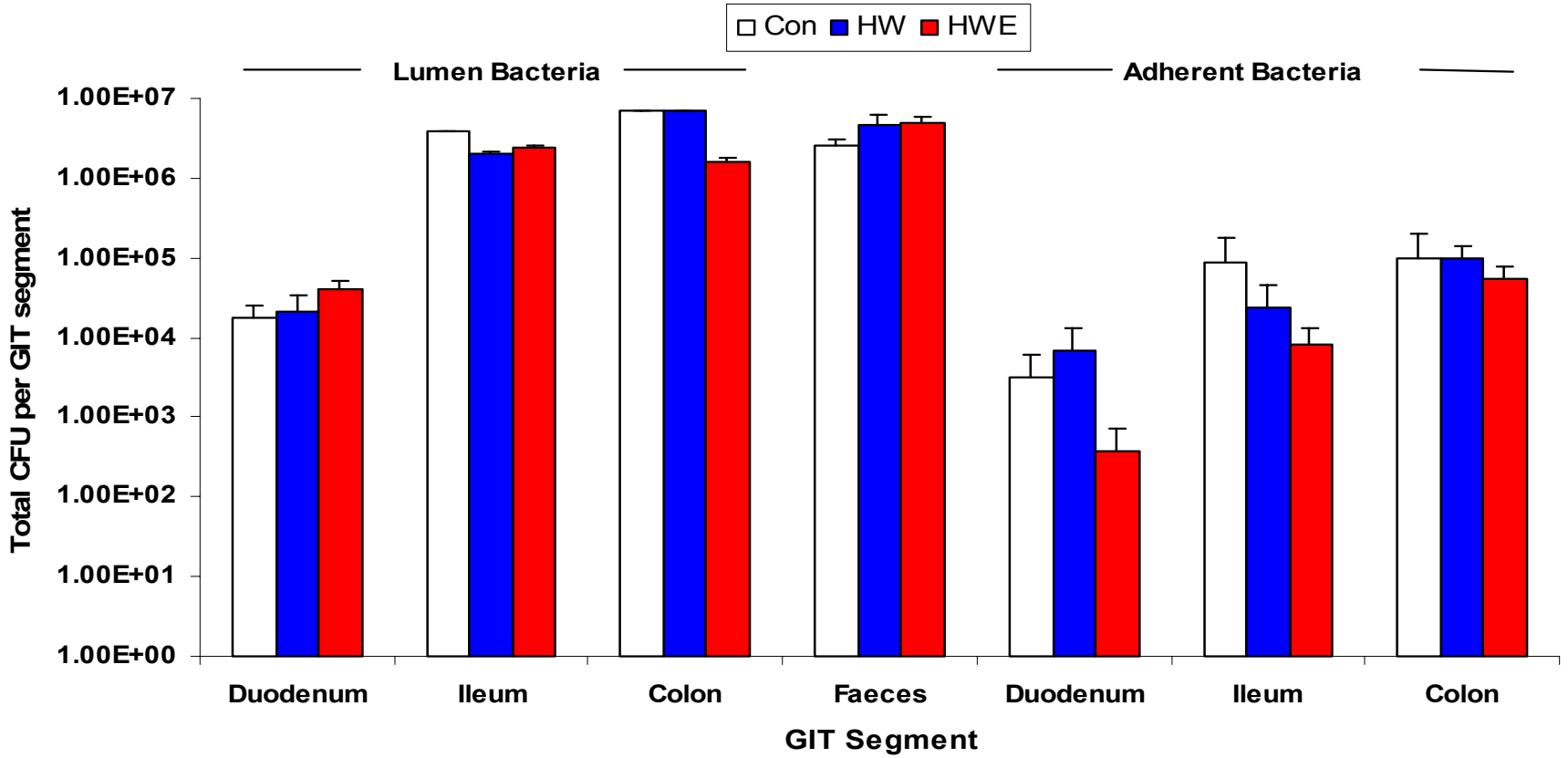


FIG 2.7 Effect of feeding extracts from the fruiting bodies of *Ganoderma* on lumen and adherent total bacterial populations enumerated on blood agar. Mice were euthanized after 21 days of extract feeding. Bars represent crude (HW) and purified (HWE) *Ganoderma* extracts. Control (Con) mice were fed drinking water without extracts. Values are means ± standard error of 12 mice in each treatment group.

bacterial populations in the colon of HW (1.01 x 10^4) and HWE (6.14 x 10^4) treated mice were not significantly different from those of the controls (4.93 x 10^4).

No significant difference was observed between total bacterial counts in the faeces of HE treated mice (4.63 x 10^6) or HWE treated mice (4.71 x 10^6) compared to the control group (2.59 x 10^6).

2.3.7 Lactic Acid Bacterial Enumeration

Figure 2.8 shows the mean total colony counts of lumen and adherent bacteria from the duodenum, the ileum and the colon on LAB selective MRS agar following feeding of HW and HWE polysaccharide extracts from the fruiting body of *Ganoderma*.

In the duodenum, lumen populations in HW (5.59 x 10^5) and HWE (4.02 x 10^5) treated mice were not significantly different from those of the controls (2.28 x 10^5). Adherent populations in the duodenum of HW (2.63 x 10^5) and HWE (3.21 x 10^3) treated mice as compared to controls (1.50 x 10^4) were not significantly different.

Lactic acid populations in the lumen of the ileum of HW (9.23 x 10^5) and HWE (4.19 x 10^6) treated mice were not significantly different from those of the control group (8.98 x 10^5). Adherent bacterial populations in the ileum of HW (8.11 x 10^4) and HWE (5.56 x 10^4) treated mice did not differ significantly from those of the controls (6.82 x 10^4).

Lumenal populations in the colon of HW treated mice (1.66 x 10^6) showed a significant reduction compared to controls (2.87 x 10^6) ($p = 0.003$). HWE treated mice however, had an increased LAB population (6.42 x 10^6) that was significantly different from that of controls ($p = 0.008$). The lumenal

population of LAB was significant different from the two treatment groups ($p < 0.001$). Adherent populations in the colon of HW (2.47×10^5) and HWE (1.19×10^5) did not differ significantly from those of the control group (7.61×10^4).

HW treated mice (2.02×10^7) and HWE treated mice (2.03×10^7) did not show significant difference in their faecal LAB populations compared to the control group (2.22×10^7).

2.3.8 *Streptococcal* Enumeration

Figure 2.9 shows the mean total colony counts of lumen and adherent bacteria from the duodenum, the ileum and the colon after culture on Streptococci selective KEA agar following feeding of HW and HWE polysaccharide extracts from the fruiting body of *Ganoderma*.

Streptococcal populations in the lumen of the duodenum of HW (1.97×10^5) and HWE (4.61×10^4) were not significantly different from those of the controls (1.54×10^5). Adherent streptococcal populations in the duodenum of HW (2.63×10^4) and HWE (2.67×10^2) treated mice did not show a significant difference compared to controls (8.13×10^3).

In the ileum, lumen populations in HW (8.45×10^5) and HWE (5.09×10^5) treated mice were not significantly different from those of the controls (6.93×10^5). Adherent populations in the ileum of HW (1.60×10^4) and HWE (1.49×10^3) treated mice did not differ significant from the controls (9.51×10^3). However, populations of adherent bacteria in the ileum of HW treated mice were observed to be significantly different ($p = 0.047$) from that of the population in HWE treated mice.

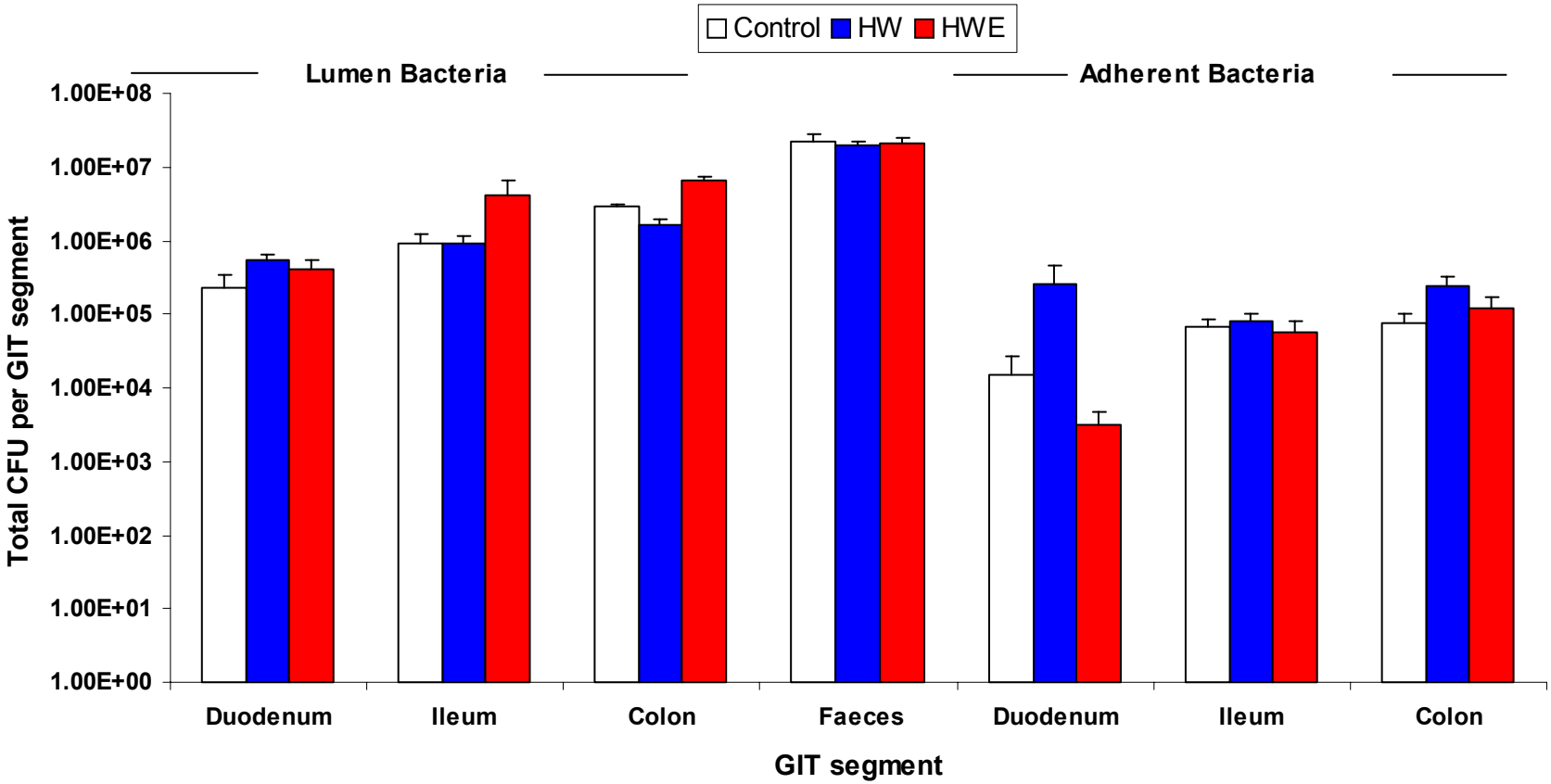


FIG 2.8 Effect of feeding extracts from the fruiting bodies of *Ganoderma* on lumen and adherent lactic acid bacterial bacterial populations recovered from intestinal segments and faeces of mice when enumerated on MRS agar. Mice were euthanized after 21 days of extract feeding. Bars represent crude (HW) and purified (HWE) *Ganoderma* extracts. Control (Con) mice were fed drinking water without extracts. Values are means ± standard error of 12 mice in each treatment group.

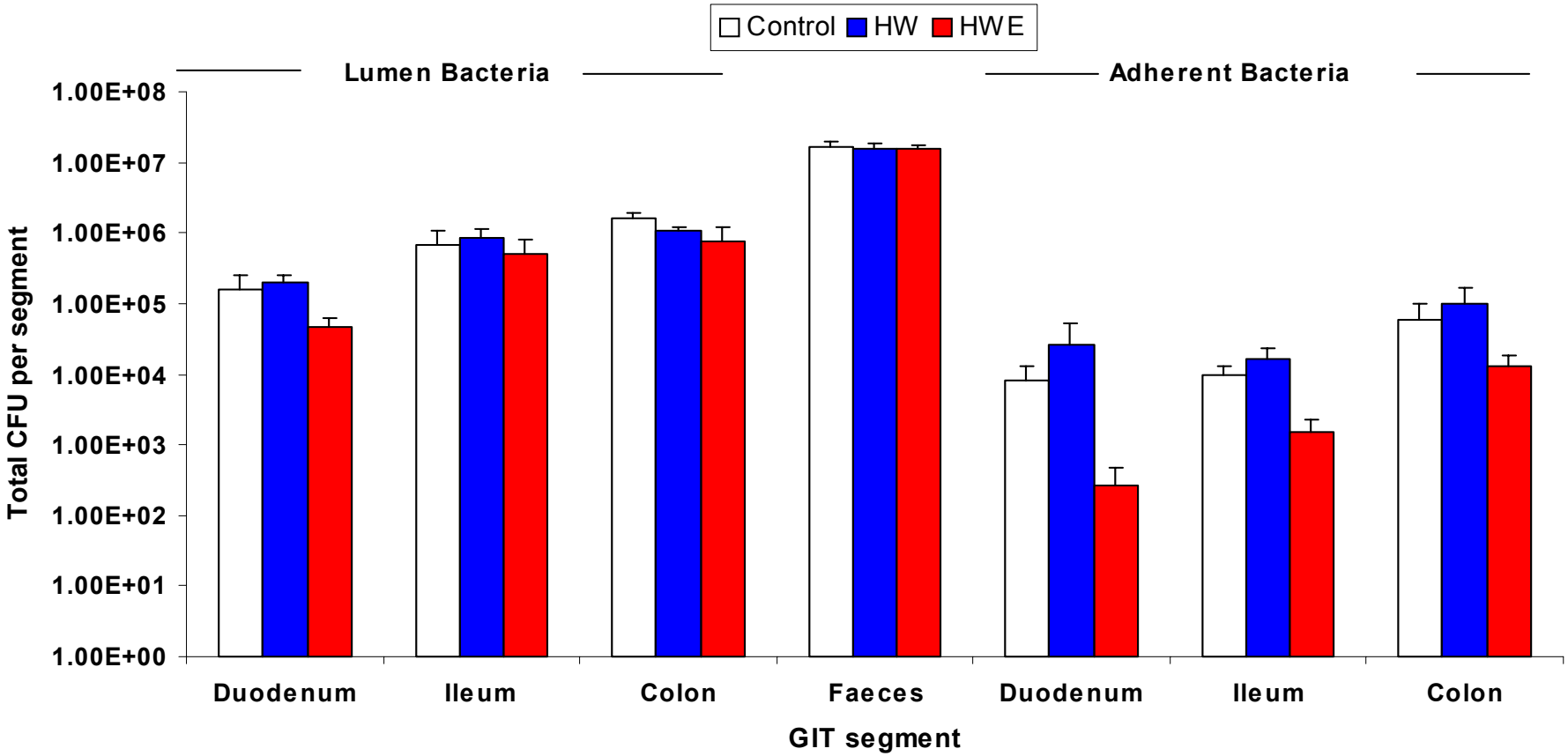


FIG 2.9 Effect of feeding extracts from the fruiting bodies of *Ganoderma* on lumen and adherent *Streptococci* bacterial populations recovered from intestinal segments and faeces of mice when enumerated on KEA agar. Mice were euthanized after 21 days of extract feeding. Bars represent crude (HW) and purified (HWE) *Ganoderma* extracts. Control (Con) mice were fed drinking water without extracts. Values are means ± standard error of 12 mice in each treatment group.

Lumenal populations in the colon of HW (1.08×10^6) and HWE (7.38×10^5) were not significantly different from those of the controls (1.63×10^6). Adherent streptococcal populations in the duodenum of HW (9.82×10^4) and HWE (1.28×10^4) treated mice did not show significant difference compared to controls (6.00×10^4).

HW treated mice (1.54×10^7) and HWE treated mice (1.59×10^7) did not show significant difference in their faecal streptococcal populations compared to the control group (1.63×10^7).

2.3.9 *Enterococcal* Enumeration

Figure 2.10 shows the mean total colony counts of lumen and adherent bacteria from the duodenum, the ileum and the colon after culture on *Enterococci* selective CATC agar following feeding of HW and HWE polysaccharide extracts from the fruiting body of *Ganoderma*.

Lumenal bacteria populations in the duodenum of HW (4.43×10^4) and HWE (4.00×10^4) treated mice did not show significant difference to the controls (1.13×10^4). Compared to HW and HWE treated mice that had no colonies, control mice (1.13×10^4) showed a significant amount of adherent enterococcal bacteria.

In the ileum, luminal bacteria of HW (3.87×10^5) and HWE (2.20×10^5) treated mice were not significantly different from the controls (5.04×10^4). No adherent enterococci from the ileum were observed on the CATC agar.

In the colon, no significant difference was observed in the average populations of enterococci in HW (2.30×10^5) and HWE (5.04×10^5) compared to control mice (8.69×10^5). Adherent populations of enterococci from the colon were not observed on the CATC agar.

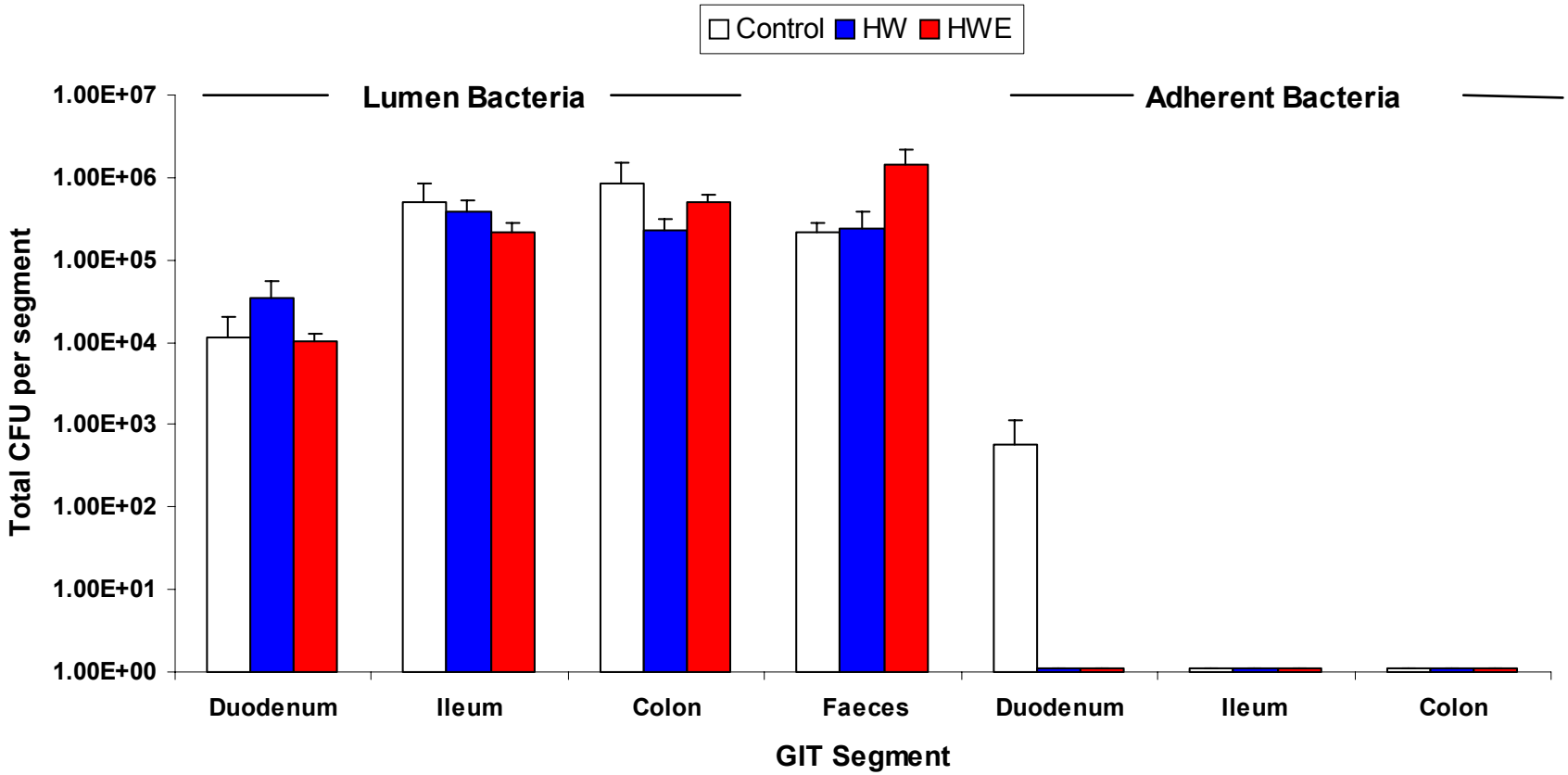


FIG 2.10 Effect of feeding extracts from the fruiting bodies of *Ganoderma* on lumen and adherent *Enterococci* bacterial populations recovered from intestinal segments and faeces of mice when enumerated on CATC agar. Mice were euthanized after 21 days of extract feeding. Bars represent crude (HW) and purified (HWE) *Ganoderma* extracts. Control (Con) mice were fed drinking water without extracts. Values are means ± standard error of 12 mice in each treatment group.

Enterococci populations in the faeces showed no significant difference between HW (2.42×10^5) and HWE (1.44×10^6) compared to controls (2.14×10^5). However, populations of enterococci in the faeces of HW treated mice were observed to be significantly different ($p = 0.03$) from that of the population in the HWE treated mice.

2.3.10 *Enterobacteriaceae* Enumeration

Figure 2.11 shows the mean total colony counts of lumen and adherent bacteria from the duodenum, the ileum and the colon after culture on *Enterobacteriaceae* selective MAC agar following feeding of HW and HWE polysaccharide extracts from the fruiting body of *Ganoderma*.

Lumen *Enterobacteriaceae* populations in the duodenum of HW (3.38×10^4) and HWE (1.17×10^4) treated mice were not significantly different from the controls (1.16×10^4). Compared to the control in which no *Enterobacteriaceae* were being observed, there was observed an increase in average populations of *Enterobacteriaceae* in the HW (1.69×10^3) treated mice. No *Enterobacteriaceae* were observed in the HWE treated mice.

Lumenal bacteria in the ileum of HW (5.89×10^5) and HWE (2.42×10^5) treated mice were not significantly different from those in controls (4.19×10^5). No adherent *Enterobacteriaceae* was observed in the ileum of the HW and HWE treated mice. Control mice showed an average population count of 2.93×10^4 CFU.

In the colon, lumenal bacteria of HW treated mice showed an average population count (2.88×10^5) that was significantly lower than the control group ($1.042.12 \times 10^6$) ($p = 0.017$). HWE treated mice (1.16×10^6) did not show significant difference compared to the controls. Adherent populations in HW

(3.66×10^2) and HWE (4.22×10^2) compared to the control group (3.94×10^2) did not show significant difference. However, populations of *Enterobacteriaceae* in the colon of HW treated mice were observed to be significantly different ($p = 0.028$) from that of the population of HWE treated mice.

Enterobacteriaceae populations in the faeces showed no significant difference between HW (4.52×10^5) and HWE (1.52×10^6) as compared to the controls (2.77×10^5).

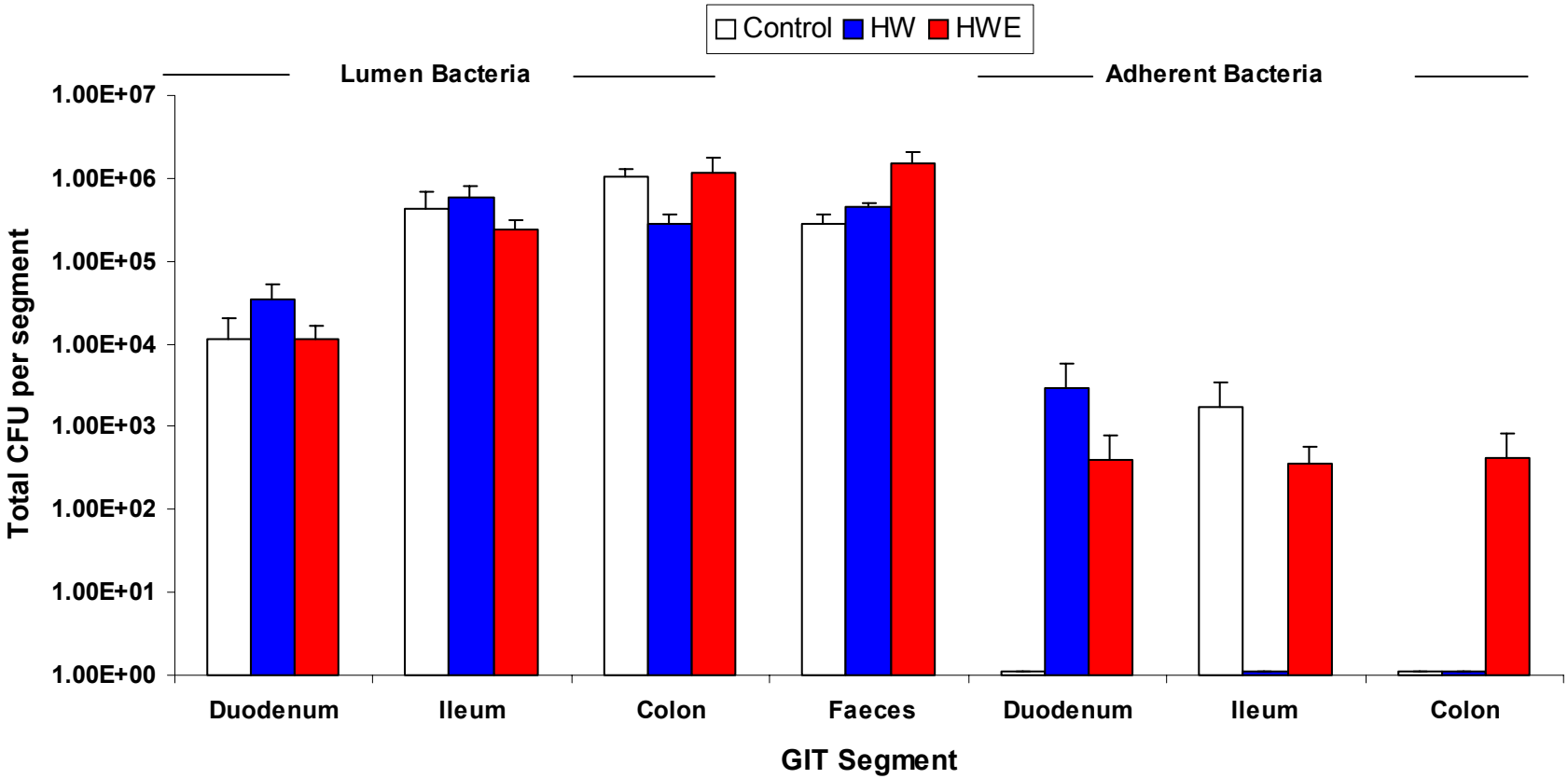


FIG 2.11 Effect of feeding extracts from the fruiting bodies of *Ganoderma* on lumen and adherent *Enterobacteriaceae* bacterial populations recovered from intestinal segments and faeces of mice when enumerated on MAC agar. Mice were euthanized after 21 days of extract feeding. Bars represent crude (HW) and purified (HWE) *Ganoderma* extracts. Control (Con) mice were fed drinking water without extracts. Values are means ± standard error of 12 mice in each treatment

2.4 Discussion

The present study was performed to investigate the composition of two different extracts made from *Ganoderma* and their biological effect on the gastrointestinal microflora. To assess the biological activity these polysaccharides might have, mice were fed with the extracted polysaccharides for a certain period of time. The main findings from this experiment were that the HWE extract seemed to be most successful in altering the population dynamics of the cultivable bacteria in the gut.

Majority of previous studies on activity of medicinal mushrooms have been undertaken with purified isolated components *in vitro* and by using animal models (Hsu *et al.,* 1997). Most of these have been carried out to demonstrate the anti-tumour and immune stimulation effects, but also other activities have been shown (Wasser and Weis 1999). None of these studies has, however, attempted to demonstrate the dietary modulator properties these mushrooms might have.

It is a well-established fact that resistance to pathogens and immune stimulation can be achieved by nutraceutical bacteria (Fooks *et al.,* 2002). Exogenous bacteria (i.e. probiotics) are the best known for this. However, the probiotics are confronted by a number of physical and chemical barriers upon ingestion. Thus only a small proportion of them are able to reach and establish themselves in the colon. Prebiotics, partly overcome these limitations by stimulating the growth and/or activity of a limited number of commensal bacteria in the colon. At the moment most searches for dietary modulators that show prebiotic activity is directed toward the growth of LAB producing microorganisms (Roberfroid, 2001). In the present study, however, the

mushrooms' effect on the pathogenic flora components was also evaluated. In addition, this study evaluated the population dynamics in the internal environment, compared to previous studies where microorganisms mainly have been isolated from faeces. It was further attempted to distinguish between transitory populations in the lumen and adherent populations that might have preferentially established a colonizing relationship with the intestinal surface. Such quantitative estimations are impossible to conduct without sacrificing animals and can only be carried out with smaller groups of experimental subjects. Hence, this is the first report ever seeking to compare the effects of different extracts made from *Ganoderma* on cultivable bacterial populations from distinct parts of the GIT, but also two distinct populations representing transitory (lumen) and colonizing (adherent) bacteria.

Data obtained from weight recordings showed that mice in all treatment groups gained weight over the duration of the experiment. No statistical significance was reached between the different treatment groups, however the trend was that the mice fed the extracts gained less weight than the controls. The treatment groups were too small to get reliable results for weight gain. To obtain more accurate data, a longer measuring period and bigger treatment groups could have been used. It is interesting to note, however, that the control group that gained the most weight consumed the least amount of water. Initially, there were some leaking problems due to pressure equilibration resulting in some water loss. Changing the water delivery system was found to fix this problem.

Enumeration of the frequency of haemolytic bacteria relative to total recoverable bacteria from either the lumen or adherent populations was

attempted on blood agar. Although haemolysis is a well known property of many pathogenic bacteria, no attempt was done to identify the haemolytic species. The trend was that the total numbers of bacteria were increased in mice treated with both HW and HWE extracts. However, the most interesting finding regarding promising beneficial health effect was the significant reduction in haemolytic lumen bacteria recovered from the colon of HWE treated mice. This suggests that haemolytic bacteria were more vulnerable to the action of HWE extract in the colon. Since haemolytic bacteria are considered to have a detrimental effect, a reduction in the frequency of haemolytic colonies may induce less stress on the gut mucosal immune system in having to counter the pathogenic strategies of potentially virulent bacteria. An important point to note from these findings is the amount of variation seen in total colony counts, and especially in the frequency of haemolytic bacteria from all GIT segments. Similar studies previously performed at EMAI did not have such variations. Hence, the differences are most likely due to operator subjectivity.

Overall adherent bacteria were always lower in numbers per intestinal segment compared to the lumen population. This result confirms that the washing protocols were sufficiently rigorous to remove non-adherent populations and that adherent populations could only be released with detergent treatment that did not affect the viability of the species isolated on selective media.

The use of selective media showed that the most abundant bacterial populations were members LAB and *Streptococci* groups. *Enterobacteriaceae*, represented by species such as *E. coli*, and *Enteroccocci* occurred at relatively lower frequencies. A positive finding from the bacterial enumeration

experiments was that the HWE extract induced a significant increase in the numbers of LAB in the colon compared to the controls. These findings are in agreement with previous studies regarding prebiotic activity, where a preferential growth of bacteria such as *Bifidobacteria* and *Lactobacilli* is encouraged following prebiotic feeding (Bouhnik *et al.*, 1996)

Chapter 3

Development of a Multiplex PCR Assay for the Characterisation of Colicinogenic Commensal *E. coli* Strains Inhibitory to Pathogenic *E. coli*

3. 1 Introduction

The primary role of the human gastrointestinal tract (GIT) is to digest food and to absorb nutrients. Gut microflora resident in the lumen as well as those that attach to epithelial cells that line involuting villi also play a major role in this process (Falk *et al.,* 1998). These commensal organisms can colonize various ecological niches in different compartments of the GIT. The lumen inhabitants are not in a free-living state but are usually attached to digesta as it progresses through the intestinal tube (Berg, 1996). Others colonize the mucin interphase while some have evolved specialized structures such as pili, fimbriae and adhesins that allow them to form intimate attachments with the enterocyte surface (Berg, 1996). The GIT microflora is vast, both quantitatively and in terms of diversity, with the presence of an estimated 400-500 different species of bacteria (Hart *et al.,* 2002). Consequently, these large complex bacterial communities have to compete with each other for nutrients and a microhabitat. There are several mechanisms used by bacteria in this antagonism. These include the competition for nutrients and attachment sites in the GIT or the production of anti-microbial compounds (Guarner and Malagelada, 2003).

E. coli is a major commensal of the normal GIT microflora of humans and other mammals (Nataro and Kaper, 1998). Paradoxically, *E. coli* is also capable of causing a wide variety of intestinal and extra-intestinal diseases such as diarrhoea, urinary tract infections, septicaemia and neonatal meningitis (Clermont *et al.,* 2000). Pathogenic *E. coli* and commensal *E. coli* typically differ with respect to phylogenetic background and virulence attributes. Extra-intestinal pathogenic *E. coli* strains derive chiefly from phylogenetic group B2

(and to a lesser extent group D), as defined by multilocus enzyme electrophoresis and Clermont polymerase chain reaction (PCR). In contrast, most commensal *E. coli* are characteristically from phylogenetic group A (Clermont *et al.,* 2000; Johnson and Stell, 2000). In addition, pathogenic *E. coli* possess specialized virulence factors that confer pathogenic potential and characteristically, are infrequent amongst commensal strains (Nataro and Kaper, 1998).

Colicins are bacteriocins, produced by *E. coli* and other members of the family *Enterobacteriaceae* such as *Shigella* and *Salmonella*, that specifically inhibit *E. coli* and closely related strains (Kuhar and Zgur-Bertok, 1999; Riley, 1998). Colicins are defined by (i) a narrow spectrum of inhibitory activity, (ii) the presence of an essentially active protein moiety, (iii) a bactericidal mode of action, (iv) attachment to specific receptors, (v) plasmid-borne genetic determinants of both production and host cell immunity, (vi) induced (SOS) release of the bacteriocin from the producer cell associated with death of that cell (Jack *et al.*, 1995). The characterized colicins, numbering over 20, can be divided into two major classes, the pore-former and nuclease colicins (Riley and Wertz, 2002). Pore-former colicins kill by creating channels in the cytoplasmic membrane whereas nuclease colicins kill by non-specific degradation of DNA or specific cleavage of RNA (Kleanthous and Walker, 2001; Stroud *et al.*, 1998). Under conditions of stress, such as nutrient depletion or overcrowding, a small proportion of colicin-producer cells in a population are induced to produce colicin resulting in rapid release of colicin into the environment (Eraso *et al.*, 1996; Kuhar and Zgur-Bertok, 1999).

Colicin-sensitive cells have colicin-specific receptors located in the outer membrane of the cell envelope and are killed by colicins that attach to those receptors. Cells may be insensitive to colicins because of (i) immunity, (ii) resistance, or (iii) tolerance mechanisms (Murinda *et al.*, 1996). Immunity is mediated by a homologous protein that binds the colicin, preventing the colicin from killing the target bacterial strain (Kleanthous *et al.*, 1998). Colicin resistant bacteria are defective in their capacity to adsorb a particular colicin, whereas tolerant bacteria adsorb the colicin but are blocked in some further step in the mode-of-action sequence (Riley and Gordon, 1999).

The anti-microbial properties of colicins make them excellent candidates for use by commensal bacteria as weaponry against pathogenic bacteria in the GIT. Several authors have identified colicin producing *E. coli* with the potential to inhibit pathogenic *E. coli*. Zhao *et al.*, (1998) reported the first use of colicin producing probiotics isolated from cattle that could inhibit pathogenic 0157:H7 *E. coli* in cattle. Recently, Schamberger and Diez-Gonzalez (2004) have reported the selection of 14 colicin producing *E. coli* strains inhibitory to 0157:H7. Despite these promising results, surveys of colicin production in enteric *E. coli* have relied on faecal isolates. Although the faecal microbiota appears to be a good qualitative indicator of the distal colonic microbiota, it does not reflect the intestinal microbiota as a whole, and certainly not that of the small intestine. Recently, Dixit *et al*. (2004) have demonstrated that different GIT regions represent different ecological niches for *E. coli* with clonal composition of upper GIT (duodenum, ileum) being significantly different from the lower GIT (colon, faeces). This is because strains of the same species are

more likely to have a similar ecological niche than are strains of different species, due to competition with each other for nutrients and a microhabitat.

Studies to demonstrate the existence of colicins have traditionally been based on functional assays involving agar-spotting and other overlay techniques (Pugsley and Oudega, 1987). These are laborious to undertake and frequently it is not possible to process multiple samples on a single plate at any one time. The molecular approach has accumulated a large number of colicin gene sequences including those that encode for the active peptide as well as factors responsible for immunity and receptor function. As a result, PCR techniques are increasingly being used as alternatives to these traditional methods. Nandiwada *et al.* (2004), and Schamberger and Diez-Gonzalez (2004) have successfully used this technique to screen for the presence of colicin genes in *E. coli* isolates. A welcome improvement to this method would be PCR multiplexing, a technique that has the advantage of allowing one to simultaneously detect more than one gene in an isolate.

Although PCR can identify which colicin gene is present and is capable of determining if multiple genes are present, PCR cannot determine which colicin protein, if any are present are being expressed. So far, a number of methods have been used to assess the inhibitory activity of colicinogenic bacteria against pathogenic bacteria *in vitro*. The agar well diffusion assay and agar spot test have been the most extensively used in the majority colicin antimicrobial assays (Murinda *et al.,* 1996; Schamberger and Diez-Gonzalez 2004). Whilst there is no doubt that this method is effective in identifying inhibitory bacterial strains against target bacterial strains, it does have some disadvantages. For example, this method is limited by the fact that large

numbers of plates have to be used for assessing such effects on a large panel of indicator strains as the producer strain is rarely tested against a limited number of indicator organisms. Secondly, there is the human error factor coming into play when quantifying zones of inhibition on a large number of agar plates. Finally, this assay is limited in that it yields qualitative rather than quantitative determination of activity. To address these deficiencies, there is need for a quantitative assay for assessing the anti-microbial activity of colicins.

3.1.1 Aims

Commensal *E. coli* are normally the primary colonizers of the porcine GIT. Pathogenic *E. coli* must compete against this population to survive. The competition between these 2 classes of *E. coli* in different intestinal compartments *in vivo* has never been examined. One of the main aims of this work was to develop an *in vitro* model to assess whether commensal isolates from the small and large intestine of the pig differ in their ownership of colicin genes and to compare this with pathogenic *E. coli* isolates known to cause post-weaning diarrhea (PWD) in pigs. Based on this information, the second aim was to analyze *in vitro*, the relationship between ownership of colicin genes and the functional capacity of an isolate to express these genes to gain a competitive advantage over pathogenic strains.

Multiplex PCR was the method applied to screen for colicin genes while the method called Kinetic Inhibitory Microtitre Assay or KIMA (I would like to acknowledge Sameer Dixit, a former PhD student at EMAI, for developing and optimised this assay) was used to evaluate the activity of different *E. coli* strains against other gram-negative pathogens. *E. coli* reference strains that have been

shown to carry plasmids harbouring colicin genes were initially assessed for their inhibitory activity against these pathogens using KIMA. The ability of commensal strains isolated from different porcine GIT compartments to antagonize growth of a selected pathogenic panel was also assessed similarly. The results obtained from this work will facilitate a better understanding of antimicrobial activity of commensal *E. coli* strains. Commensal strains showing high inhibitory ability can be scrutinized further to establish their effectiveness as probiotic strains.

3.2 Materials and Methods

3.2.1 Selection of Bacterial and Colicinogenic Strains

E. coli strains carrying previously characterized colicins (Table 3.1) were kindly provided by David Gordon (Australian National University, Canberra, Australia). These colicins were chosen to represent the two main groups with different mechanisms: those with hydrolysable targets in the cytoplasm (RNAse or DNAse), and those that form pores in the inner membrane. The DNAses were represented by colicins E2 and E7 while the RNAses were represented by colicins D and E6. Colicins A, E1, Ia and V represented the pore-formation group. In addition, according to Riley and Gordon (1999) these colicins are most likely to be found in *E. coli* isolates. Strain, BZB1011, known to be a non-colicin producer as well as colicin sensitive (Pugsley and Oudega, 1987) was included to be used as the negative control.

E. coli isolates used for colicin gene screening were kindly provided by Toni Chapman, Sameer Dixit and Xi-Yang Wu (Elizabeth McArthur Agricultural Institute (EMAI), Camden, Australia). They included a total of 152 commensal *E. coli* isolated from the duodenum, ileum, colon and faeces of the pig GIT (Appendix 3). Pathogenic *E. coli* isolates numbered 107 and were isolated from pigs with (PWD) (68 in total) and from humans with diarrhoea, haemolytic-uremic syndrome or urinary-tract infections (39 in total) (Appendix 3). These were chosen based on the following criteria:

- Possession of virulence genes or virulence gene combinations that are characteristic of the infective species
- Display of a pathogenic phenotype such as hemolysis
- Clinical strains isolated from diseased animals or humans.

Eight sets of primers that specifically amplify colicins A, D, E1, E2, E6, E7, Ia and V were published previously and their sequences are listed in Table 3.2. All eight sets of oligonucleotide primers were synthesized at Sigma-Aldrich (Castle Hill, Australia). The primers were made into 200 µl volumes in TE buffer and stored at –20 °C. The concentration of working primer solutions was made to 200 µM in TE buffer and stored at –20 °C.

Table 3.1. List of *E. coli* strains carrying plasmids encoding specific colicin genes. BZB1011 is a non-colicin producer and was included for use in negative controls

Strain	Colicin	Plasmid	Activity
BZB2101	**A**	**pColA-CA31**	**Pore-formation**
BZB2104	**E1**	**pColE1-K53**	**Pore-formation**
BZB2114	**Ia**	**pColIa-CA53**	**Pore-formation**
PAP222	**V**	**pColV-K270**	**Pore-formation**
BZB2125	**E2**	**pColE2-P9**	**DNAse**
BZB2110	**E7**	**pColE7-K317**	**DNAse**
BZB2103	**D**	**pColD-CA23**	**RNAse**
BZB2109	**E6**	**pColE6-CT14**	**RNAse**
BZB2011	**Nil**	**Nil**	**Nil**

Table 3.2. Primer pairs used for detection of colicin genes by PCR. Forward primers are represented by the letter f and reverse primers are represented as r.

Please see print copy for Table 3.2

3.2.2 Recovery of Bacteria and Preparation of Template DNA

All bacteria used in this study were stored frozen at –80 °C. These stocks were prepared by mixing 500 µl of an overnight culture of each bacterium with an equal volume of Luria broth (LB+30 % glycerol) in 1.5 ml eppendorf tubes (Sarstedt, Ingle farm, Australia). To recover the bacteria, a sterile toothpick was used to chip the surface of the –80 °C stocks and the chipped piece spread on the surface of a Luria agar (LA) plate using a sterile wire loop. The plates were then incubated for 16 hours at 37 °C.

Template DNA was prepared according to Johnson and Brown (1996). A single freshly grown colony was picked from a LA plate with a sterile toothpick and resuspended in 2 ml LB in 5 ml falcon tubes (Sarstedt, Ingle farm, Australia). The tubes were then incubated for 16 hours (37 °C, 200 rpm) after which 1 ml of the overnight culture was aliquoted into 2 ml thick walled eppendorf tubes (Sarstedt, Ingle farm, Australia). The bacterial cells were recovered by centrifugation (14,000 rpm, 3 min) in a bench centrifuge (Eppendorf, 5415D, Westbury, USA). The supernatant was discarded and the pellet re-suspended in 200 µl milli Q H_2O (MQ H_2O) by gentle vortexing. The tubes were then placed on a heat block (Thermolyne, Australia) and heated at 102 °C for 15 min after which they were stored on ice for 5 min. Cellular debris was pelleted by centrifugation (14,000 rpm, 3 min) in a bench centrifuge (Eppendorf, 5415D, Westbury, USA). 180 µl of the boiled lysate was aliquoted into sterile 1.5 ml eppendorf tubes (Sarstedt, Ingle farm, Australia) and stored at –20 °C until use

Template DNA for commensal and pathogenic *E. coli* was kindly provided by Toni Chapman and Xi-Yang Wu (EMAI).

3.2.3 Validation of Primer Pairs

Primers for each colicin gene were first validated by use of template DNA from each colicin reference strain. The products of the boiled lysate were used for the template DNA for colicins A, D, E1, E2, E6, E7, Ia and V. Reaction mixtures contained 2 μl template DNA 5 μl 10 X PCR buffer, 1.5 mM $MgCl_2$, 200 μM each of 4 dNTPs (dATP, dCTP, dGTP, dTTP), 50 pmol of each primer and 0.2 units of Taq polymerase. The volume was made up to 50 μl using MQ H_2O. The 10 X PCR buffer, dNTPs, $MgCl_2$ and Taq polymerase were all purchased from Invitrogen (Victoria, Australia). Control reactions with template DNA from non-colicin producer strain BZB1011 were also included.

3.2.3.1 PCR Amplification Conditions

PCR amplification conditions for colicins A, E1, E2, E6, E7 and Ia followed the method of Gordon *et al.* (1998) and consisted of: 5 cycles of 92 °C for 10 s, 40 °C for 2 min and 72 °C for 1.5 min; then 35 cycles of 92 °C for 5 s, 55 °C for 25 s and 72 °C for 1.5 min; and 1 cycle of 92 °C for 10 s, 45 °C for 20 s and 72 °C for 5min.

The amplification conditions for colicin D followed the method of Hofinger *et al.* (1998). The conditions were: 1 cycle of 4 min at 94 °C followed by 30 s at 94 °C; 1.5 min at 57 °C and 1 min at 72 °C for 30 cycles; and a final extension step at 72°C for 5 min.

Colicin V gene was amplified according to the method of Johnson and Stell (2000) and consisted of: 1 cycle of 4 min at 94 °C followed by 30 s at 94 °C; 30 s at 63 °C and 3 min at 72 °C for 25 cycles; and a final extension step at

72 °C for 10 min. The PCR reactions were carried out in an automated thermal cycler (PC-960, Corbett research, Sydney, Australia).

3.2.3.2 Visualization of PCR Products by Gel Electrophoresis

A 1 % agarose gel was prepared by dissolving 1 g agarose (Seakem, Australia) in 100 ml 0.5 X TBE buffer (5.4 g/L Trisbase, 2.75 g/L Boric Acid, 2 ml/L 0.5M EDTA pH 8, in 1 L MQ H_2O). The agarose was melted in a microwave, and then cooled in a 65 °C water bath for 5 min. Ethidium bromide (3.5 µl of 10 mg/ml) was added to the agarose and mixed using a magnetic stirrer. The gel was poured, well combs put in place and left for 1 hour at room temperature to set. The gel was then submerged in 600 ml 0.5 X TBE buffer. DNA samples (3 µl) were mixed with 1 µl loading dye (bromophenol blue 0.25 % with 15 % ficoll) and loaded onto the gel.

Gels were then run under constant electrophoretic conditions of 80 volts, 500 milli-amps for 2.5 hours. The gels were then photographed by use of an ultraviolet transilluminator and digital capture system (Gel Doc; Biorad, Hercules, California). The sizes of the amplicons were determined by comparing them with λ/*Hind* III (MBI fermentas, Hanover, USA) and 100-bp DNA ladder (Gibco/BRL, Gaithersburg, USA) markers run in lanes on the same gel.

3.2.4 Multiplex PCR

A multiplex PCR was designed for the simultaneous amplification of all colicin genes. This has the advantage of detecting more than one colicin gene at once, making the technique not only faster but also time saving especially

during screening of these genes in a large number of *E. coli* isolates. DNA templates and primer pairs were sorted and mixed into three pools according to primer compatibility and amplicon sizes (Table 3.3). This was accomplished using the AMPLIFY programme (Department of Genetics, University of Wisconsin, USA), which showed that there were no primer dimers and that these primers were compatible for each of the three pools. Each primer pool was first validated by use of pooled control DNA containing all relevant colicins. Reaction mixtures contained 2 μl of pooled template DNA, 5 μl 10 X PCR buffer, 1.5 mM or 3.0 mM $MgCl_2$, 200 μM each of 4 dNTPs (dATP, dCTP, dGTP, dTTP), 50 pmol of each primer and 0.2 units of HotStarTaq polymerase. All reagents were purchased from Qiagen (Victoria, Australia). The volume was made up to 50 μl using MQ H_2O. Control reactions included DNA from the non-colicin producer strain BZB1011.

Table 3.3. Primer pools used for multiplex colicin gene polymerase chain reaction assay sorted according to compatibility and amplicon size.

Multiplex Pool 1	size (bp)	**Multiplex** Pool 2	size (bp)	**Multiplex** Pool 3	size (bp)
Col E2	2221	Col Ia	2510	Col E6	862
Col E7	1894	Col A	2025	Col V	680
		Col E1	650	Col D	587

The new PCR conditions were 1 cycle of initial denaturation at 94 °C for 4min, followed by denaturation at 94 °C for 30 s; annealing at 55 °C or 60 °C for 1 min; extension at 72 °C for 3 min for 30 cycles and 1 cycle of final extension at 72 °C for 5 min. The PCR reactions were carried out in an automated gradient thermal cycler (PC-960G, Corbett research, Sydney, Australia) and the PCR amplicons visualized on 1 % agarose gel as outlined before.

3.2.4.1 Reassessment of Multiplex PCR Conditions

The multiplex PCR amplification conditions were improved by varying the concentrations of $MgCl_2$ and primers. The $MgCl_2$ concentrations were adjusted to 2 mM for pool 1, 4 mM for pool 2 and 3 mM for pool 3. In pool 1, the primer pair concentrations for colicins E2 and E7 were reduced from 50 pmol to 25 pmol while in pool 2 the primer pair concentrations for colicins E1 and Ia were increased from 50 pmol to 100 pmol. The primer pair concentrations for colicin A remained unchanged at 50 pmol. In pool 3, the primer pair concentrations for colicin V remained unchanged at 50 pmol while those for colicins D and E6 were adjusted to 5 pmol. These conditions are summarised in Table 1 (Appendix 4). In addition, HotStarTaq polymerase was used in place of normal Taq polymerase.

The cycling protocol was performed in an automated thermal cycler (PC960, Corbett Research, Sydney, Australia) using the following conditions: 1 cycle of 15 min at 95 °C to activate the HotStarTaq polymerase, then 30 cycles that each consisted of denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min, and extension at 72 °C for 3 min. The samples were then heated at 72 °C

for 5 min for the final extension reaction. The PCR amplicons were visualized on 1 % agarose gel as outlined before.

3.2.5 Preparation of Colicin Supernatant Extracts from Reference and Producer Strains

Growth of all *E. coli* strains representing colicin E1, E2, E7 and Ia reference strains and seven commensal colicin producers was carried out using a modification of the method described by Gordon *et al.* (1998). Strains were propagated in 4 ml LB from single colonies on plate culture. After growth for 18 hours under shaking (37 °C, 200 rpm), 2 ml of the overnight culture was inoculated into 20 ml LB medium in a 50 ml falcon tube (Sarstedt, Ingle farm, Australia). Tubes were shaken for 1 hour in an orbital shaker (37 °C, 200 rpm), and 0.2 μg of mitomycin C (Sigma, St. Louis, USA) per ml was added. Control cultures did not receive any mitomycin C. Cultures were then shaken for an additional 4 hours.

Culture supernatant was prepared by first centrifuging bacterial culture at 10,000 rpm for 20 min (Beckman, Gladesville, Australia). The culture supernatant was then filtered through a 0.22 μm membrane (Sartorius, Waverley, Australia) and stored at –80 °C. Maintenance of supernatants at this temperature eliminates the possibility of phage contribution to any observed inhibitory activity (David Gordon, personal communication).

3.2.6 Growth of Indicator Strains

All isolates were purity streaked onto LB Agar plates. A single freshly grown colony was inoculated onto 1 ml LB broth culture and grown overnight with agitation (37 °C, 200 rpm). Following overnight incubation, 200 µl of the culture was inoculated into 10 ml fresh LB broth and allowed to incubate under shaking until the Optical Density (OD) at an absorbance of 650 nm reached 1. An initial dilution series was carried out on the cultures for drop plating to confirm bacterial Colony Forming Units (CFU) per ml at OD of 1 at an absorbance of 650 nm. Bacterial CFU was adjusted to the required concentrations following optimization assays for all subsequent analysis.

3.2.7 Experimental Design for Assay

The assay utilized sterile 96-well microtitre plates (Figure 3.1). The target bacteria CFU was adjusted to a starting density of $1x10^7$ CFU/well by diluting in LB and 75 µl of these bacterial cells were seeded onto wells of 96-well microtitre plate. Next, 75 µl of 1 % LB-low melt agarose (Seakem, Waverley, Australia) was added on top of the indicator bacteria cells and the microtitre plate incubated for 15 min at 4 °C in a fridge to allow the agarose to set.

The colicin supernatants were recovered from the –80 °C stocks and allowed to thaw at room temperature. 50 µl of the supernatants were used as top overlay using undiluted (neat) and diluted supernatant (1/5, 1/10 and 1/20). The dilutions were carried out using LB media. Gentamycin sulphate (Sigma Aldrich, St. Louis, USA) supplemented LB broth was used as an internal control at a concentration of 50 µg/ml. The target bacteria were also overlaid with LB

media to ensure that O.D. readings truly reflected only the growth of bacteria. Plate design facilitated use of quadruplet wells for each treatment condition.

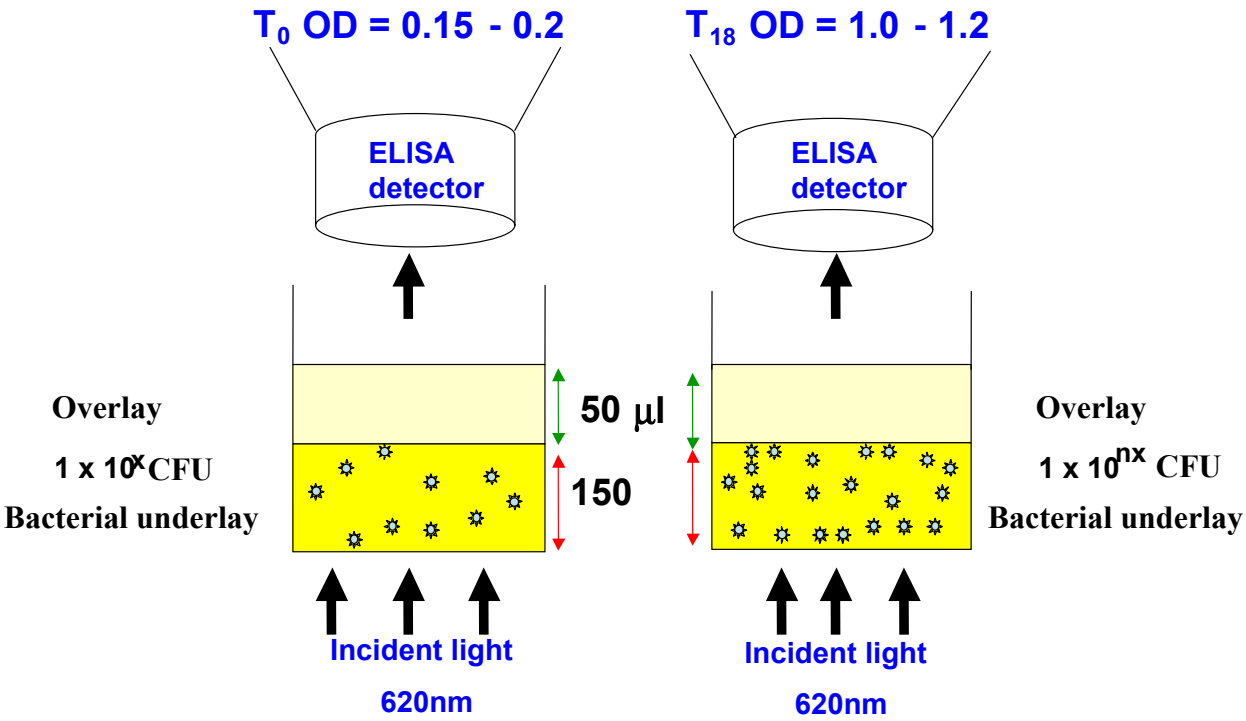


FIG 3.1 Schematic diagram of assessment of growth of bacteria in a microtitre plate well using an ELISA detector. The longitudinal cross section of the well on the left represents 0 hour reading while the same on the right represents 18 hour reading. The initial bacterial CFU is designated as 1 x 10^x where "x" refers to 7. The final CFU is designated as 1 x 10^{nx} where "nx" refers to a final CFU value. The top overlay is set 50 µl whilst the bottom bacterial underlay is fixed at 150 µl final volume. The amount of bacterial cells in the wells is turbidimetrically assessed under a wavelength of 620 nm.

3.2.8 Calculations to Assess Effect of Bacterial Supernatant

Since mitomycin C was present in some treatment conditions but not in controls, two sets of calculations had to be carried out to accommodate these two parameters.

Growth of bacterial strains in control wells not containing mitomycin C were identified as G_{Lb}, where

$$G_{Lb(-M)} = G18_{(-M)} - G0_{(-M)}$$

$$G_{Lb(+M)} = G18_{(+M)} - G0_{(+M)}$$

Growth of bacterial strains in treatment wells containing mitomycin C were identified as G_{CS}, where

$$G_{CS(-M)} = G18_{(-M)} - G0_{(-M)}$$

$$G_{CS(+M)} = G18_{(+M)} - G0_{(+M)}$$

In all conditions, M refers to mitomycin C and "+" or "-" refers to presence or absence of mitomycin C in growth culture. Growth inhibition (% GI), if applicable, for each test culture supernatant was expressed as follows, and this calculation was applied separately to treatment and control groups:

$$\% \text{ GI} = [(G_{LB} - G_{CS}) / G_{LB}] \times 100$$

The presence or absence of mitomycin C in broth medium was taken into account when calculating % inhibition or promotion. Values from treatment containing mitomycin C were calculated against those obtained from respective controls containing mitomycin C. Values from treatments not receiving mitomycin C were calculated against those obtained from respective controls not receiving mitomycin C. The contribution of mitomycin C in the overall inhibitory effects of treatments could be negated this way.

3.3 Results

The first task of this chapter was to develop and validate a multiplex PCR assay that would correctly amplify eight common colicin genes. This technique would then be applied to assess the prevalence of these colicin genes in commensal and pathogenic *E. coli* clones. The ability of colicin reference strains to inhibit growth of a known colicin sensitive strain *in vitro* was assessed using KIMA. Culture supernatants from reference strains were used as overlays in the assay with selected *E. coli* strains used as target underlay. Supernatants were obtained from two sets of *E. coli* growth cultures- those that were propagated in the presence of mitomycin C, and those that were not. Based on the results obtained, the ability of four reference *E. coli* strains to antagonize growth of five pathogenic *E. coli* strains was assessed. KIMA was once again used as a tool to assess the ability of the supernatants from the reference strain to inhibit growth of the selected pathogens. Further, supernatants from porcine commensal *E. coli*, isolated from different GIT sections was assessed for possible inhibitory ability against the same panel of selected *E. coli* pathogens. This was carried out with the view of identifying possible colicin producer commensal strains with antimicrobial ability.

3.3.1 Validation of Primer Pairs

As a first step, to ensure that the newly synthesized primer pairs were working correctly, each primer pair was tested against its control template DNA. Primer pairs for each of the eight colicin genes were validated by assessing the size of amplicons generated following PCR. The correct expected amplicon size for each gene is shown in Table 3.2. Visualisation of the PCR products by agarose

gel electrophoresis revealed that these genes were correctly amplified and that the products resolved were of the expected sizes (Figure 3.2).

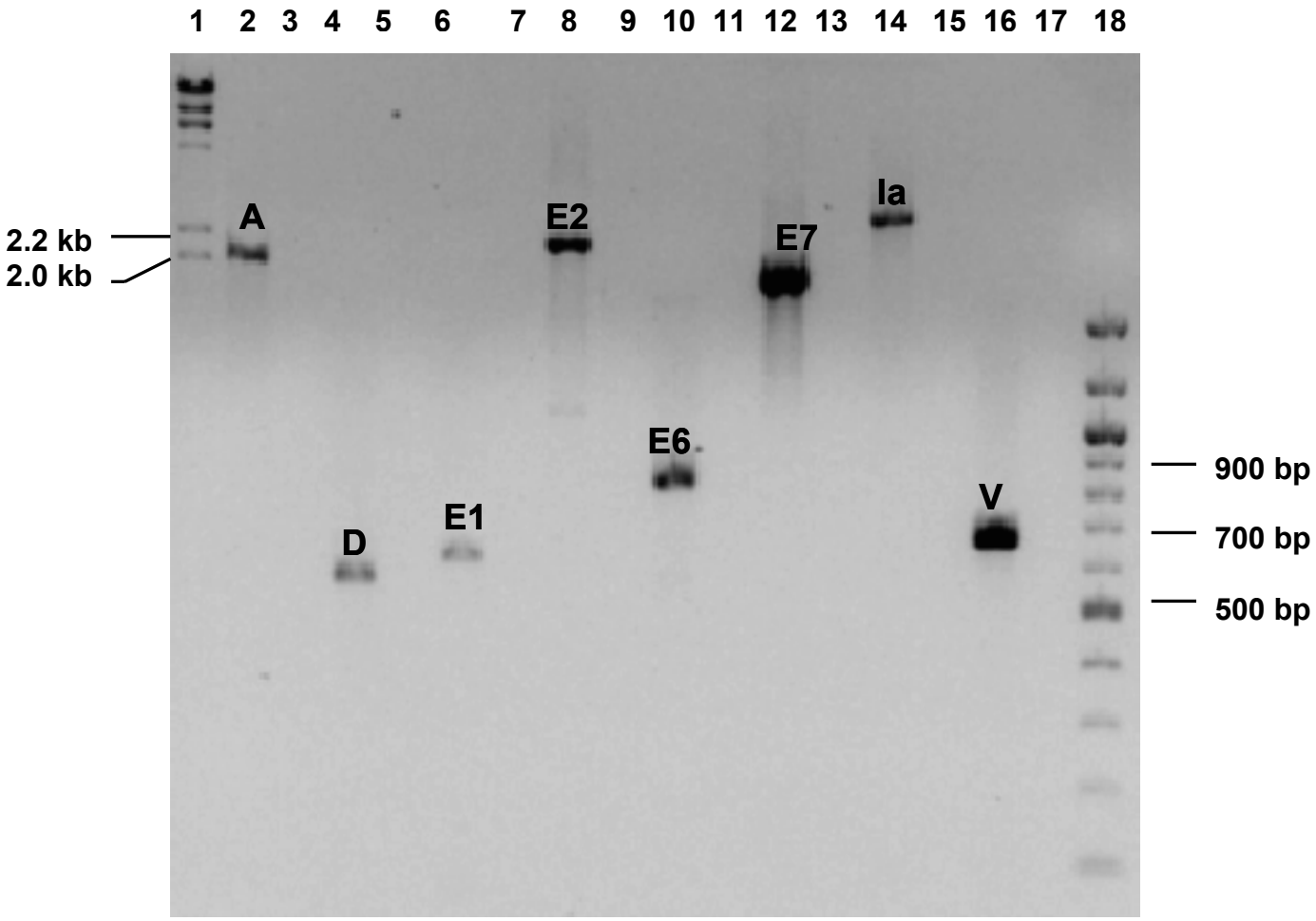
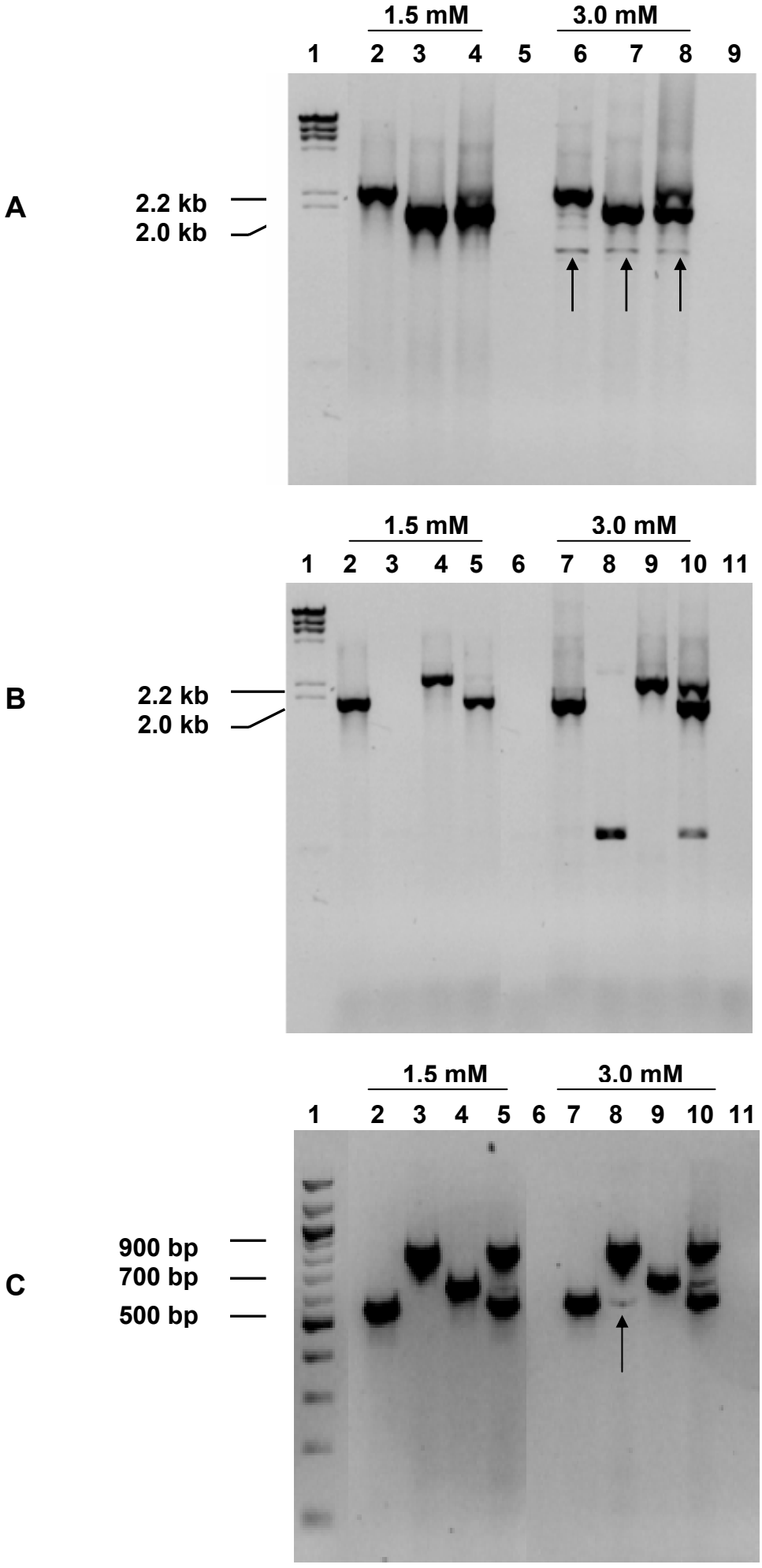


FIG 3.2 Validation of primer pairs used for the PCR amplification of colicin genes. The DNA templates were derived from released genomic DNA and were amplified as described in the methods section. PCR amplicons for colicins A, D, E1, E2, E6, E7, Ia and V are shown in lanes 2, 4, 6, 8, 10, 12, 14 and 16 respectively. Lanes with 3, 5, 7, 9, 11, 13,15 and 17 are negative controls using DNA template derived from BZB1011. λ/Hind III and 100 bp molecular weight markers are shown in lanes 1 and 18respectively

3.3.2 Multiplex PCR

Following confirmation that primers pairs for all eight colicin genes were working correctly, the next task was to sort these primer pairs into separate pools that would allow their simultaneous amplification. DNA templates and primer pairs for each colicin gene were sorted and mixed into three pools according to primer compatibility and amplicon sizes (Table 3.3). The initial amplification conditions were found to be non-optimal with preferential amplification of some target sequences over others and also presence of non-specific products (Figure 3.3). In pools 1 and 3, non-specific PCR products were present (Figure 3.3-A and Figure 3.3-C respectively) while in pool 2, PCR product bands for colicins E1 and Ia were missing in the presence 1.5 mM $MgCl_2$ (Figure 3.3-B). Possible factors responsible for this include suboptimal concentrations of $MgCl_2$ and primer pairs in the PCR reactions. To address these problems, the concentrations of primer pairs and $MgCl_2$ in each pool were varied since it has been found that alteration of these two components in multiplex PCR usually results in considerable improvement in the specificity of the test (Markoulatos *et al.,* 2002). By changing the two parameters as described in section 3.2.4.1 in the Materials and Methods, all desired products could be amplified in three separate PCR reactions and the expected product sizes from each multiplex reaction could be resolved by size in gel electrophoresis (Figure 3.4). In addition, non-specific PCR products were eliminated in all three pools. This optimized PCR technique was then used to survey the prevalence of colicin genes in commensal and pathogenic *E. coli* clones.

FIG 3.3 Amplification products obtained by multiplex PCR using primers sorted into three pools according to primer compatibility. Pooled genomic DNA was amplified in the presence of 1.5 mM or 3.0 mM $MgCl_2$ **(A)** Multiplex pool 1 for amplification of colicin E2 and E7. Lanes 4 and 8 are pooled DNA, lanes 2, 6, are E2 controls while lanes 3, 7 are E7 controls included to show successful amplification of these genes in the multiplex pool. Lanes 5 and 9 are negative controls using DNA template from BZB1011. Non-specific PCR products are indicated by arrows **(B)** Multiplex pool 2 for amplification of colicin A, E1 and E7. Lanes 5 and 10 are pooled DNA, lanes 2, 7 are A controls, 3, 8 are E1 controls, 4, 9 are Ia controls included to show successful amplification of these genes in the multiplex pool. Lanes 6 and 11 are negative controls using template DNA from BZB1011. **(C)** Multiplex pool 3 for amplification of colicin D, E6 and V. Lanes 5 and 10 are pooled DNA, lanes 2, 7 are D controls, 3, 8 are E6 controls, 4, 9 are V controls included to show successful amplification of these genes in the multiplex pool. Lanes 6 and 11 are negative controls using template DNA from BZB1011. Arrow indicates non-specific PCR product. λ/Hind III molecular weight marker is shown in lane 1(Figures A and B) while lane 1 (Figure C) shows 100 bp molecular weight marker.


A
1.5 mM
3.0 mM
1 2 3 4 5 6 7 8 9
2.2 kb
2.0 kb
B
1.5 mM
3.0 mM
1 2 3 4 5 6 7 8 9 10 11
2.2 kb
2.0 kb
C
1.5 mM
3.0 mM
1 2 3 4 5 6 7 8 9 10 11
900 bp
700 bp
500 bp

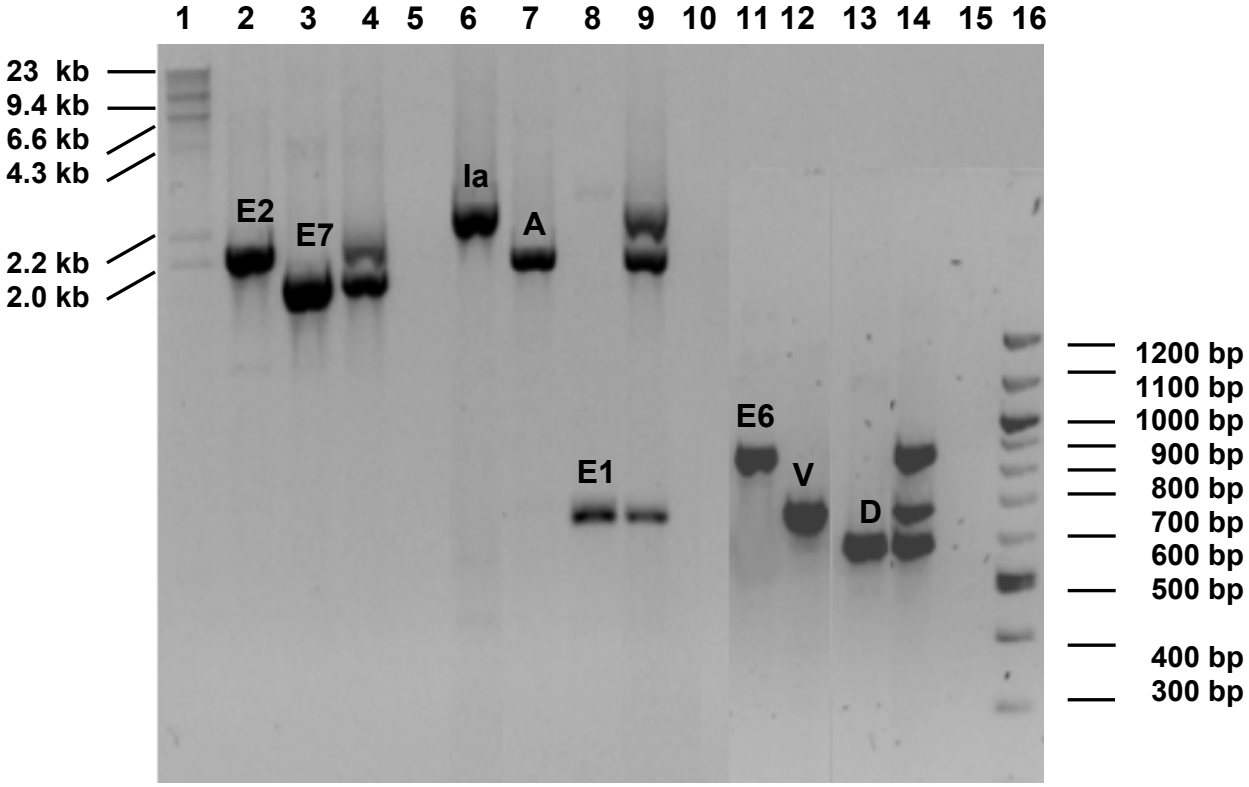


FIG 3.4 Agarose gel showing colicin amplicons represented by three multiplex PCR pools shown in lanes 4, 9 and 14. Lane 4 represents multiplex reaction for colicins E2 and E7, lane 9 represents multiplex reaction for colicins A, E1 and Ia and lane 14 shows the multiplex reaction for colicins D, E6 and V. Single PCR amplicons showing the correct sizes for each multiplex pool are shown in lanes 2, 3, 6, 7, 8, 11, 12 and 13. λ/*Hind III* marker and 100bp ladder DNA markers are shown in lanes 1 and 16 respectively. Lanes 5, 10 and 15 are negative controls using DNA template from BZB1011.

3.3.3 Prevalence of Colicin Genes in Porcine Commensal *E. coli* Isolates

Application of the multiplex PCR technique to screen for the eight colicin genes in the 152 commensal isolates revealed that in total, about 36 % of all the commensal clones were positive for a colicin gene (Figure 3.5). The frequency of these colicin genes in the different GIT segments ranged from 30 % in the

duodenum to 51.3 % in the ileum. The frequency in the colon and faeces was 35.9 % and 38.6 % respectively.

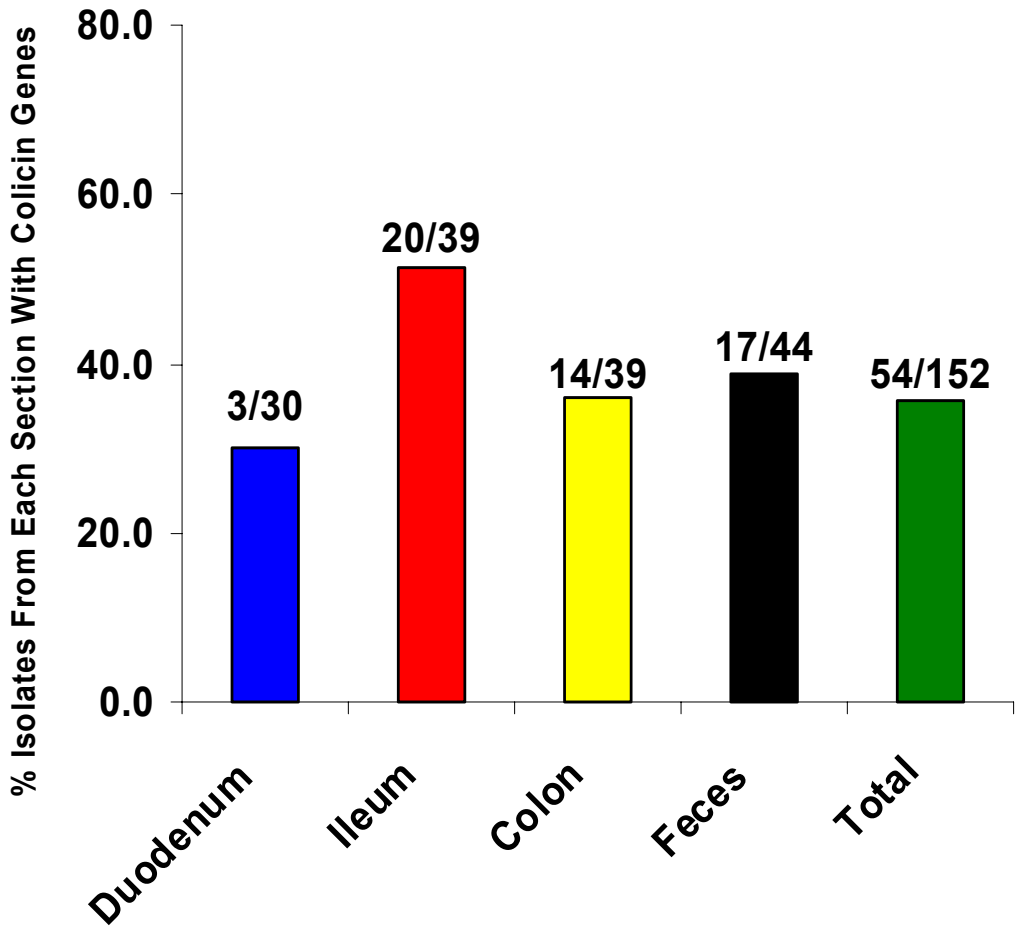


FIG 3.5 Frequency of colicin bearing commensal clones from each gastrointestinal compartment. The compartments represented were the duodenum, ileum, colon and faeces. Values above bars are number of clones positive for a colicin gene out of the total number of clones in that GIT compartment.

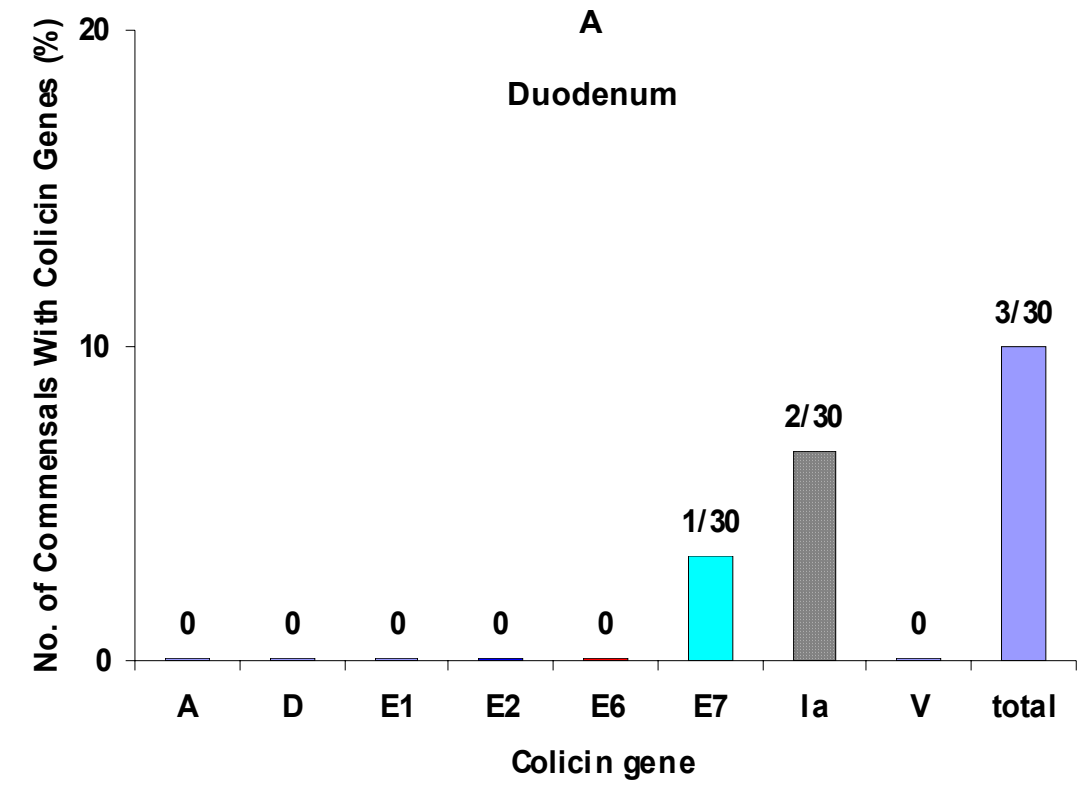
3.3.4 Prevalence of Colicin Genes in Different Intestinal Compartments

Approximately 10 % of clones in the duodenum possessed colicin genes (Figure 3.6-A). Of these, 6.7 % carried colicin Ia while 3.3 % carried colicin E7. In the ileum, about 51.3 % of clones were found to possess colicin genes with colicins Ia (20.5 %) and E1 (15.4 %) being the most common (Figure 3.6-B). Colicin E7 occurred at a lower frequency of 7.7 %. In addition, three multiple colicins, E1/E2, E2/E7 and E2/Ia were found in about 2.6 % of clones.

In the colon, around 35.9 % of clones carried a colicin gene (Figure 3.7-A). Colicins E1 and E7 were found in about 12.8 % of clones respectively whereas colicin Ia occurred at a lower frequency of around 2.6 %. One multiple colicin, E1/E7 was found in about 5.2 % of clones. In the faeces, approximately 38.6 % of clones possessed a colicin gene (Figure 3.7-B). Of these, colicins E1 and E7 were the most common occurring in frequencies of 13.7 % and 22.7 % respectively. Finally, one multiple colicin, E2/E7 was found in 2.3 % of clones.

Of the 54 porcine commensal isolates positive for a colicin gene, the most common colicin types detected were E1 and E7 (Table 3.4). The two colicins represented 31.5 % and 35.2 % of all colicin types detected in these isolates. The next most common colicin was colicin Ia (20.3 %) with colicins E2, E1/E2, E1/E7, E2/E7 and E2/Ia occurring at much lower frequencies of between 1.9 % and 3.8 %. Colicin E7 was the only colicin to be detected in all four intestinal sections while colicins E1 and Ia were present in all compartments except the colon and faeces respectively. The remaining colicin types were present in only one or two sections.

FIG 3.6 Frequency of colicin genes in commensal *E. coli* isolates from the duodenum and ileum. Values above bars represent number of isolates positive for a colicin gene out of a total of 30 in the duodenum **(A)** and 39 in the ileum **(B)**.


A
Duodenum
No. of Commensals With Colicin Genes (%)
20
10
0
0
0
0
0
0
1/30
2/30
0
3/30
A
D
E1
E2
E6
E7
Ia
V
total
Colicin gene

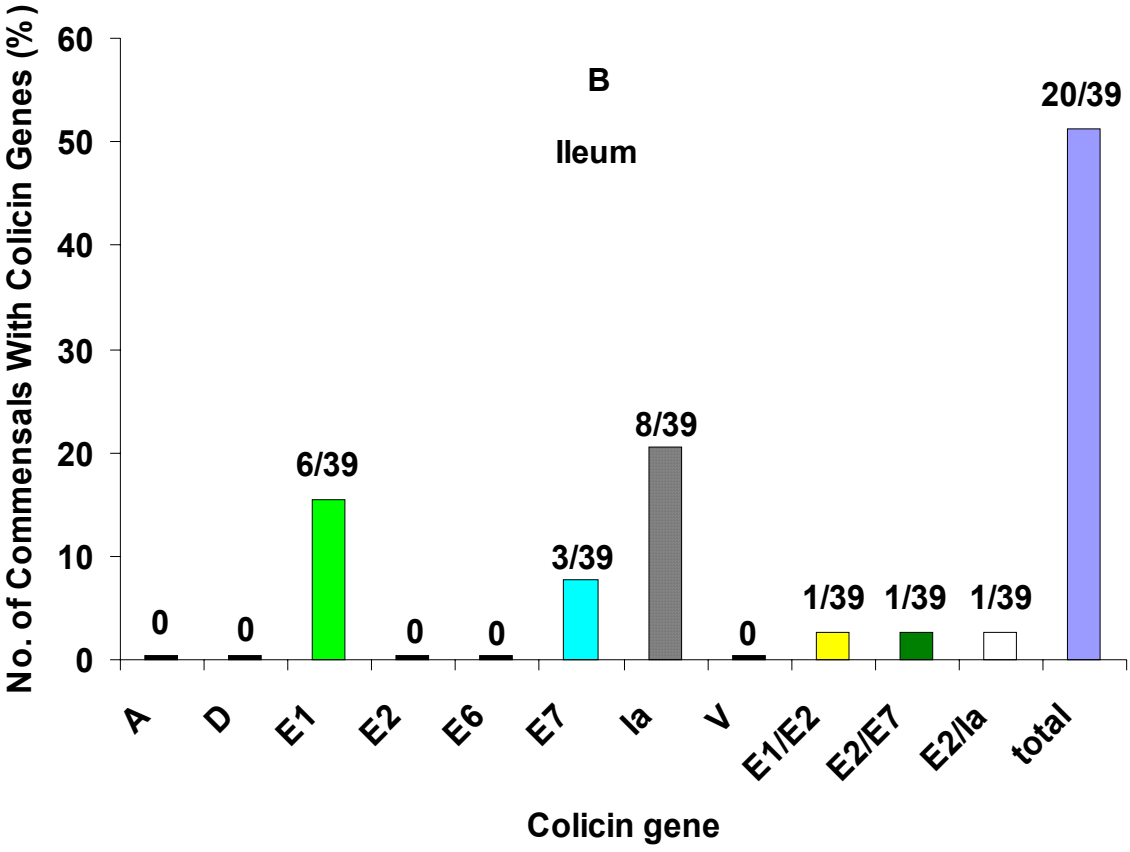
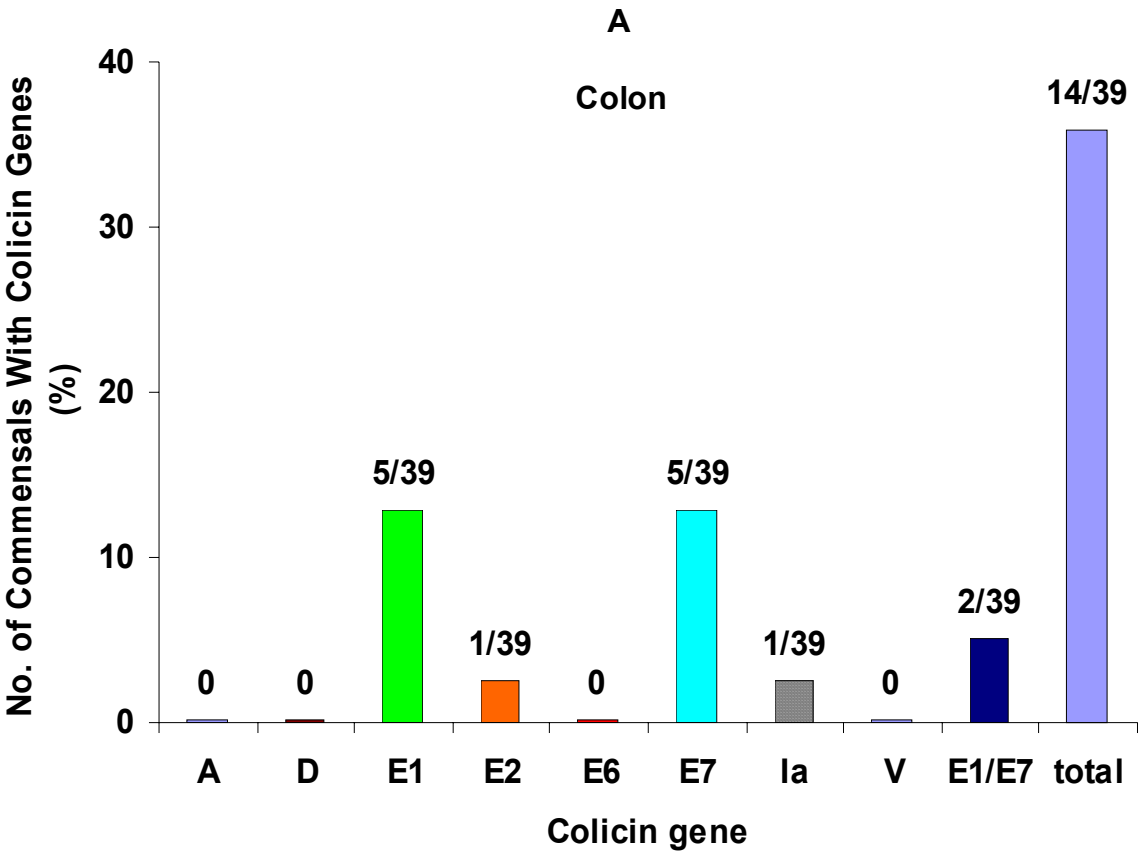

B
Ileum
No. of Commensals With Colicin Genes (%)
60
50
40
30
20
10
0
0
0
6/39
0
0
3/39
8/39
0
1/39
1/39
1/39
20/39
A
D
E1
E2
E6
E7
Ia
V
E1/E2
E2/E7
E2/Ia
total
Colicin gene

FIG 3.7 Distribution of colicin genes in commensal *E. coli* isolates from the colon and faeces. Values above bars represent number of isolates positive for a colicin gene out of a total of 39 in the colon **(A)** and 44 in the faeces **(B)**.


A
Colon
No. of Commensals With Colicin Genes (%)
40
30
20
10
0
0
0
5/39
1/39
0
5/39
1/39
0
2/39
14/39
A
D
E1
E2
E6
E7
Ia
V
E1/E7
total
Colicin gene

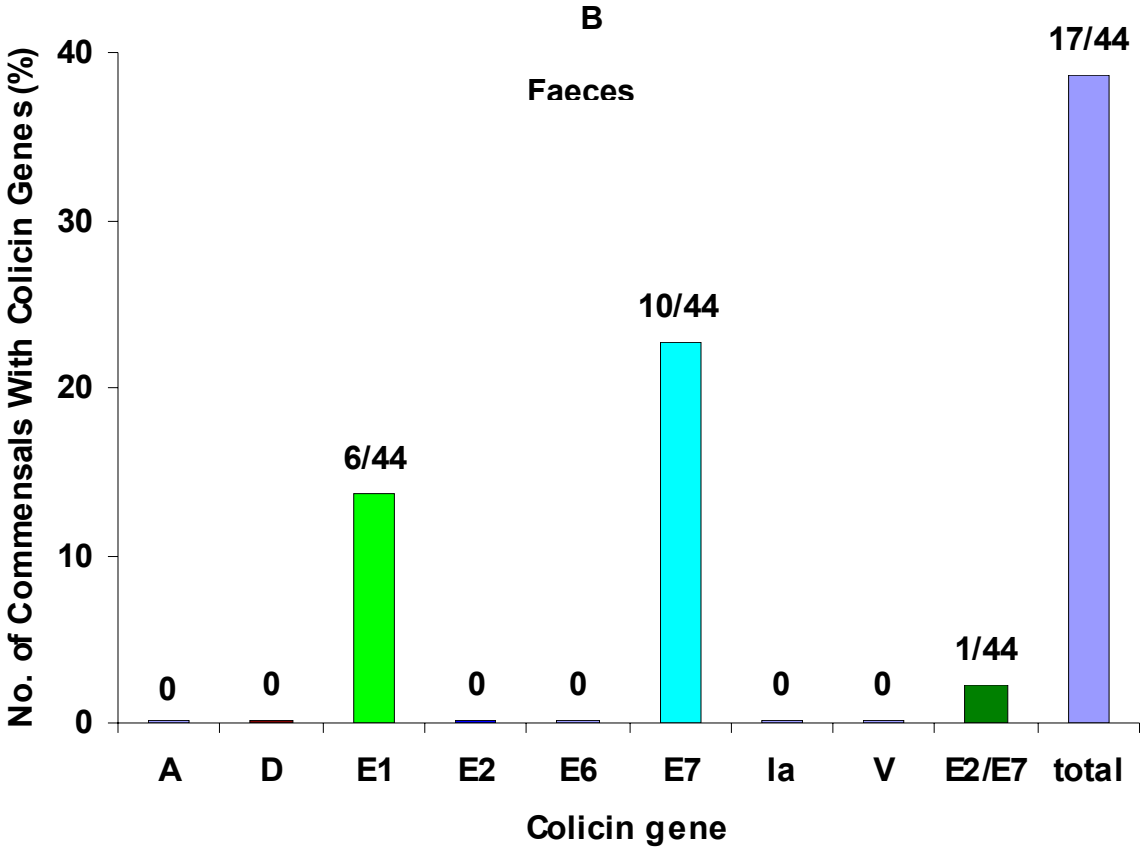

B
Faeces
No. of Commensals With Colicin Genes (%)
40
30
20
10
0
0
0
6/44
0
0
10/44
0
0
1/44
17/44
A
D
E1
E2
E6
E7
Ia
V
E2/E7
total
Colicin gene

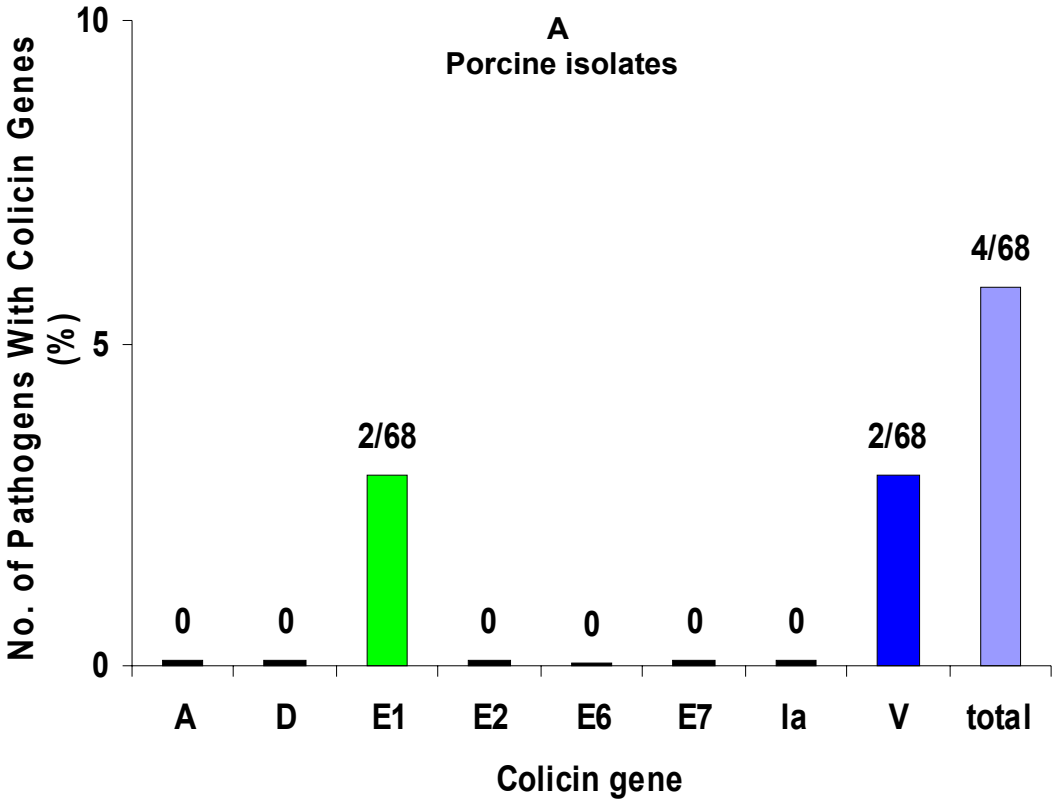
Table 3.4. Frequency and presence of each colicin type in different GIT compartments of porcine commensal *E. coli*.

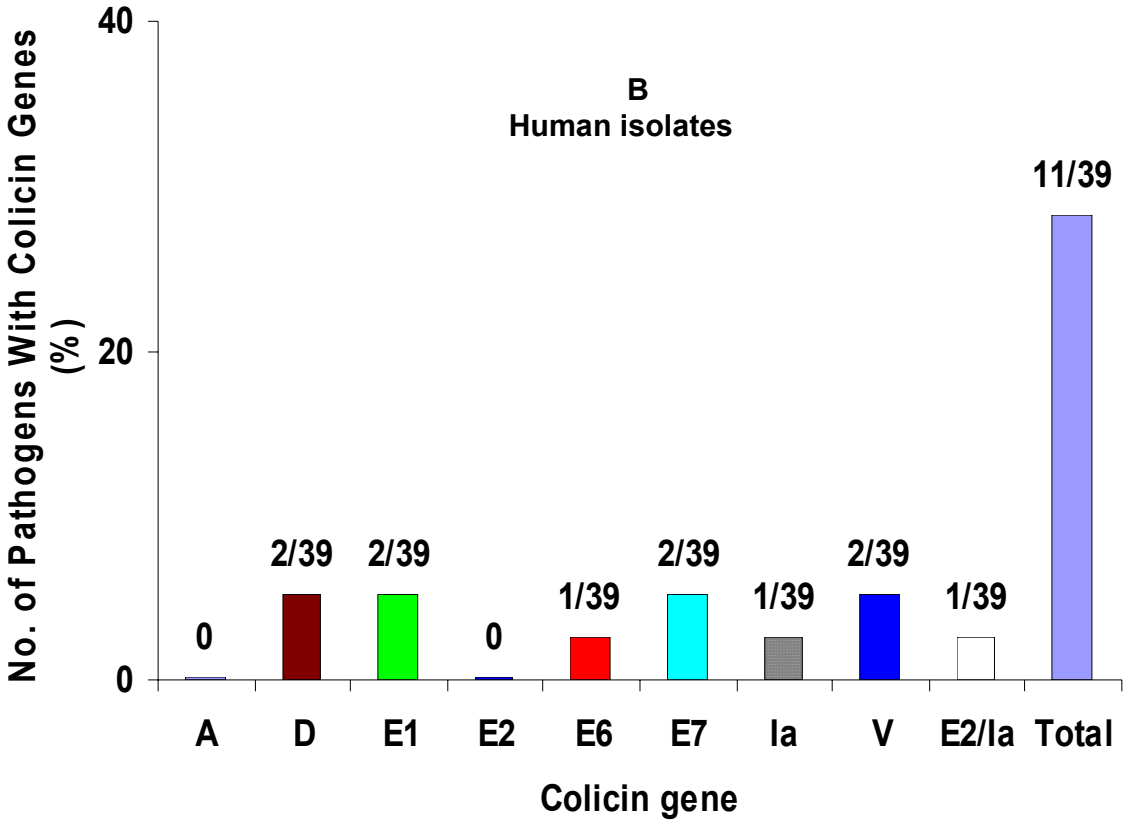
Colicin genotype	Intestinal compartment	% frequency
A	Nil	Nil
D	Nil	Nil
E1	Ileum, duodenum, faeces	31.5
E2	faeces	1.9
E6	Nil	Nil
E7	duodenum, Ileum, colon, faeces	35.2
Ia	duodenum, Ileum, colon	20.3
V	Nil	Nil
E1/E2	Ileum	1.9
E1/E7	Colon	3.8
E2/E7	Ileum, faeces	3.8
E2/Ia	Ileum	1.9

3.3.5 Frequency of Colicin Genes in Porcine and Human Pathogenic *E. coli* Isolates

The distribution of colicin genes in porcine and human pathogenic *E. coli* is shown in Figure 3.8. Approximately 6 % of porcine pathogenic *E. coli* clones possessed a colicin gene with colicins E1, V occurring at frequencies of 3 % each (Figure 3.8-A). The frequency of colicin genes in human isolates was about 28.2 % (Figure 3.8-B). Colicins D, E1, E7 and V were the most common occurring at frequencies of 5.1 % each. Colicins E6, Ia and E2/Ia were less frequent and were found in about 2.6 % of clones.

FIG 3.8 Frequency of colicin genes in pathogenic *E. coli* isolates from porcine and human sources. Values above bars represent number of isolates positive for a colicin gene out of a total of 68 in the porcine pathogens **(A**) and 39 in the human isolates **(B)**.


A
Porcine isolates
No. of Pathogens With Colicin Genes (%)
10
5
0
0
0
2/68
0
0
0
0
2/68
4/68
A
D
E1
E2
E6
E7
Ia
V
total
Colicin gene

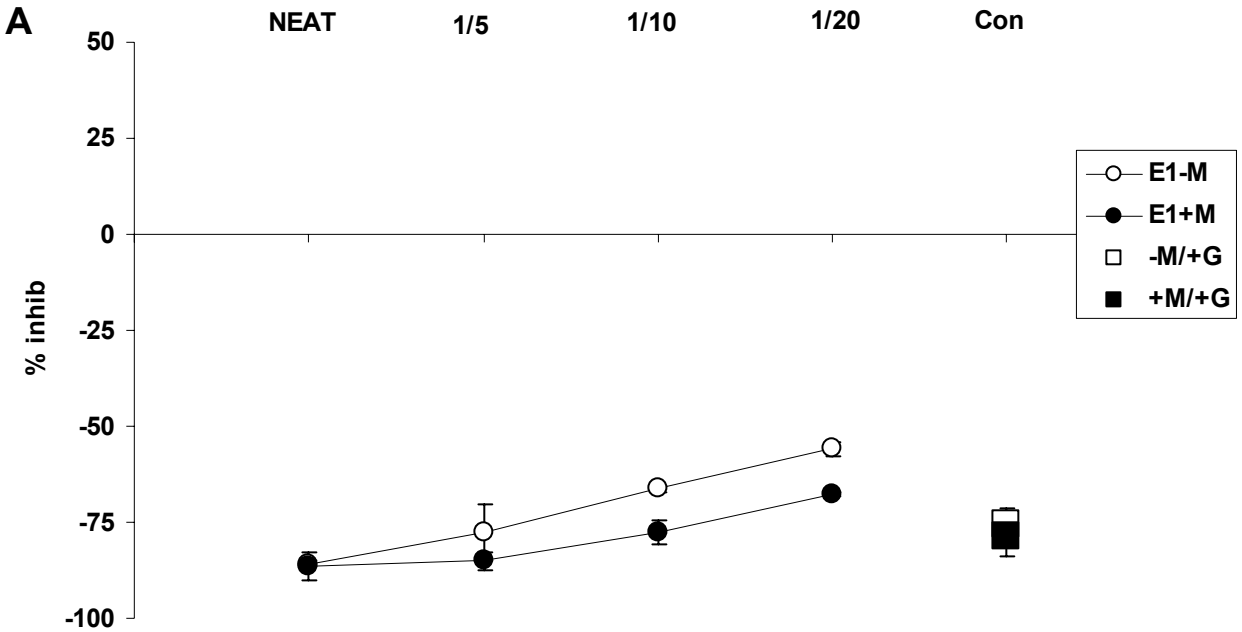

B
Human isolates
No. of Pathogens With Colicin Genes (%)
40
20
0
0
2/39
2/39
0
1/39
2/39
1/39
2/39
1/39
11/39
A
D
E1
E2
E6
E7
Ia
V
E2/Ia
Total
Colicin gene

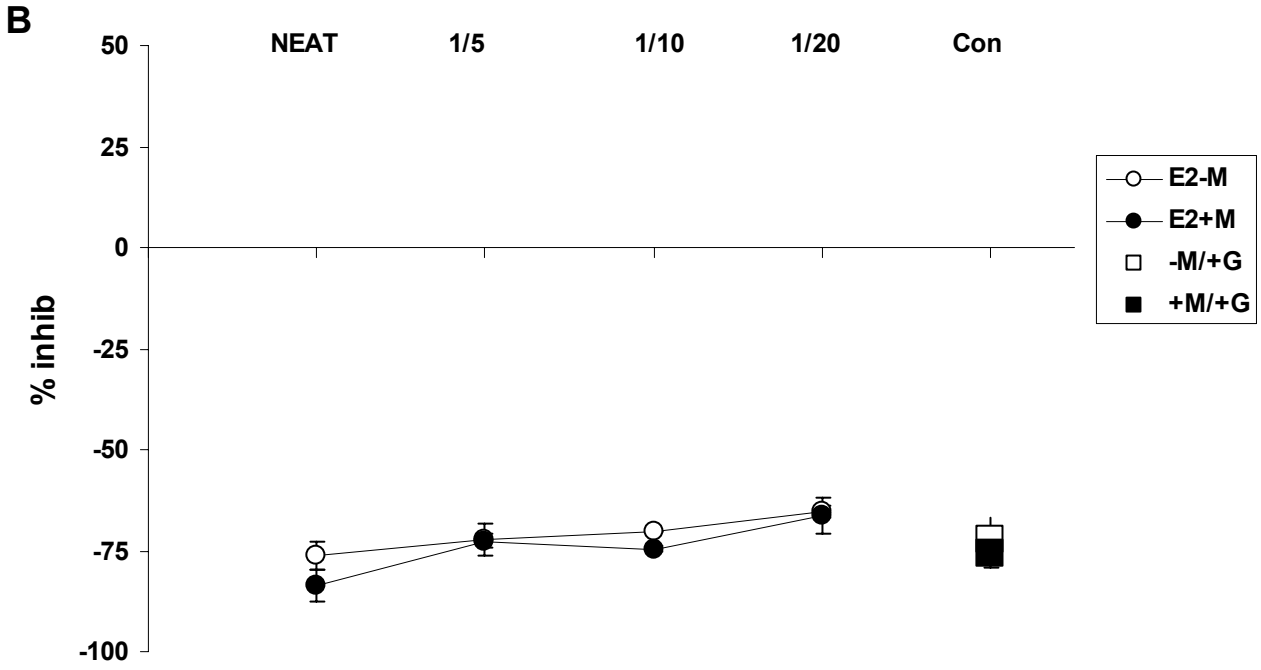
3.3.6 Inhibitory Ability of Colicin Reference Strains on Colicin Sensitive Strain BZB1011

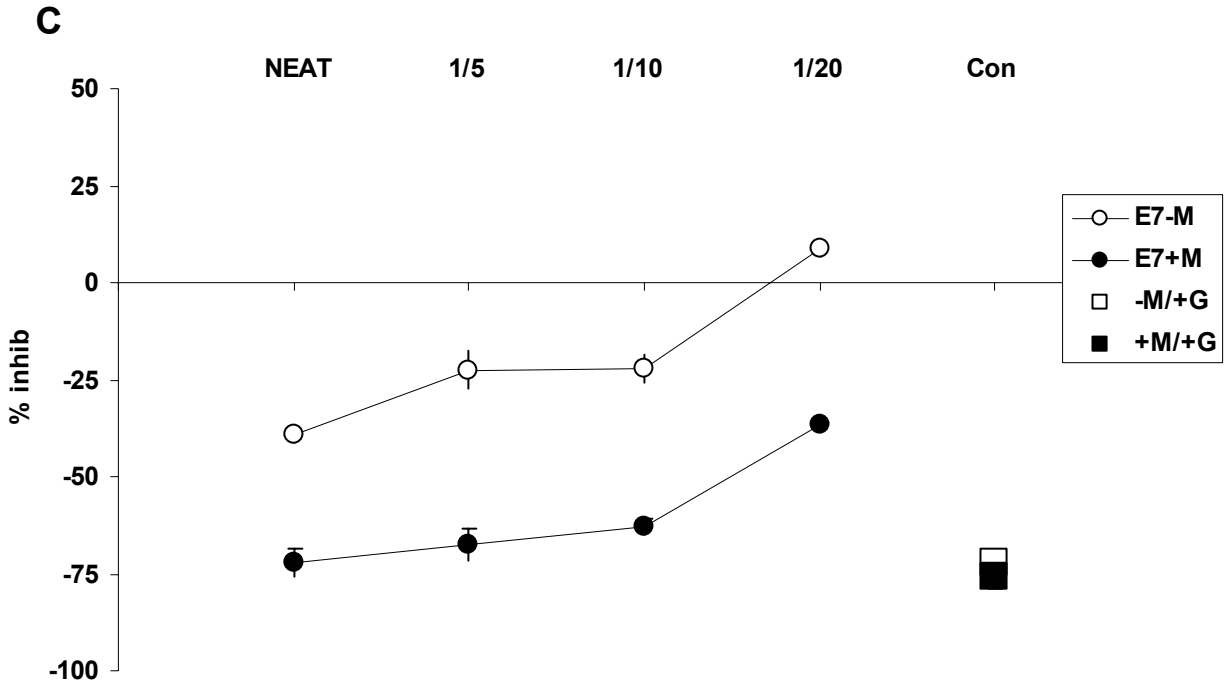
E. coli strain BZB1011 has been reported to be colicin sensitive due to receptors present in the cell surface of that strain (Pugsley and Oudega, 1987). This strain was used as a target in order to assess the inhibitory ability of these colicin reference *E. coli* strains. Culture supernatants of the reference *E. coli* strains were obtained from growth cultures that were either induced with 0.2 µg/ml mitomycin C or not induced. Using the method of KIMA, the supernatants were used as overlay on BZB1011 underlay for assessment of inhibition.

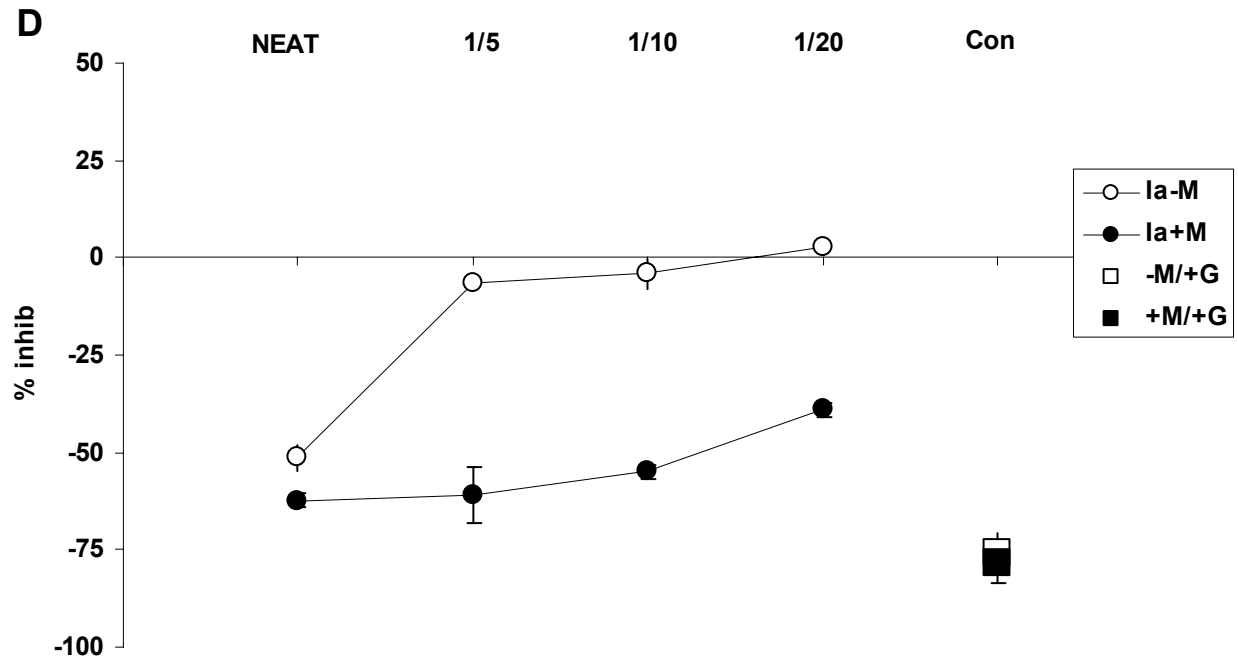
All four colicin reference strains were able to repress growth of BZB1011 regardless of whether those were mitomycin C treated or untreated (Figure 3.9 A-D). Amongst the four reference strains, undiluted supernatant from the colicin E1 reference strain displayed the highest inhibitory ability (Figure 3.9-A) while neat un-induced supernatant from colicin E2 reference strain showed the least inhibition (Figure 3.9-D). Supernatants from mitomycin C induced cultures displayed higher inhibitory profiles compared to supernatants from non-induced cultures.

FIG 3.9 Effects of colicin supernatant from growth cultures of *E. coli* reference strains E1, E2, E7, and Ia (**A-D**) on growth of colicin sensitive strain BZB1011. Colicins supernatant were obtained from cultures, which had either been treated with 0.2 µg/ml mitomycin (+M) C or not treated (-M). Adopting the method of KIMA, the colicin supernatants were used as overlay on top of BZB1011 underlay to assess difference in growth compared to LB broth overlay control. Colicin supernatants were either undiluted (neat) or diluted 1/5, 1/10 and 1/20 in LB broth. Results are presented as percent inhibition (% inhib) compared to LB broth overlay controls. LB broth control (Con) containing Gentamicin/ + mitomycin C (+M/+G) or Gentamicin/ - mitomycin C (-M/+G) were included on all assay as negative controls to facilitate plate reproducibility. Experiments were done in triplicates and the values represented are means.


A
NEAT
1/5
1/10
1/20
Con
50
25
0
-25
-50
-75
-100
% inhib
E1-M
E1+M
-M/+G
+M/+G


B
NEAT
1/5
1/10
1/20
Con
50
25
0
-25
-50
-75
-100
% inhib
E2-M
E2+M
-M/+G
+M/+G


C
NEAT
1/5
1/10
1/20
Con
50
25
0
-25
-50
-75
-100
% inhib
E7-M
E7+M
-M/+G
+M/+G


D
NEAT
1/5
1/10
1/20
Con
50
25
0
-25
-50
-75
-100
% inhib
Ia-M
Ia+M
-M/+G
+M/+G

3.3.7 Growth Effect of Colicin Reference Strains on Selected *E. coli* Pathogens

Previous results (section 3.3.6) showed that KIMA allowed testing of inhibitory activity of reference strains against a target sensitive *E. coli* BZB1011 strain. The next step was to then assess whether these strains could also inhibit growth of harmful pathogens implicated in human and animal diseases of the GIT. A panel of target bacterial strains were therefore chosen as representative gram-negative pathogens.

These were chosen based on the following criteria:

- Possession of virulence genes that are characteristic of the infective species;
- Display of a pathogenic phenotype such as hemolysis;
- Clinical strains isolated from diseased animals or humans.

Four of these were *E. coli* pathogens that cause PWD in pigs and included serotypes (08:G7 {P127/1}, 0149:K88 {P139/1}, 141:K85 {154/3} and 0141:K88 {043/3}). The panel also included one pathogenic *E. coli* strain (0157:H7 {DES1643}) causing diarrhoea in humans. (I would like to acknowledge the help of Toni Chapman and Kent Wu, PhD candidates at EMAI who carried out the experimental work leading to isolation and characterization of these strains).

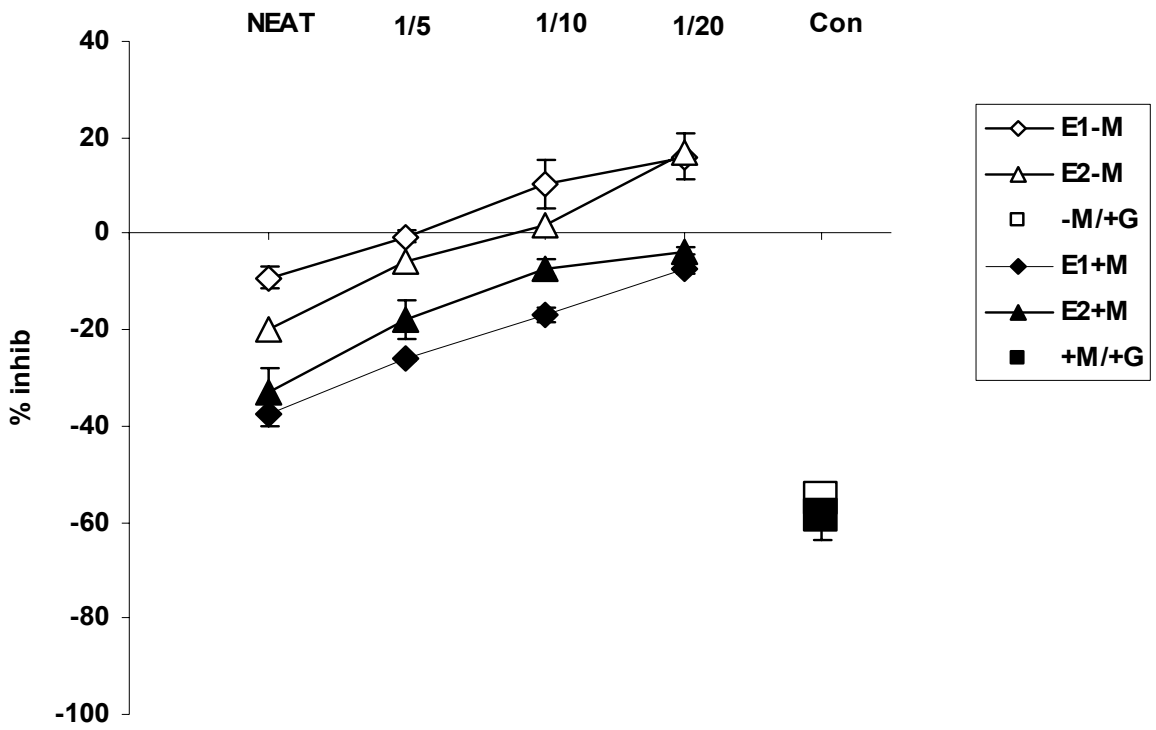
The culture supernatants of the four colicin reference strains were assessed for inhibitory activity against these pathogens. Results of inhibition

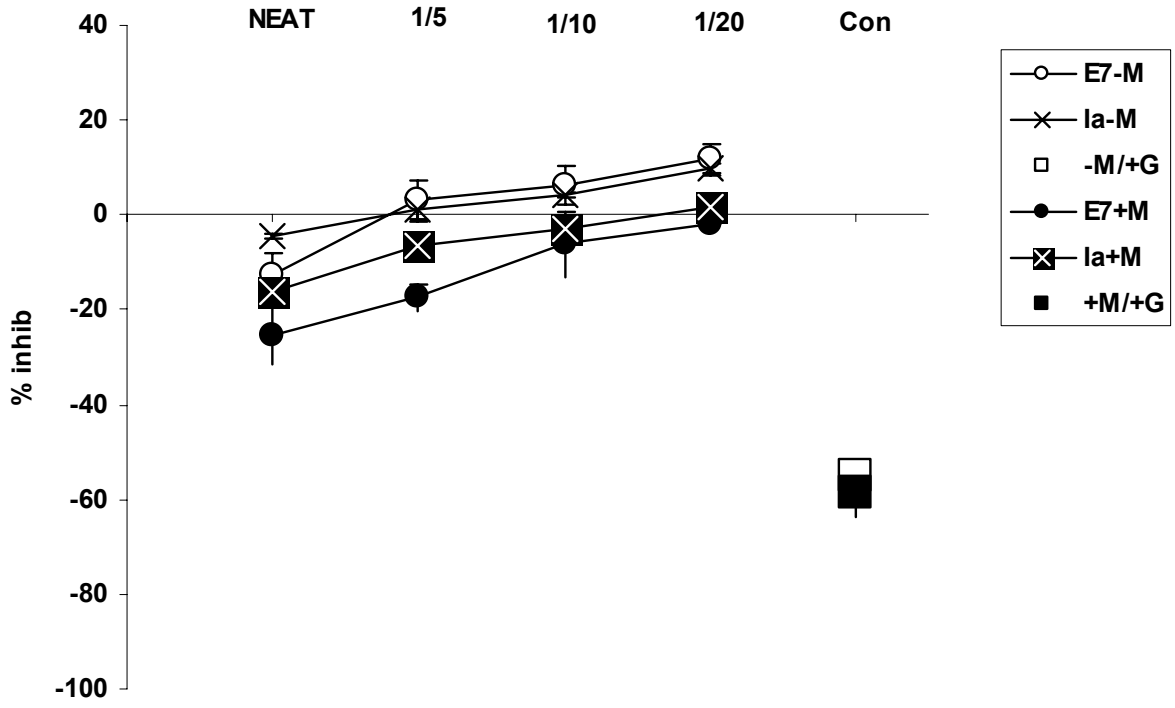
showed that the four reference strains were not able to inhibit growth of all five pathogens in the manner observed against the sensitive strain BZB1011. All reference strains assessed appeared to not greatly inhibit growth of most of the pathogens assessed (Figure 3.10 A-E). However, these inhibitory activities were variable depending on the pathogen used. Moreover, supernatant from Mitomycin C treated culture appeared to inhibit better than supernatant from untreated culture.

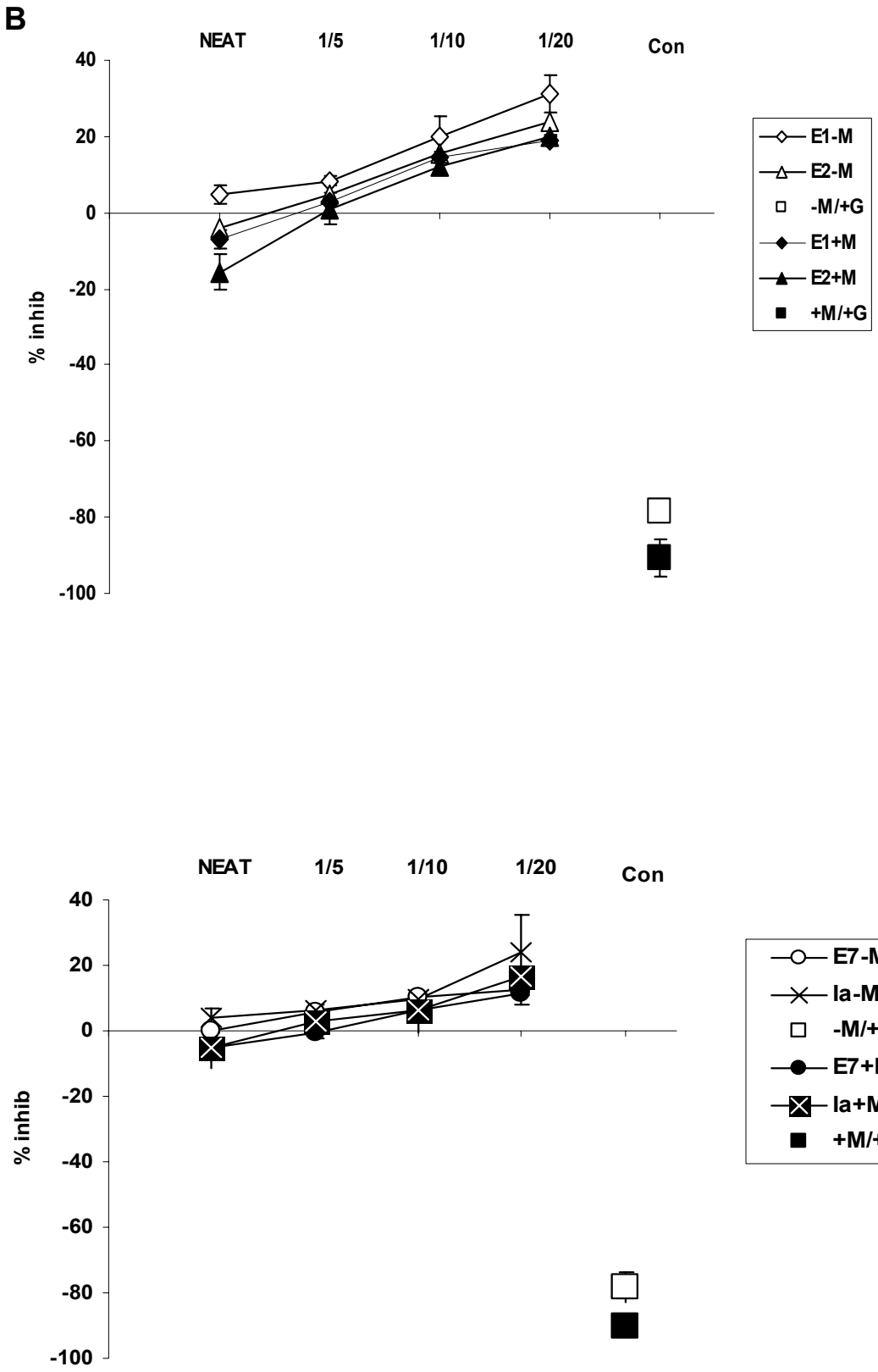
There were three instances of higher percentage inhibition shown by reference strains against some of the pathogens. Strains E1, E2 and E7 were able to inhibit growth of pathogen *E. coli* O141: K85 (154/3) by more 70 % using undiluted supernatants (Figure 3.10-D). In addition, the inhibitions were higher in the presence of mitomycin C. The same strains were however unable to show the same level of inhibition against all other target strains. This result was observed in more than one instance, suggesting *E. coli* O141:K85 (154/3) is susceptible to colicins E1, E2 and E7 but appears to be resistant to all other colicins. These results serve to demonstrate the contrasting ability of different colicin producers to inhibit growth of pathogenic organisms. Based on the results, the pathogenic strains 08:G7 (P127/1) and 0149:K88 (P139/1) seemed to very resistant to all reference strains even when neat supernatant from mitomycin C induced cultures were used.

FIG 3.10 Assessment of inhibition of selected pathogenic strains **(A-E)** by *E. coli* reference strains E1, E2, E7, and Ia. Culture supernatants were obtained from producer strains that had been propagated in LB/+ Mitomycin C (+M; dark circle) or LB/ - Mitomycin C (-M; light circle). Colicin supernatants were either undiluted, or diluted 1/5, 1/10 and 1/20 in LB broth. Inhibition is represented on the Y-axis on a scale of -100% (maximum inhibition) to +40% (minimum inhibition). Colicins supernatant were obtained from cultures, which had either been treated with 0.2 µg/ml mitomycin (+M) C or not treated (-M). **A**. *E. coli* O157:H7 (DES1643). **B.** *E. coli* O8:G7 (p127/1). **C**. *E. coli* O149: K88 (p139/1). **D.** *E. coli* 0141:K85 (154/3). **E.** *E. coli* O141:K88 (043/3) LB broth control (Con) containing Gentamicin/ + mitomycin C (+M/+G) or Gentamicin/ - mitomycin C (-M/+G) were included on all assay as negative controls to facilitate plate reproducibility. Experiments were done in triplicates and the values represented are means.

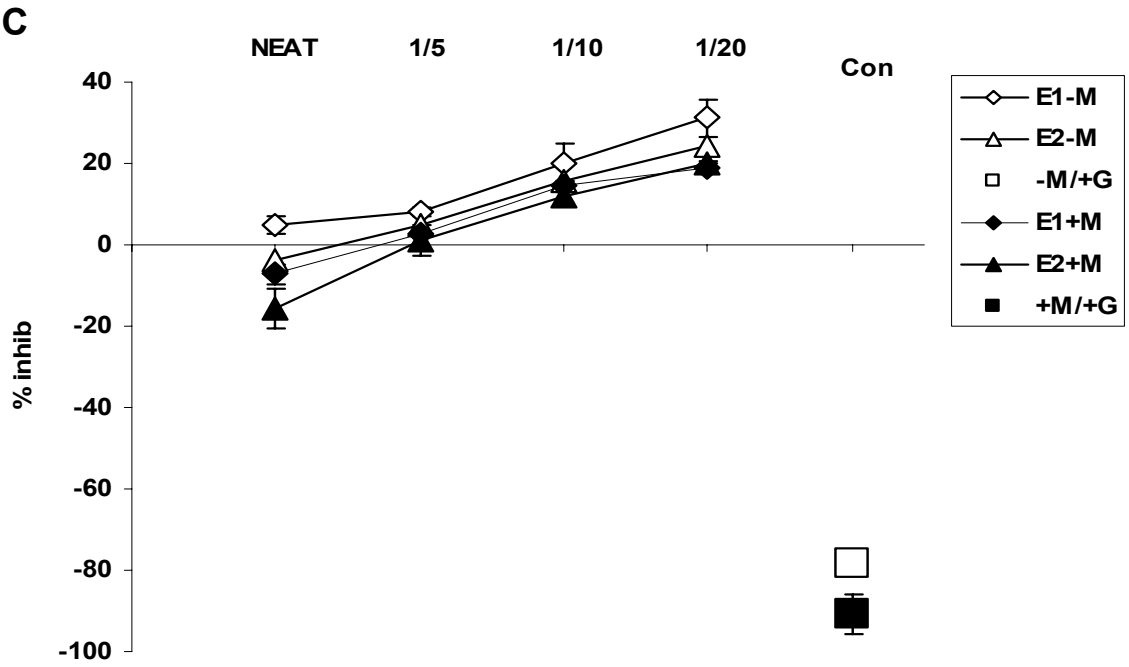
A

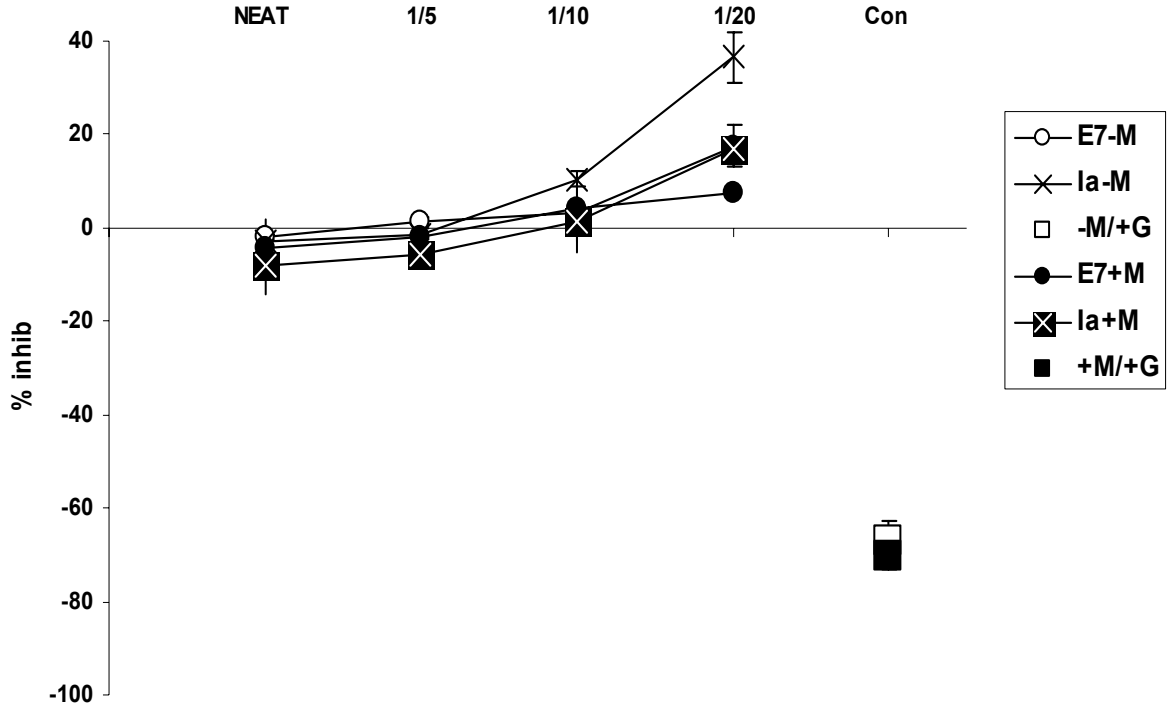

NEAT
1/5
1/10
1/20
Con
40
20
0
-20
-40
-60
-80
-100
% inhib
E1-M
E2-M
-M/+G
E1+M
E2+M
+M/+G


NEAT
1/5
1/10
1/20
Con
40
20
0
-20
-40
-60
-80
-100
% inhib
E7-M
Ia-M
-M/+G
E7+M
Ia+M
+M/+G

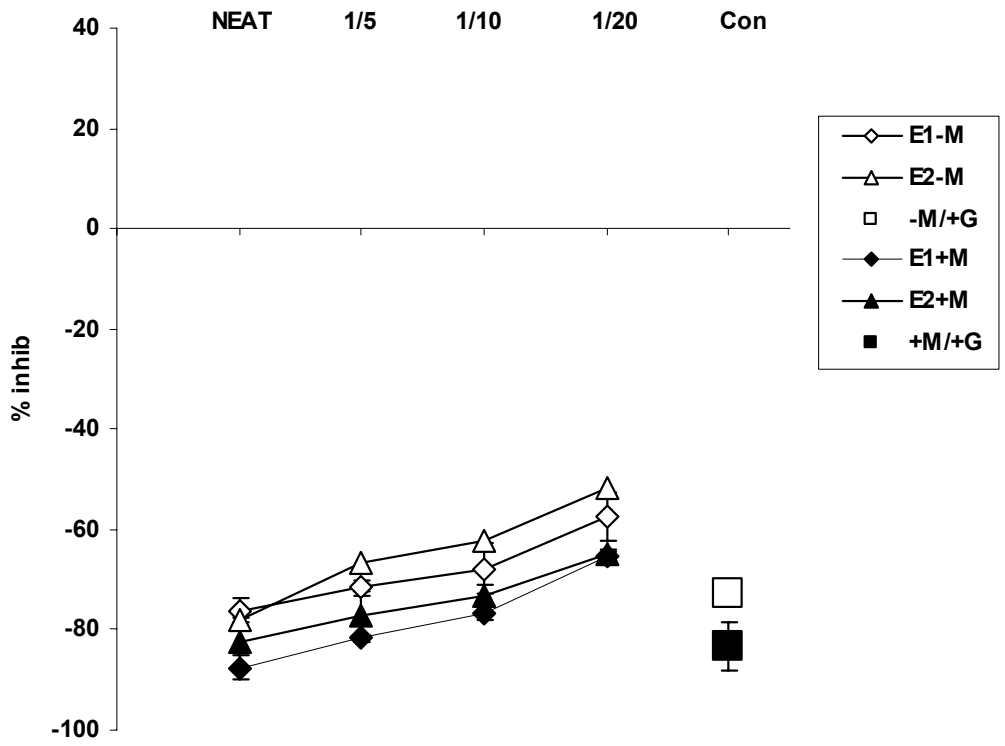

B
NEAT
1/5
1/10
1/20
Con
40
20
0
-20
-40
-60
-80
-100
% inhib
E1-M
E2-M
-M/+G
E1+M
E2+M
+M/+G
NEAT
1/5
1/10
1/20
Con
40
20
0
-20
-40
-60
-80
-100
% inhib
E7-M
Ia-M
-M/+G
E7+M
Ia+M
+M/+G

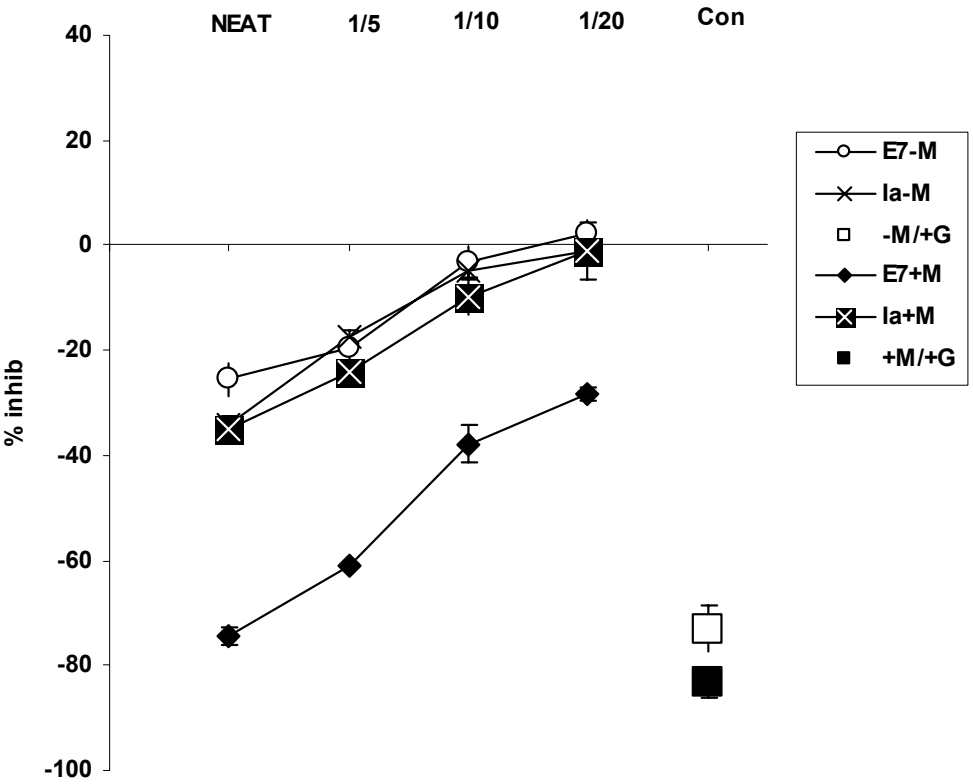
C


NEAT
1/5
1/10
1/20
Con
40
20
0
-20
-40
-60
-80
-100
% inhib
E1-M
E2-M
-M/+G
E1+M
E2+M
+M/+G

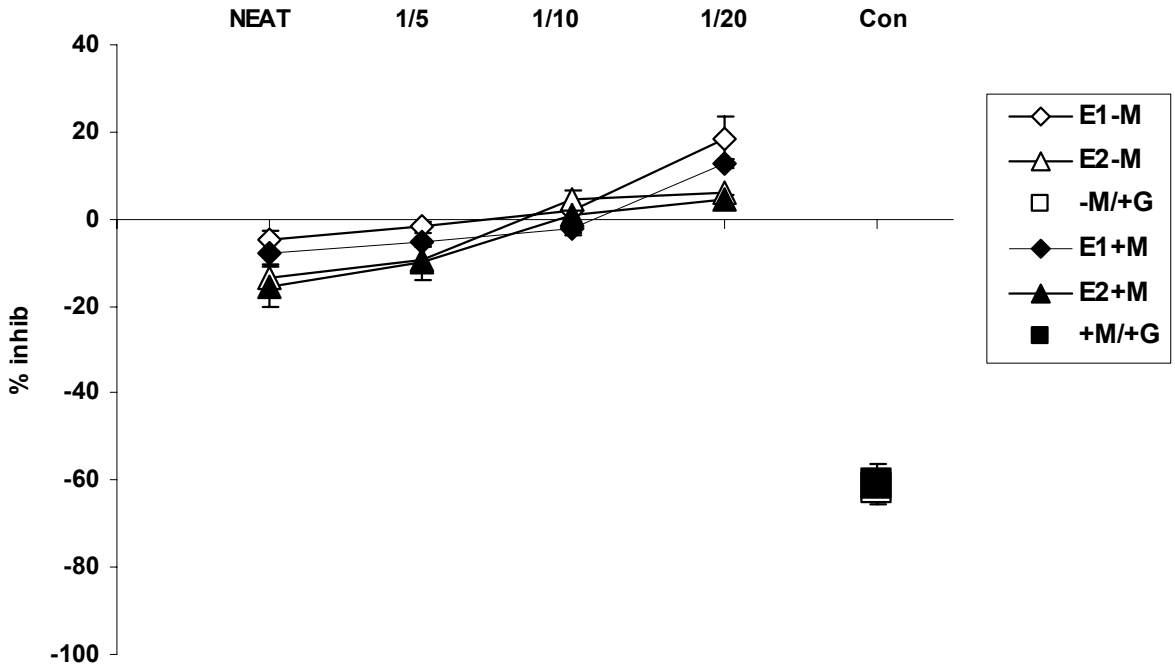

NEAT
1/5
1/10
1/20
Con
40
20
0
-20
-40
-60
-80
-100
% inhib
E7-M
Ia-M
-M/+G
E7+M
Ia+M
+M/+G

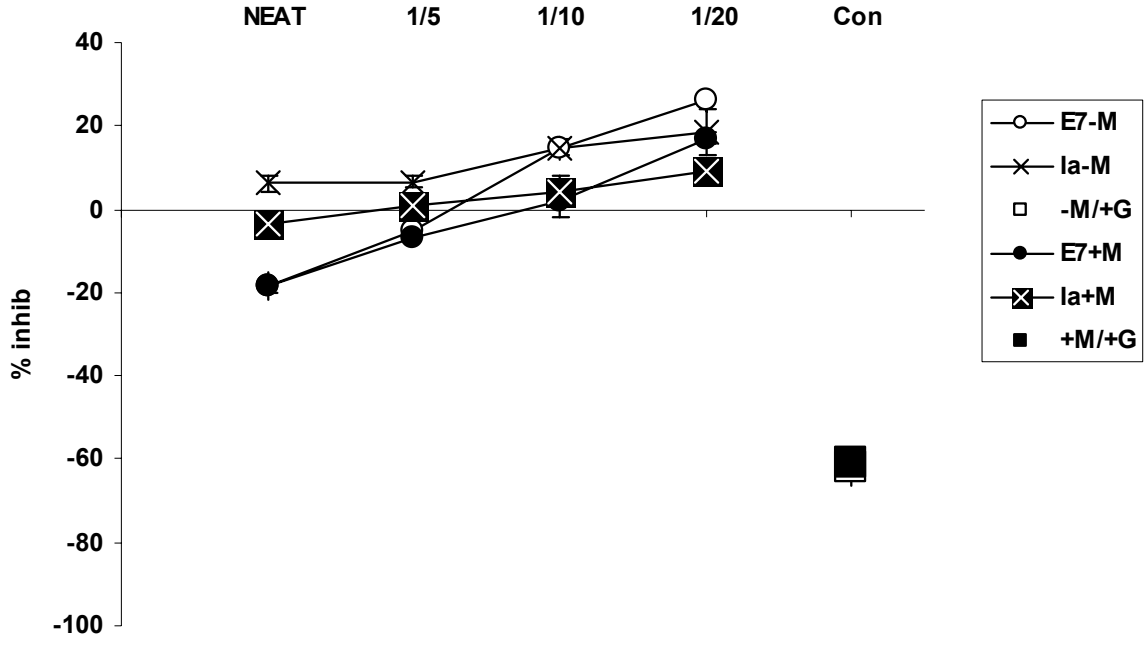
D


NEAT
1/5
1/10
1/20
Con
40
20
0
-20
-40
-60
-80
-100
% inhib
E1-M
E2-M
-M/+G
E1+M
E2+M
+M/+G


NEAT
1/5
1/10
1/20
Con
40
20
0
-20
-40
-60
-80
-100
% inhib
E7-M
Ia-M
-M/+G
E7+M
Ia+M
+M/+G

E


NEAT
1/5
1/10
1/20
Con
40
20
0
-20
-40
-60
-80
-100
% inhib
E1-M
E2-M
-M/+G
E1+M
E2+M
+M/+G


NEAT
1/5
1/10
1/20
Con
40
20
0
-20
-40
-60
-80
-100
% inhib
E7-M
Ia-M
-M/+G
E7+M
Ia+M
+M/+G

3.3.8 Assessment of Effect of Commensal Strains on Colicin Sensitive Strain BZB1011

One of the requirements for potential probiotic strains is their ability to antagonize growth of pathogenic bacteria *in vitro* and *in vivo*. The ability of novel bacterial strains to antagonize growth of pathogenic strains *in vitro* was assessed by KIMA using strains isolated from different porcine GIT compartments. The presence of colicin genes in these strains was detected using the multiplex PCR technique. They included two strains from the duodenum (DC07 and DC13), two from the ileum (ILC 32 and ILC 33), two from the colon (CC59 and CC89), and one from the faeces (FC115). They carried colicin genes for E1 (ILC33 and CC59), E7 (DC13 and FC115), Ia (DC07) and the dual colicins E1/E7 (CC89) and E2/E7 (ILC32).

It would be prudent to first examine their inhibitory actions on the colicin sensitive strain BZB1011. Supernatants from all strains grown in LB broth, with and without mitomycin C treatment were used for all assessments. Based on the results obtained using the reference strains, it was decided to use only neat supernatants because they displayed appropriate inhibitory levels. All commensal strains were able to inhibit the sensitive strain with the inhibitions being higher in supernatants from mitomycin C induced cultures (Figure 3.11). Strains ILC33, CC89 and FC115 displayed the highest inhibitory activity while strain DC13 showed the least inhibition. An interesting observation was that although both strains ILC33 and CC59 carried colicin E1 gene, strain ILC33 showed a higher inhibitory action (-83 %) than CC59 (-56 %).

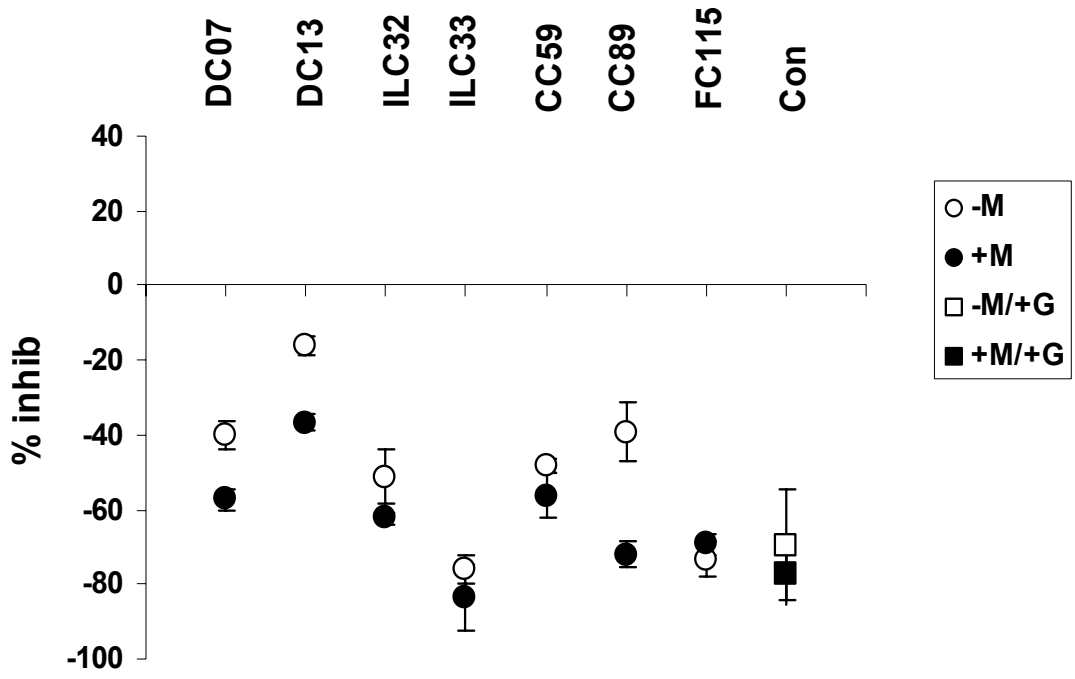


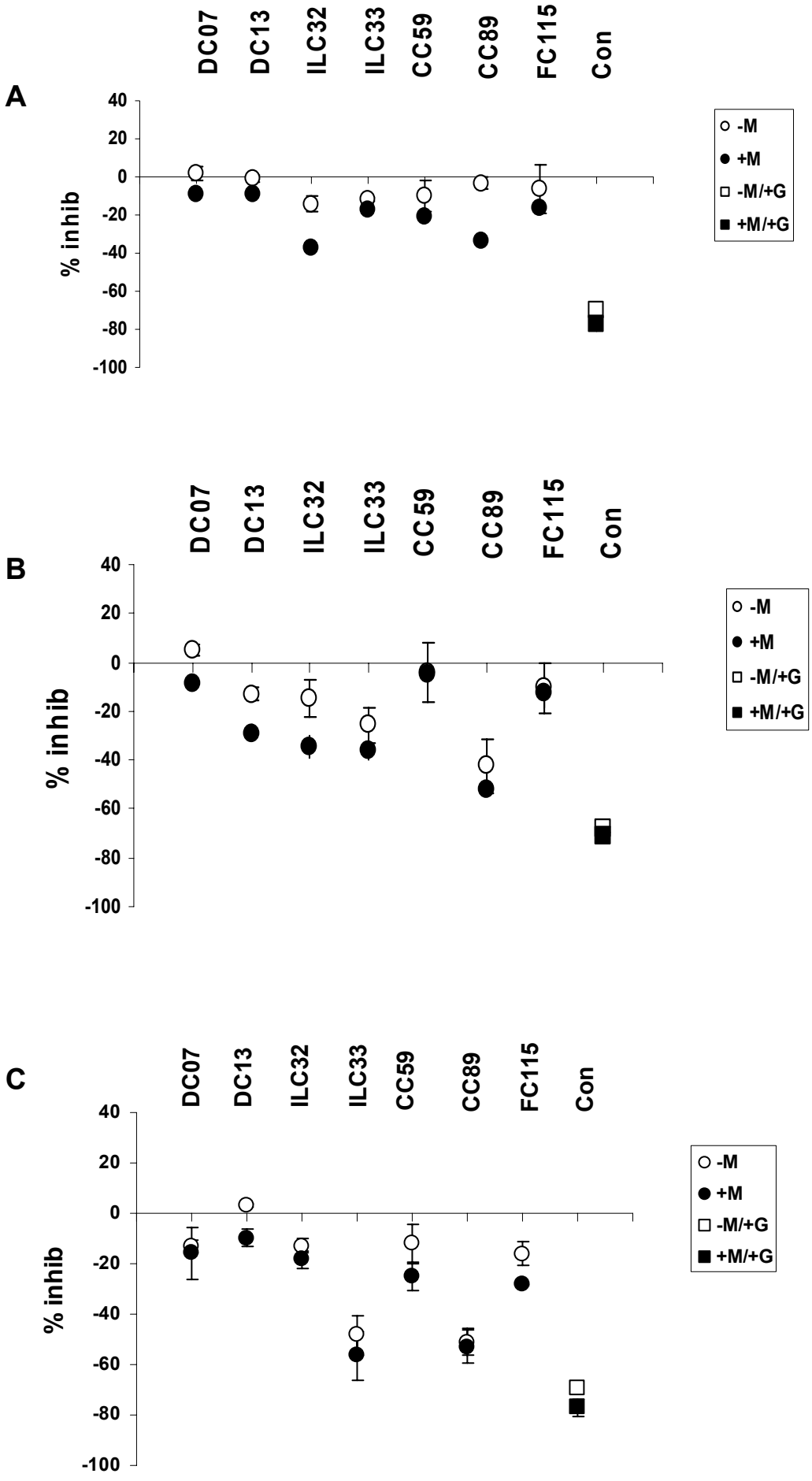
FIG 3.11 Assessment of inhibition of colicin sensitive strain BZB1011 by *E. coli* commensal strains DC07, DC13, ILC32, ILC33, CC59, CC89 and FC115. Colicins supernatant were obtained from cultures, which had either been treated with 0.2 µg/ml mitomycin (+M) C or not treated (-M). Adopting the method of KIMA, the colicin supernatants were used as overlay on top of BZB1011 underlay to assess difference in growth compared to LB broth overlay control. Results are presented as percent inhibition (% inhib) compared to LB broth overlay controls. LB broth control (Con) containing Gentamicin/ + mitomycin C (+M/+G) or Gentamicin/ - mitomycin C (-M/+G) were included on all assay as negative controls to facilitate plate reproducibility. Experiments were done in triplicates and the values represented are means.

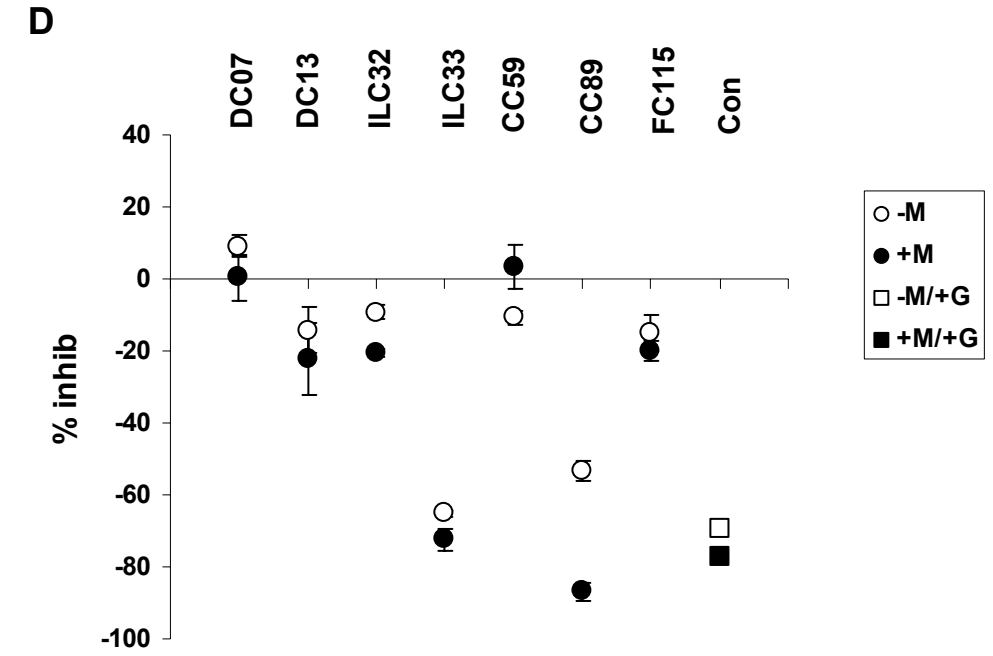
3.3.9 Assessment of Inhibitory Effect of Commensal Strains on Selected Pathogenic Strains

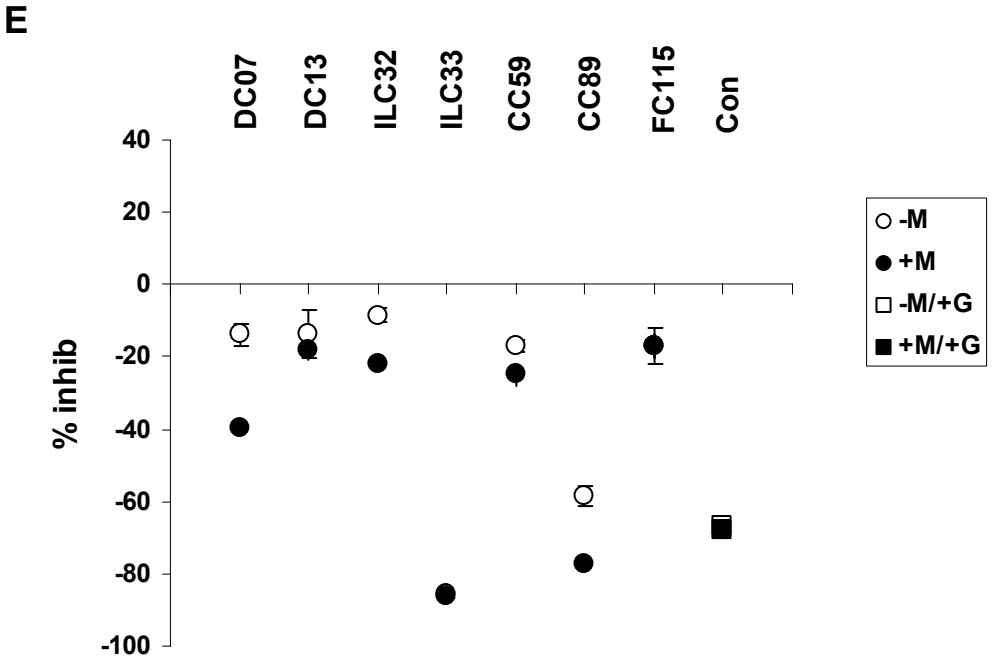
The same panel of pathogenic *E. coli* used as targets strains against the four colicin reference strains were again utilized. They were used undiluted with controls represented by LB broth overlay, which were either devoid of mitomycin C or contained 0.2 µg/ml mitomycin C. Internal gentamicin controls were included as overlays with and without incorporation of mitomycin C to LB broth containing gentamicin.

The results varied between target pathogens tested. Undiluted mitomycin treated supernatants from strains ILC33 and CC89 appeared to be the most inhibitory against 0149:K88 (P139/1) (Figure 3.12-C), 0141:K85 (154/3) (Figure 3.12-D) and 0141:K88 (043/3) (Figure 3.12-E) compared to controls. Mitomycin C supplemented supernatants appeared to have a greater inhibitory ability than those not supplemented with mitomycin C. The pathogenic strains 0157:H7 (DES1643) (Figure 3.12-A) and 08:G7 (P127/1) (Figure 3.12-B) showed low susceptibility to supernatants from most of the treatment groups. These results would suggest that either colicin was not produced by the commensal strain assessed. If colicin was produced, the class of colicin produced was non-inhibitory against the strains assessed.

FIG 3.12 Assessment of inhibition of selected pathogenic strains **(A-E)** by *E. coli* commensal strains DC07, DC13, ILC32, ILC33, CC59, CC89 and FC115. Culture supernatants were obtained from producer strains that had been propagated in LB/+ Mitomycin C (+M; dark circle) or LB/ - Mitomycin C (-M; light circle). Colicin supernatants were either undiluted, or diluted 1/5, 1/10 and 1/20 in LB broth. Inhibition is represented on the Y-axis on a scale of -100% (maximum inhibition) to +40% (minimum inhibition). Colicins supernatant were obtained from cultures, which had either been treated with 0.2 µg/ml mitomycin (+M) C or not treated (-M). **A**. *E. coli* O157:H7 (DES1643). **B.** *E. coli* O8:G7 (P127/1). **C**. *E. coli* O149: K88 (P139/1). **D.** *E. coli* 0141:K85 (154/3). **E.** *E. coli* O141:K88 (043/3) LB broth control (Con) containing Gentamicin/ + mitomycin C (+M/+G) or Gentamicin/ - mitomycin C (-M/+G) were included on all assay as negative controls to facilitate plate reproducibility. Experiments were done in triplicates and the values represented are means.


A
B
C
DC07
DC13
ILC32
ILC33
CC59
CC89
FC115
Con
% inhib
40
20
0
-20
-40
-60
-80
-100
-M
+M
-M/+G
+M/+G


D
DC07
DC13
ILC32
ILC33
CC59
CC89
FC115
Con
40
20
0
-20
-40
-60
-80
-100
% inhib
-M
+M
-M/+G
+M/+G


E
DC07
DC13
ILC32
ILC33
CC59
CC89
FC115
Con
40
20
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-20
-40
-60
-80
-100
% inhib
-M
+M
-M/+G
+M/+G

3.4 Discussion

The first aim of this study was to develop an *in vitro* assay for the identification of colicin genes. To aid in the identification of eight common colicin genes in *E. coli* isolates, a multiplex PCR assay was developed. Multiplex PCR is a variant of PCR in which two or more target sequences can be simultaneously amplified by including more than one pair of primers in the same reaction (Markoulatos *et al.,* 2002). The ability to detect and distinguish multiple gene targets rather than one target makes this method not only cost effective but also time saving (Caterina *et al.,* 2004).

The multiplex PCR was developed to detect the eight colicin genes in three separate reactions according to primer specificity and amplicon size. The initial amplification conditions were found to be non-optimal with preferential amplification of some target sequences over others and also presence of non-specific products (Figure 3.3). To address these problems, the concentrations of primer pairs and $MgCl_2$ in each pool were varied since it has been found that alteration of these two components in multiplex PCR usually results in considerable improvement in the specificity of the test (Markoulatos *et al.,* 2002). In addition, a HotStarTaq polymerase was used instead of normal Taq polymerase. HotStarTaq polymerase is a thermostable enzyme that remains inactive at temperatures up to 75 °C, but is activated at a higher temperature of 95 °C (Markoulatos *et al.,* 2002). The enzyme improves performance of multiplex PCR because it eliminates non-specific priming (Brownie *et al.,* 1997). Preferential amplification of one target sequence over another in multiplex PCR is known as PCR selection (Elnifro *et al.,* 2000). PCR selection is caused by a number of factors including primer pair concentration. Initially equimolar primer

concentrations were used in all multiplex pools. When there is uneven amplification, with some of the products barely visible, changing the proportion of the various primers is required, with an increase in the amount of primers for the "weak" loci and a decrease in the amount for the "strong" loci (Markoulatos *et al.,* 2002). Reducing the primer pair concentrations for colicins A, D, E2, E6 and E7 while leaving the ones for colicins E1, Ia and V unchanged improved the amplification of all colicin genes in the three pools (Figure 3.4).

The overall percentage (36.2 %) of colicins in the 152 porcine commensal *E. coli* strains was very close to the colicinogeny estimates of 35 % and 38 % reported by Riley and Gordon (1992) and Schamberger and Diez-Gonzalez (2002). This suggests that the multiplex PCR assay is capable of producing similar results generated using traditional methods of colicin detection such as the agar spot method. When the percentage was calculated for each intestinal compartment, the frequency of these colicin genes ranged from 30 % in the duodenum to 51.3 % in the ileum (Figure 3.5). It has been suggested that the concentrations of major proteases such as trypsin and chymotrypsin are lower in the ileum, colon and faeces compared to the duodenum and it may be possible that these enzymes may be degrading some colicins in the duodenum (Hofinger *et al.,* 1998).

The relative abundance of different colicin types has been found to vary substantially between strain collections (Riley and Gordon, 1996). The results of this study reinforce this observation. Pore-forming colicins are usually the dominant type with colicin E1 and Ia regularly observed (Tan and Riley, 1996) although the study of Gordon *et al.* (1998) found colicin E7 to be a common type of colicin present in *E. coli* isolates from mice. Results of this study showed

that colicin E7 was the most common colicin and also the only colicin present in all four GIT compartments (Table 3.4). Colicins E1 and Ia were also both found to be prevalent and were present in three intestinal compartments (Table 3.4). Interestingly, colicin Ia was more prevalent in the upper GIT (duodenum, ileum) (Figure 3.6) compared to the lower GIT (colon, faeces) (Figure 3.7). Multiple colicin types that were detected in this collection were of the E1/E2, E2/E7 and E2/Ia types, which were found in six strains. These colicins appear to be hybrids between colicins E1 and E2 (E1/E2), colicins E2 and E7 (E2/E7), and colicins E2 and Ia (E2/Ia). Tan and Riley (1996) reported recombinant E2/E7 colicins from an *E. coli* recovered from Australian feral mice and an *E. coli* strain isolated from France, referring to them as E2 type 2 colicins. Recently, Nandiwada *et al.* (2004) has also characterized another E2/E7 hybrid from a human *E. coli* isolate. The E2/E7 hybrid is thought to be derived from a recombination event between the colicin E2 gene cluster and the colicin E7 plasmid (Gordon *et al.,* 1998). However, no one has as yet reported colicins of the E1/E2 and E2/Ia type but it is possible that they are formed in the same way as the E2/E7 hybrid.

The prevalence of colicins in the porcine pathogenic strains was lower than in the porcine commensal strains. Compared to 36.2 % in the commensal strains, only 6 % (Figure 3.8-A) of the porcine pathogenic isolates possessed a colicin and these were exclusively colicins E1 and V. Colicin V plasmids have been proposed to confer enhanced virulence to *E. coli* pathogenic isolates through their carriage of specific virulence determinants, including the aerobactin iron assimilation system, adhesion properties and serum resistance genes, such as *traT* and *iss* (Johnson and Stell, 2000). The increased virulence

of colicin V producing strains might be due to these determinants since colicin V itself does not seem to act as a pathogenic determinant (O'Brien *et al.,* 1996). The carriage of colicin genes by the human pathogens was much higher (Figure 3.8-B) with 28.2 % of isolates possessing a colicin gene. This agrees with Bradley (1991) who demonstrated that 24 % of human intestinal pathogenic *E. coli* strains synthesized colicins. The presence of colicin genes in the human pathogens suggests that human pathogenic *E. coli* possessing colicin genes, in addition to virulence gene factors may have more firepower to attain higher levels of fitness survival compared to the porcine pathogens that carry less colicin genes.

It has been established in literature that colicins from *E. coli* can antagonise other gram-negative strains (Jordi *et al.,* 2001; Murinda *et al.,* 1996). However, these studies have relied on laborious qualitative assays. To address this issue, a quantitative assay, KIMA was employed. To test this method, the inhibitory activity of four colicin reference strains known to express four different colicin genes (E1, E2, E7 and Ia) were investigated for their ability to inhibit the colicin sensitive strain BZB1011. As expected, all four reference strains were shown to inhibit the growth of the sensitive strain especially in the presence of mitomycin C (Figure 3.9 A-D). The next step was to see whether the reference strains could inhibit a panel of pathogenic *E. coli* isolates from pigs with PWD and the human pathogen 0157:H7. It was shown that the reference strains did not inhibit the growth of these pathogens even with undiluted supernatants from cultures induced with mitomycin C (Figure 3.10 A-E). The exception was for serotypes 0141:K85 (154/3) and to a lesser extent the human pathogen 0157:H7 (DES1643) (Figure 3.10-A and Figure 3.10-D). This result contrasts

with that of Murinda *et al.* (1996) who found that *E. coli* O157:H7 was highly susceptible reference to *E. coli* reference strains producing E1, E2, E7 and Ia colicins in an agar spot assay. However, microtitre assays were not carried out to assess the inhibition.

Consequently, colicin-containing commensal *E. coli* strains were screened for their ability to inhibit pathogenic *E. coli*. Seven porcine commensal *E. coli* strains from the duodenum, ileum, colon and faeces and identified as possessing colicin genes by multiplex PCR were assessed for inhibitory activity against the same panel of pathogenic *E. coli* using KIMA.The seven commensal strains were found to inhibit the colicin sensitive strain BZB1011, showing that they possessed inhibitory activity (Figure 3.11). The inhibitory action of these commensal strains against the panel of pathogenic *E. coli* was found to be variable (Figure 3.12). Commensal strains ILC33 and CC89 were shown to be highly inhibitory to PWD serotypes 0149:K88 (P139/1), 0141:K85 (154/3) and 0141:K88 (042/3) (Figure 3.12-C, Figure 3.12-D and Figure 3.12-E) but their inhibitory action was much reduced against the other pathogenic strains especially 0157:H7 (DES1643) and 08:G7 (P127/1) (Figure 3.12-A and Figure 3.12-B). ILC33 is a colicin E1 producer while CC89 is a colicin E1/E7 producer. This result agrees with previous data indicating that colicin E1 displayed antimicrobial activity against PWD strains 0149:K88 and 0141:K85 (Stahl *et al.,* 2004). On the other hand, they do not agree with other studies indicating that 0157:H7 is also sensitive to colicin E1 (Murinda *et al.,* 1996; Callaway *et al.,* 2004). However, other studies have indicated that the sensitivity of *E. coli* 0157:H7 to any single colicin can be variable (Schamberger and Diez-Gonzalez, 2002). It was interesting to note that possession of a dual colicin did

not necessarily equate with greater firepower as evidenced by low antagonistic action of ILC32, positive for the dual colicin E2/E7 (Figure 3.12 A-E).

Several factors could account for the observed insensitivities. There are three mechanisms of defence that *E. coli* cells can use to protect themselves against colicin action (Schamberger & Diez-Gonzalez, 2004). The first method of resistance is mediated by possession of a plasmid-encoded immunity protein, which inactivates the colicin protein (Kleanthous *et al.,* 1998). Mutation of a colicin receptor at the outer membrane of sensitive cells is another common mechanism of resistance found in *E. coli* populations (Lazdunski *et al.,* 1998). The third protective measure against colicins is a change in a colicin translocation protein on the cell membrane that can prevent colicin internalization (Alonso *et al.,* 2000). Colicin resistance appears to be quite common in natural *E. coli* populations. It has been estimated that more than 90 % of *E. coli* strains are resistant to at least one colicin and resistance to all tested colicins was observed in 25–40 % of *E. coli* strains (Gordon *et al.,* 1998). Finally, Nandiwada *et al.* (2004) have recently demonstrated that overnight induction with mitomycin C increases levels of colicin inhibition. It may be possible to increase the inhibitory activity of these colicins with longer induction times.

The contributions of inhibitory factors other than colicins could be largely discounted for these strains. Bacteriophages and microcins have been shown to be inhibitory to *E. coli* and other members of the family *Enterobacteriaceae* (Gordon *et al.,* 1998). Microcins are low-molecular weight channel-forming bacteriocin peptides produced by members of family *Enterobacteriaceae* (Gillor *et al.,* 2004). Microcin synthesis is non-lethal for producing strains, unlike colicin

production, and not mediated by conditions inducing SOS system (Gillor *et al.,* 2004). Since some results from commensal strains showed the absence of inhibition regardless of the whether SOS system was induced with mitomycin C, the contribution of microcins can be ignored. Additionally, freezing of bacterial culture supernatants at -80˚C and thawing is known to inactivate phage activity (David Gordon, personal communication). Since the experimental procedure of obtaining supernatants involved freezing and thawing, bacteriophage activity could be considered non-existent.

An important outcome of this research is the use of gene screening protocols for colicins to identify and select commensal isolates in possession of desired combinations of colicin genes. KIMA can then be used to validate the functionality of selected commensals in terms of their capacity to inactivate pathogenic strains that cause post-weaning diarrhoea with a view to their use as probiotics.

Chapter 4

Conclusions and Future Directions

The primary aims of the experiments described in this thesis were:

- To test the hypothesis that polysaccharides isolated from the medicinal mushroom, *Ganoderma lucidium,* are able to alter the population dynamics of cultivable good and bad bacteria in the GIT;
- To develop and validate a multiplex PCR for colicin detection;
- To apply the multiplex PCR to survey the presence of colicins in commensal and pathogenic *E. coli* isolates; and
- To bioassay the inhibitory spectrum of colicin producing commensal *E. coli* strains against pathogenic *E. coli*

The first aim used a mouse model to examine the effect of *Ganoderma lucidium* extracts on the population dynamics of cultivable intestinal bacteria. Experiments testing this hypothesis were carried out by feeding separately housed groups of mice with two extracts; crude HW and ethanol purified HWE extracts in their drinking water with control mice being fed *ad libitum* without any supplement in their water. Results showed that HW and HWE extracts were not able to significantly alter the population dynamics of cultivable intestinal bacteria. The exception was a significant reduction in haemolytic lumen bacteria and an increase in LAB lumen bacteria recovered from the colon of HWE treated mice. Since haemolytic bacteria are considered to have a detrimental health effect while LAB have a positive health promoting effect, further feeding

studies may be carried out to examine the prebiotic potential of this extract in these enteric populations.

A multiplex PCR was optimised and applied to survey the prevalence of eight common colicin genes (Colicins A, D, E1, E2, E6, E7, Ia and V) in commensal and pathogenic *E. coli* isolates. Results revealed that there was a higher carriage of colicin genes in commensal *E.* coli isolates compared to pathogenic *E. coli* isolates. There have been few studies applying a quantitative assay on the inhibitory action of colicins. This project utilized a KIMA assay to assess the inhibitory activity of a previously identified set of porcine commensal colicinogenic *E. coli* that against five pathogenic *E. coli* strains. The assay was found to be both sensitive and reproducible. Taken together, an important outcome of this research was the use of gene screening protocols for colicins to identify and select commensal isolates in possession of desired combinations of colicin genes. KIMA can then be used to validate the functionality of selected commensals in terms of their capacity to inactivate pathogenic strains that cause post-weaning diarrhoea with a view to their use as probiotics. Future feeding studies could be developed to determine their *in vivo* inhibitory action against pathogenic *E. coli* including common serotypes responsible for post-weaning diarrhoea in pigs. From this, the ability of pathogenic *E. coli* strains to develop resistance could be further studied, to understand the mechanisms involved and prevention. The occurrence of colicin V in pathogenic *E. coli* could also be studied to better understand the association between this colicin and pathogenicity.

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Appendix

Appendix 1. Bacteriological media for the cultivation of bacteria

Blood Agar

Suspend 40g agar in 1L deionized water. Bring to the boil to dissolve completely. Sterilise by autoclaving at 121°C for 15 minutes. Cool to 50°C and add 7% sterile blood.

Mix with gentle rotation and pour into sterile dishes

CATC Agar

Suspend 56g agar in 1L deionized water. Bring to the boil to dissolve completely. Sterilise by autoclaving at 121°C for 15 minutes. Cool to 50°C and add:

- 20ml 10 % sodium carbonate solution
- 10ml 1 % 2,3,5-triphenyltetrazolium chloride solution
- 4ml 10 % sodium azide solution

each filter-sterilized

Mix with gentle rotation and pour into sterile dishes

KEA Agar

Suspend 47.5g agar in 1L deionized water. Bring to the boil to dissolve completely. Sterilise by autoclaving at 121°C for 15 minutes. Cool to 50°C.

Mix with gentle rotation and pour into sterile dishes

Low melt agarose (1.5%)

To 1L deionized water add 15g low melt agar. Sterilise by autoclaving at 121°C for 15 minutes.

Luria-Bertani (LB) Broth

To 1L deionized water add:

Bacto-Tryptone	10.0 g
Bacto yeast extract	5.0 g
NaCl	10.0 g

Sterilise by autoclaving at 121°C for 15 minutes

LB Agar

To 1L deionized water add:

Bacto-Tryptone	10.0 g
Bacto yeast extract	5.0 g
NaCl	10.0 g
Agar	15.0 g

Sterilise by autoclaving at 121°C for 15 minutes. Cool to 50°C and pour into sterile petri dishes

MAC Agar

Suspend 50g agar in 1L deionized water. Bring to the boil to dissolve completely. Sterilise by autoclaving at 121°C for 15 minutes. Cool to 50°C.

Mix with gentle rotation and pour into sterile dishes

MRS Agar

Suspend 66.2g agar in 1L deionized water. Bring to the boil to dissolve completely. Sterilise by autoclaving at 121°C for 15 minutes. Cool to 50°C.

Mix with gentle rotation and pour into sterile dishes

Appendix 2. Buffers

Phosphate Buffered Saline (PBS)

To 1L deionized water add:

137 mM NaCl
3 mM KCl
8 mM Na_2HPO_4
1 mM KH_2PO_4

Final pH 7.2

Sterilise by autoclaving at 121°C for 15 minutes. Cool at room temperature and store at 4°C

Tris-Borate-EDTA (TBE)

To 1L deionized water add:

5.4g/L Trisbase
2.75g/L Boric Acid
2ml/L 0.5M EDTA pH 8

Sterilise by autoclaving at 121°C for 15 minutes. Cool and store at room temperature

TE

To 100mL deionized water add:

5.4g/L Trisbase
EDTA 0.25 M

Adjust pH to 8.0 with HCl

Sterilise by autoclaving at 121°C for 15 minutes. Cool and store at room temperature

Appendix 3. *E. coli* isolates screened for colicin genes using multiplex PCR

Table 3.1. Porcine commensal *E. coli* isolates sourced from the duodenum. DC: duodenum commensal.

Identifying code	Serotype	Intestinal section
DC 01	Ont:H45	Duodenum
DC 02	Ont:H45	Duodenum
DC 03	Ont:H45	Duodenum
DC 04	Ont:H45	Duodenum
DC 05	Ont:H45	Duodenum
DC 06	Ont:H45	Duodenum
DC 07	Ont:H-	Duodenum
DC 08	O20/R:H-	Duodenum
DC 09	O20:H-	Duodenum
DC 10	Ont:H-	Duodenum
DC 11	Ont:H32	Duodenum
DC 12	O5:H-	Duodenum
DC 13	O106:H-	Duodenum
DC 14	O114:H-	Duodenum
DC 15	O105/O120:H19	Duodenum
DC 16	Ont:H2	Duodenum
DC 17	O88:H25	Duodenum
DC 18	Ont:H45	Duodenum
DC 19	Ont:H-	Duodenum
DC 20	Ont:H-	Duodenum
DC 21	Ont:H-	Duodenum
DC 22	O40:H-	Duodenum
DC 23	Ont:H25	Duodenum
DC 24	Ont:H25	Duodenum
DC 25	O77:H-	Duodenum
DC 26	Ont:H45	Duodenum
DC 27	O80/R:H26	Duodenum
DC 28	O75:H32	Duodenum
DC 29	Ont:H-	Duodenum
DC 30	O16:H48/HR	Duodenum

Table 3.2. Porcine commensal *E. coli* isolates sourced from the ileum. ILC: ileum commensal.

Identifying code	Serotype	Intestinal section
ILC 01	O40:H25	Ileum
ILC 02	O8:H-	Ileum
ILC 03	Ont:H9	Ileum
ILC 04	O40:H25	Ileum
ILC 05	O40:H25	Ileum
ILC 06	Ont:H-	Ileum
ILC 07	Ont:H-	Ileum
ILC 08	O100:H-	Ileum
ILC 09	O100:H-	Ileum
ILC 10	Ont:H-	Ileum
ILC 11	Ont/R:H-	Ileum
ILC 12	Ont/R:H-	Ileum
ILC 13	Ont/R:H-	Ileum
ILC 14	Ont/R:H-	Ileum
ILC 15	Ont:H27	Ileum
ILC 16	Ont:H27	Ileum
ILC 17	O5:H-	Ileum
ILC 18	Ont:H34	Ileum
ILC 19	Ont:H-	Ileum
ILC 20	Ont:Hnt	Ileum
ILC 21	O18 a,b:H-	Ileum
ILC 22	Ont:H3	Ileum
ILC 23	Ont:H-	Ileum
ILC 24	Ont:H-	Ileum
ILC 25	Ont:H39	Ileum
ILC 26	O82:H8	Ileum
ILC 27	O88:H8	Ileum
ILC 28	O91:H38	Ileum
ILC 29	O84:Hnt	Ileum
ILC 30	O25:H32	Ileum
ILC 31	O2:H8	Ileum
ILC 32	O2:H8	Ileum
ILC 33	O2:H8	Ileum
ILC 34	O2:H8	Ileum
ILC 35	Ont:H45	Ileum
ILC 36	Ont:H27	Ileum
ILC 37	OR:H48	Ileum
ILC 38	Ont:H45	Ileum
ILC 39	0152:H51	Ileum

Table 3.3. Porcine commensal *E. coli* isolates sourced from the colon. CC: colon commensal.

Identifying code	Serotype	Intestinal section
CC 01	O152:H51	Colon
CC 02	Ont/R:H9	Colon
CC 03	O40:H25	Colon
CC 04	O40:H25	Colon
CC 05	O25:H-	Colon
CC 06	Ont:H-	Colon
CC 07	Ont:HR	Colon
CC 08	Ont:Hnt	Colon
CC 09	O5:H45	Colon
CC 10	Ont:H2	Colon
CC 11	O8:H-	Colon
CC 12	Ont:HR	Colon
CC 13	Ont:Hnt	Colon
CC 14	Ont:HR	Colon
CC 15	Ont:HR	Colon
CC 16	O114:H-	Colon
CC 17	Ont:H-	Colon
CC 18	Ont:H-	Colon
CC 19	Ont:H-	Colon
CC 20	Ont:H-	Colon
CC 21	O89:H38	Colon
CC 22	Ont:Hnt	Colon
CC 23	Ont:Hnt	Colon
CC 24	Ont:Hnt	Colon
CC 25	Ont:H-	Colon
CC 26	Ont:Hnt	Colon
CC 27	O5(related):H26	Colon
CC 28	O93:H-	Colon
CC 29	O82:H8	Colon
CC 30	O82:H8	Colon
CC 31	O82:H8	Colon
CC 32	Ont:H-	Colon
CC 33	O105/O120:H19	Colon
CC 34	Ont:H-	Colon
CC 35	O71:H-	Colon
CC 36	O51:H27	Colon
CC 37	O51:H27	Colon
CC 38	O5(related):H26	Colon
CC 39	O5(related):H26	Colon

Table 3.4. Porcine commensal *E. coli* isolates sourced from the colon. FC: Faecal commensal.

Identifying code	Serotype	Intestinal section
FC 01	O40:H25	Faeces
FC 02	O40:H25	Faeces
FC 03	O40:H25	Faeces
FC 04	O40:H25	Faeces
FC 05	O40:H25	Faeces
FC 06	O40:H25	Faeces
FC 07	O40:H25	Faeces
FC 08	O40:H25	Faeces
FC 09	O40:H25	Faeces
FC 10	O40:H25	Faeces
FC 11	O40:H25	Faeces
FC 12	O40:H25	Faeces
FC 13	Ont:H-	Faeces
FC 14	O40:H25	Faeces
FC 15	OR:H-	Faeces
FC 16	OR:H-	Faeces
FC 17	OR:H38	Faeces
FC 18	O21:H25	Faeces
FC 19	O89:H38	Faeces
FC 20	OR:H-	Faeces
FC 21	O114:H-	Faeces
FC 22	Ont:H-	Faeces
FC 23	O105/O120:H19	Faeces
FC 24	O8:H9	Faeces
FC 25	O114:H-	Faeces
FC 26	O5(related):H26	Faeces
FC 27	O8:H-	Faeces
FC 28	O51:H-	Faeces
FC 29	O114:H-	Faeces
FC 30	O51:H-	Faeces
FC 31	Ont:H10	Faeces
FC 32	O5(related):H26	Faeces
FC 33	O5(related):H26	Faeces
FC 34	Ont:H-	Faeces
FC 35	O25:HR	Faeces
FC 36	O71:H-	Faeces
FC 37	O153:H-	Faeces
FC 38	Ont:H26	Faeces
FC 39	O71:H-	Faeces
FC40	O25:H32	Faeces
FC 41	O8:H8	Faeces
FC 42	O25:H32	Faeces
FC 43	Ont:H-	Faeces
FC 44	O82:H8	Faeces

Table 3.5. Characteristics of pathogenic *E. coli* isolated from diseased human subjects. EPEC: enteropathogenic *E. coli*, EHEC: enterohemorrhagic *E. coli*, D: diarrhea, F; faeces, HUS: hemolytic uremic syndrome, UTI: urinary tract infection, NT: not tested.

Strain	Serotype	Category	Source
DES B1	O26:H11	EHEC (EPEC)	D
DES H24	O86:K61:H-	EPEC	D
DES H7	O91:H10	EHEC	D
DES A1	O113:H21	EHEC	D
DES H14	O123:H-	NT	D
DES H8	O128:H2	EHEC (EPEC)	D
DES H19	O128:H2	EHEC (EPEC)	D
DES 2004	Ont:H-	NT	D
DES 1644	O111:H-	EHEC (EPEC)	D
DES 1643	O157:H7	EHEC	D
5485	O55:H-	EPEC	D
2665	O111:H2	EHEC (EPEC)	D
9090(1)	O111:H4	NT	D
5480	O119:H2	NT	D
5483	O126:H12	NT	D
7413	O111:H-	EHEC (EPEC)	HUS
5768	O128:H2	EHEC (EPEC)	HUS
8469	O75:H7	UPEC	D
8982	O2:H-	UPEC	UTI
1616	O2:H4	UPEC	UTI
8985	O4:H5	UPEC	UTI
5584	O6:H1	UPEC	UTI
8990	O75:H-	UPEC	UTI
9983	O7:H6	UPEC	D
2766	O25:H1	UPEC	D
DG1-10	NT	NT	F
JA1-18	NT	NT	F
STJ1-1	NT	NT	F
FF1-78	NT	NT	F
TA165	Ont:H-	NT	Possum
STJ1-K4	NT	NT	Septic tank
ECOR60	O4:HN	ECOR collection	UTI
ECOR66	O4:H40	ECOR collection	Ape
ECOR68	ON:NM	ECOR collection	Giraffe
S. flexneri 1	serotype 1	NT	F
S. flexneri 2	serotype 2a	NT	F
TW4393	NT	NT	F
TW6395	NT	NT	F

Table 3.6 Characteristics of pathogenic *E. coli* isolated from piglets post-weaning diarrhea (PWD). NSW: New South Wales, QLD: Queensland.

Strain	Serotype	Disease syndrome	Origin
438/3	O8:F41	PWD	NSW
453/3	O8:K88	PWD	NSW
603/2	O8:K88	PWD	NSW
231/3	O8:K88	PWD	NSW
580/2	O8:K99	PWD	NSW
439/3	O8:K99	PWD	NSW
552/2	O9:F41	PWD	NSW
590/2	O9:F41	PWD	NSW
690/2	O9:F41	PWD	NSW
121/3	O9:987P	PWD	NSW
581/2	O20:K99	PWD	NSW
634/2	O20:K99	PWD	NSW
635/2	O101:F41	PWD	NSW
734/3	O157:K88	PWD	NSW
P61/3	O8:F-	PWD	Vietnam
P137/2	O64:k99	PWD	Vietnam
P208/1	O101:k88	PWD	Vietnam
287/3	O8:G7	PWD	NSW
557/2	O8:G7	PWD	NSW
03/01	O8:G7	PWD	NSW
256/3	O139	PWD	NSW
0964/2	O141:K85ac	PWD	NSW
757/2	O141:K85ac	PWD	NSW
286/3	O141:K85ac	PWD	NSW
039/2	O141:K85ac	PWD	NSW
154/3	O141:K85ac	PWD	NSW
145/3	O141:K85ac	PWD	NSW
706/00	0141ab	PWD	NSW
045/01	0141ac	PWD	NSW
0354/2	O149:K88	PWD	NSW
0489/2	O149:K88	PWD	NSW
077/3	O149:K88	PWD	NSW
043/3	O149:K88	PWD	NSW
0962/1	O149:K88	PWD	NSW
0586/2	O149:K88	PWD	NSW
283/01	O149:K88	PWD	NSW
P127/1	O8:G7	PWD	Vietnam
P6	O149:K91:K88	PWD	Vietnam
P139/1	O149:K91:K88	PWD	Vietnam
P140/2	O149:K91:K88	PWD	Vietnam
P140/3	O149:K91:K88	PWD	Vietnam
VPC2	O149:K91:K88	PWD	Vietnam
VPC5	O149:K91:K88	PWD	Vietnam
VPC55	O149:K91:K88	PWD	Vietnam
VPC63	O149:K91:K88	PWD	Vietnam
CDT15	O149:K91:K88	PWD	Qld
CDT28	O149:K91:K88	PWD	Qld
CDT43	O149:K91:K88	PWD	Qld
CDT44	O149:K91:K88	PWD	Qld
CDT47	O149:K91:K88	PWD	Qld
CDT48	O149:K91:K88	PWD	Qld
CDT50	O149:K91:K88	PWD	Qld
CDT60	O149:K91:K88	PWD	Qld
CDT61	O149:K91:K88	PWD	Qld
CDT84	O149:K91:K88	PWD	Qld
CDT91	O149:K91:K88	PWD	Qld
CDT97	O149:K91:K88	PWD	Qld
CDT104	O149:K91:K88	PWD	Qld
CDT108	O149:K91:K88	PWD	Qld
CDT109	O149:K91:K88	PWD	Qld
CDT110	O149:K91:K88	PWD	Qld
CDT111	O149:K91:K88	PWD	Qld
CDT114	O149:K91:K88	PWD	Qld
CDT115	O149:K91:K88	PWD	Qld
CDT116	O149:K91:K88	PWD	Qld
CDT129	O149:K91:K88	PWD	Qld

Appendix 4: Optimised Multiplex PCR Conditions

Table 1. Volumes of PCR cocktails used for optimised multiplex PCR pools 1-3. Forward primers are represented by the letter f and reverse primers are represented as r.

Reagents		Pool 1	Pool 2	Pool 3	Final concentration during PCR
10 X PCR buffer with $MgCl_2$		5µl	5µl	5µl	1.5 mM $MgCl_2$
10 mM dNTP mix		1µl	1µl	1µl	each dNTP, 200µM
25 mM $MgCl_2$		1µl	5µl	3µl	Pool 1: 0.5 mM $MgCl_2$ Pool 2: 2.5 mM $MgCl_2$ Pool 3: 1.5 mM $MgCl_2$
Hot star Taq (5units/µl)		0.25µl	0.25µl	0.25µl	1.25 units/reaction
Primers	Working primer pair concentrations				
Af	50.0 pmol		1µl		50 pmol
Ar	50.0 pmol		1µl		50 pmol
Df	50.0 pmol			0.1µl	5 pmol
Dr	50.0 pmol			0.1µl	5 pmol
E1f	50.0 pmol		2µl		100 pmol
E1r	50.0 pmol		2µl		100 pmol
E2f	50.0 pmol	0.5µl			25 pmol
E2r	50.0 pmol	0.5µl			25 pmol
E6f	50.0 pmol			0.1µl	5 pmol
E6r	50.0 pmol			0.1µl	5 pmol
E7f	50.0 pmol	0.5µl			25 pmol
E7r	50.0 pmol	0.5µl			25 pmol
Iaf	50.0 pmol		2µl		100 pmol
Iar	50.0 pmol		2µl		100 pmol
Vf	50.0 pmol			1µl	50 pmol
Vr	50.0 pmol			1µl	50 pmol
Pooled DNA		2µl	2µl	2µl	
MQ H_20		38.75µl	26.75µl	36.35µl	
Total		50µl	50µl	50µl	