Isolation and characterization of probiotic *E. coli* strains from various sources

THESIS SUBMITTED TO THE
NATIONAL DAIRY RESEARCH INSTITUTE
KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF

MASTER OF TECHNOLOGY
IN
DAIRY MICROBIOLOGY
BY

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IN
DAIRYING
(DAIRY MICROBIOLOGY)

Approved By

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This is to certify that the thesis entitled **"Isolation and characterization of probiotic *E. coli* strains from various sources"** submitted by **Sukirti Sengupta** towards the partial fulfilment of the requirements for the award of the degree of **Master of Technology in Dairying (Dairy Microbiology)** of the **National Dairy Research Institute (Deemed University)**, Karnal (Haryana), India, is a *bonafide* research work carried out by him under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

Dated: 6th July, 2012

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Dedicated to

Maa, Baba and my beautiful hometown

HOOGHLY

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Abstract

Commensal *Escherichia coli* helps to establish a microbial barrier against potentially pathogenic invaders. They significantly contribute to the development and training of gut associated lymphoid tissue (GALT) and energy supply to the colonic mucosa. The major strains of *E.coli* exhibiting probiotic properties are *E.coli* Nissle 1917, *E.coli* Pingle, *E.coli* M17 and *Ecoli* H22. The first commercial probiotic *E.coli* Nissle 1917 was isolated by Professor Alfred Nissle and is being used as a probioitic since 1916. It is commercially available today as 'Mutaflor'. *E.coli* M17 is also commercially available today as 'Symbioflor'. This strain was identified and is being used as a probiotic for more than 70 years. However further work on the use of *E.coli* for probiotic purpose remained neglected as the focus shifted to lactobacilli and bifidobacteria. The work on *E.coli* still remains limited to few strains only. The *E.coli* isolates were isolated from human and bovine fecal materials and identified by IMViC tests.The isolates were then screened for their antimicrobial action against three gram negative pathogens namely *E.coli* O157:H7, *Salmonella typhi*, and *Shigella dysenteriae* and 15 isolates were selected. The isolates had good acid and bile tolerance attributes and cell surface hydrophobicity. The isolates were then assessed for safety. The isolates did not show multiple drug resistance, however six of the isolates showed presence of stx gene. The isolates E1, E2, E3, E8, E9, E12, E13, E14 and E15 were finally screened. These isolates showed good acid and bile tolerance and cell surface hydrophobicity. The isolates were devoid of stx gene and were not multiple drug resistant and gelatin liquefaction and haemolysis were also negative. Further studies in vivo subjects will determine the suitability of the isolates as probiotics.

सारांश

कोमेन्सल् *ई. कोलाई* रोगजनक और संभावित आक्रमणकारियों रोगजनक के खिलाफ एक सूक्ष्म बाधा स्थापित करने में मदद करता है | वे पेट जुड़े ल्यम्फोइड् ऊतक (गा आ ल ट्) के विकास और प्रशिक्षण के लिए योगदान करते हैं | उनके चयापचय उत्पाद कोलोनिक् म्युकोसा के लिए ऊर्जा आपूर्ति करते हैं । माइक्रोबियल उत्पन्न फैटी एसिड की छोटी चेन कोलोनिक् म्युकोसा के भीतर पानी अवशोषण और रक्त परिसंचरण को प्रोत्साहित करती है| *ई. कोलाई* णिस्स्ले 1917, *ई. कोलाई* पीइन्ग्ल, *ई. कोलाई* एम् 17 और एच् 22 *ई. कोलाई* के प्रमुख उपभेदों जो की प्रोबायोटिक गुण प्रदर्शित् करते है | पहला वाणिज्यिक प्रोबायोटिक *ई. कोलाई* णिस्स्ले 1917 प्रोफेसर अल्फ्रेड णिस्स्ले द्वारा पृथक किया गया और 1916 के बाद से एक प्रोबायोटिक के रूप में इस्तेमाल किया जा रहा है. यह व्यावसायिक रूप से उपलब्ध 'म्युटाफ्लोर्' के रूप में आज उपलब्ध है | *ई. कोलाई* एम् 17 भी ' सिय्म्बिओफ़्लोर्' के रूप में व्यावसायिक रूप से आज उपलब्ध है और एक प्रोबायोटिक के रूप में 70 साल से भी ज्यादा से इस्तेमाल किया जा रहा है| हालांकि प्रोबायोटिक उद्देश्य के लिए *ई. कोलाई* के उपयोग पर आगे का काम उपेक्षित रह गया है क्योकि लेक्टोबेसिलाई और बिफ़िडोबेक्टीरिय पर प्रोबायोटिक के रूप में ध्यान केंद्रित कर दिया है| *ई. कोलाई* पर काम अभी भी कुछ उपभेदों के लिए ही सीमित है | इस् काम मे *ई. कोलाई* आइसोलेट्स मानव और गोजातीय मल से अलग किये गये थे | ईन् इसोलटेस् का आई एम् वी क परीक्षण किया गया था. तत् पश्चात इन् आइसोलेट्स की तीन ग्राम-नेगटिवे रोगज़नक़ों ई. कोलाई O157: एच्7, साल्मोनेला ट्य्फि, और शिगेला ड्य्सेन्टेरिअए के खिलाफ रोगाणुरोधी जांच की गई थी | 15 आइसोलेट्स का सूक्ष्मजिवीरोधी गतिविधि के आधार पर चयन किया गया था | आइसोलेट्स का एसिड और पित्त सहिष्णुता और कोशिका की सतह के सात ह्य्ड्रोफोबिcइट्य् का अध्यन किया गया था | आइसोलेट्स का सुरक्षा कि दृष्टि से मूल्यांकन किया गया था. आइसोलेट्स ने बहुत सी ऐन्टीबायोटिक्स् के खीलाफ़् प्रतिरोध नहीं दिखाई थी, प्राप्त् आइसोलेट्स मे से छह आइसोलेट्स ने विषाक्त *stx* जीन की मौजूद्गी दिखाई थी | E1, E2, E3, E8, E9, E12, E13, E14 और E15 आइसोलेट्स की अंत में जांच की गई. इन आइसोलेट्स ने अच्छी एसिड और पित्त तथा कोशिका की सतह से ह्य्ड्रोफोबिcइट्य् सहिष्णुता प्रदर्शित् की थी | इसक अलव इन आइसोलेट्स ने विभिन्न रोगजनकों के खिलाफ रोगाणुरोधी गतिविधि दिखाई थी | आइसोलेट्स मे *stx* जीन की

उपस्थिति नहीं थी, तथा बहुत सी अन्टिबायोटिक् के खीलाफ़ प्रतिरोध और जेलाटीन द्रवीकरण और हएमोल्य्सिस्

भी नही दिखाया था|

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LIST OF ABBREVIATIONS

- **EcN**: *E.coli* Nissle 1917.
- **IMViC test** : Indole, Methyl red, Voges-Proskauer, Citrate test.
- **EMB agar**: Eosin Methylene Blue Agar.
- **PBS**: Phosphate Buffered Saline.
- **PUM buffer**: Phosphate Urea Magnesium Sulphate buffer.
- **LB broth/agar**: Luria Bertani broth/agar.
- **O.D**: Optical Density.
- **EDTA**: Ethylene Diamine Tetra Acetic Acid.
- **SDS**: Sodium Dodecyl Sulphate.
- **DNA**: Deoxyribo Nucleic Acid.
- **PCR**: Polymerase Chain Reaction.
- **MQ water**: Milli Q water
- **CIA**: Chloroform Isoamyl Alcohol.
- **STEC**: Shiga Toxin producing *E.coli.*

CHAPTER 1

Introduction

INTRODUCTION

The Greek translation of word probiotic is ‘for life’, and according to the currently adopted definition by FAO/WHO, probiotics are: "Live microorganisms which when administered in adequate amounts confer a health benefit on the host".The intestinal microflora consists of about 10^{14}microorganisms, of about 1000 species representing a complex ecological entity with diverse metabolic activities. This is about 10 times the total number of cells in our body that is 10^{13}. These purely numerical reflections point to the significance of indigenous microflora in physiological and pathophysiological process in the gut. Probiotic bacteria possess the ability to survive in the host depending on their metabolic activity, resistant to gastric acidity, adhesion to the mucosal surface, friendly to the host and protect the host against infection. Antimicrobial substances include short chain fatty acid, hydrogen peroxide and bacteriocins. Bacteriocins relatively narrow killing spectrum and are only toxic to bacteria closely related to the producing strain.

Most probiotic bacteria are Gram-positive strains. This is largely because of their ability to persist within the gut ecosystem and produce organic acids such as lactate and acetate. One difficulty with many probiotics, however, is stability within the product. For example, the bifidobacteria are strictly anaerobic, leading to processing difficulties. Attention has therefore turned to less fastidious micro-organisms and recent reports have cited the use of *E. coli* as a probiotic.

Escherichia coli strain Nissle 1917 (EcN) has come a long way since it was first described. In 1917, during World War I, the German physiologist, microbiologist and physician Alfred Nissle (1874–1965) isolated a particular *E. coli* strain that appeared to provide some protection against the intestinal disorders that were highly prevalent in South Eastern Europe at that time. Subsequent observations by Nissle, which included self-experiments, convinced him that there were no harmful side effects associated with this “antagonistically strong *E. coli* strain.” Therefore he began to fill gelatine capsules with bacteria that he had grown on agar plates, and the Mutaflor trademark was born. Since then, beneficial effects of EcN have been reported in various gastrointestinal disorders, ranging from childhood diarrhoea to Inflammatory

Bowel Diseases (IBD). More recently, basic research has identified some of the characteristics of EcN that promote its survival amongst the hostile intestinal microbiota and determine some of the underlying mechanisms mediating the clinical effects of EcN. The Escherichia coli strain Nissle 1917-designated DSM 6601 in the German Collection for Microorganisms in Brauschwieg is one of the best-examined and therapeutically relevant bacterial strains worldwide. EcN, belongs to the common O6 serogroup (serotype O6:K5:H1) which is very heterogeneous and includes non-pathogenic commensals as well as pathogenic variants (mainly diarrhoeagenic and uropathogenic). However, in contrast to many other members of this group, EcN lacks prominent virulence factors (e.g. *E.coli* α-haemolysin, P-fimbrial adhesins). Furthermore, the sequencing of the EcN has indicated that this strain, unlike other nonpathogenic strains, exhibits a specific pattern of fitness factors that promote its survival in the competitive environment of the intestine. For example, EcN exhibits at least six different iron-uptake systems for the generation of energy through ATP and through the production of microcins it excludes the pathogenic *E. coli* and other enteropathogens of the intestinal microbiota. Passage of live EcN has been demonstrated in animals and humans following oral administration.

However, the use of *E. coli* as a probiotic remains limited and except EcN few other strains like *E. coli* M17, *E. coli* Pingle and *E. coli* H22 have been investigated.. The studies on the use of *E. coli* as a probiotic still remains neglected as the major focus has been on lactobacilli and bifidobacteria. The Gram –ve bacteria can be more successful in antagonizing the closely related Gram-ve pathogens as compared to the Gram+ve lactic acid bacteria. Keeping this in view, the present study was taken with the following objectives:

- To isolate and characterize potential probiotic *E. coli* from different sources.
- Screening the potential probiotic isolates for virulence factors and susceptibility to antibiotics to evaluate them for safety.

CHAPTER 2

Review of literature

REVIEW OF LITERATURE

The intestinal microflora consists of about 10^{14}microorganisms, of about 1000 species representing a complex ecological entity with diverse metabolic activities. This is about 10 times the total number of cells in our body that is 10^{13}. These purely numerical reflections point to the significance of indigenous microflora in physiological and pathophsiological process in the gut.

E.coli is one of the most prominent colonizers of sterile gut of the neonate. The metabolic activities of the early aerobic colonizers like *E. coli* prepare the intestinal milieu for subsequent colonization by the anaerobes. Commensal *E. coli* is predominantly found in the colonic mucosa.

The commensal *E. coli* helps to establish a microbial barrier against pathogenic and potentially pathogenic invaders. They significantly contribute to development and training of gut associated lymphoid tissue (GALT). Their metabolic products support energy supply to the colonic mucosa. The short chain fatty acids of microbial origin stimulate water re-absorption, and blood circulation within the colonic mucosa. The major strains of *E. coli* exhibiting probiotic properties are:

- *E. coli* Nissle 1917.
- *E. coli* M17.
- *E. coli* Pingle.
- *E. coli* H22.

E. coli Nissle 1917 was isolated by Professor Alfred Nissle and is being used as a probiotic since 1916. It is commercially available today as ‘Mutaflor’. *E. coli* M17 is also commercially today as ‘Symbioflor’. This strain was identified and used a probiotic for more than 70 years.

2.1 Probiotics:

The gastrointestinal tract is a biologically diverse and complicated system which contains around 10^{14} bacterial cells and up to 1000 species (Savage, D.C., 1977). The microbial population consists of commensurate bacteria and opportunistic pathogens.

Some of the commensurate bacteria with beneficial effects to the host have been employed as probiotics. Probiotics are live microorganisms which when administered in adequate amount confer health benefits to the host. The major groups are *Lactobacilli*, *Bifidobacteria* and some minor groups are *Saccharomyces*, *Streptococcus*, *E. coli* Nissle 1917 and *E. coli* H22 strains have been reported as potential therapeutic agents (Gillor, O., A. Etzion and M.A. Riley, 2008). Probiotic bacteria possess the ability to survive in the host depending on their metabolic activity, resistant to gastric acidity, adhesion to the mucosal surface, friendly to the host and protect the host against infection (Klaenhammer, T.R. and M.J. Kullen, 1999). Antimicrobial substances include short chain fatty acid, hydrogen peroxide and bacteriocins. Bacteriocins relatively narrow killing spectrum and are only toxic to bacteria closely related to the producing strain (Cascales *et.al*, 2007.). Mode of action of bacteriocin produced from gram positive and gram negative microorganism differ and the immune responses activation in host against gram positive and gram negative also vary (Gillor, O., A. Etzion and M.A. Riley, 2008). Probiotics were mainly *Lactobacillus* and *Bifidobacteria*, however enteric *Escherichia coli* isolates were relatively less investigated. A nonpathogenic strain *E. coli* Nissle 1917 has been well established in human medicine since 1917(Buenau *et.al*, 2005).

2.2 *E.coli* as a probiotic:

Escherichia coli strain Nissle 1917 (EcN) has come a long way since it was first described. In 1917, during World War I, the German physiologist, microbiologist and physician Alfred Nissle (1874–1965) isolated a particular *E. coli* strain that appeared to provide some protection against the intestinal disorders that were highly prevalent in southeastern Europe at that time. Subsequent observations by Nissle, which included self-experiments, convinced him that there were no harmful side effects associated with this “antagonistically strong *E. coli* strain.” Therefore he began to fill gelatine capsules with bacteria that he had grown on agar plates, and the Mutaflor trademark was born. (Nissle A. 1916). Since then, beneficial effects of EcN have been reported in various gastrointestinal disorders, ranging from childhood diarrhoea to Inflammatory Bowel Diseases (IBD). EcN, a gram negative bacterium of the *Enterobacteriaceae* family,

belongs to the common O6 serogroup (serotype O6:K5:H1) (Wolf KM. 1997) which is very heterogeneous and includes non-pathogenic commensals as well as pathogenic variants (mainly diarrhoeagenic and uropathogenic) (Blum *et.al*, 1995). However, in contrast to many other members of this group, EcN lacks prominent virulence factors (e.g. *E. coli* α-haemolysin, P-fimbrial adhesins) (Grozdanov *et.al*, 2002). This strain was used successfully under the brand name Mutaflor for treating various gut-related diseases, e.g., chronic constipation, ulcerative colitis, Crohn's disease, or pouchitis. In addition, the strain prevented colonization of the intestine with microbial pathogens in new born infants. In Germany, *E. coli* strain Nissle 1917 is authorized under the brand name Ponsocol for the prophylaxis of neonatal calf diarrhea and has been marketed since 2001(Buenau *et.al*, 2005). *E. coli* H22 produces several antimicrobial compounds with inhibitory capabilities against pathogenic *Enterobacter* spp. and inhibited the gastrointestinal enteric infections (Cursino *et. al*, 2006). Certain strains of *E. coli* isolated from the cattle rumen was found to inhibit *E.coli* K88 and hence could be used as a probiotic in post-weaning diarrhea (Setia *et.al*, 2008). *Escherichia coli* isolates showing anti O157:h7 activity has also been isolated from bovine colon (Etcheverria *et.al*, 2005).

2.3 Isolation of probiotic *Escherichia coli*

Commensal *E. coli* predominate in colonic mucosa of mammals. They help to establish a microbial barrier against pathogenic and potentially pathogenic invaders. They significantly contribute to development and training of gut associated lymphoid tissue (GALT) (Sonnenborn, Schulze., 2004).

Faecal samples were collected from Charles Foster rats of different age groups. Samples were dissolved in 10 mL of 0.85% NaCl and agitated vigorously. Serial dilutions of the resulting suspensions were spread on MacConkey lactose agar and incubated for 24 h at 37°C. Approx 10-15 lactose-positive colonies from each sample were selected to perform IMViC tests. Isolates confirmed IMViC positives were finally transferred to HiCrome Coliform Agar (HiMedia Laboratories). Isolated strains confirmed by above tests as *E. coli* were used to screen for their probiotic ability (Kumar et.al, 2009).

The *E.coli* isolates showing anti O157:H7 activity were isolated from 200 samples of cattle colon from animals whose faeces were negative for Stx1/ Stx2 encoding E. coli as determined by polymerase chain reaction (PCR) (Woodward et al. 1992; Parma et al. 1996). Portions of distal colon (10 cm) were obtained at slaughter and transported on ice to the laboratory for later use. Mucosal swabs were plated onto the surfaces of MacConkey agar plates and then incubated overnight at 37°C. Ten to 15 colonies were randomly selected from each plate to screen their anti-E. coli O157:H7 properties.

Lysogenic, siderophore-producing and colicinogenic strains of *E.coli* were isolated , determined, and supported by Prof. V. Obdržálek (*Department of Microbiology, Faculty of Medicine, Masaryk University in Brno*). All were selected randomly during the summer of 2004 from the daily diagnostic practice of the Department of Microbiology. Each strain was taken from a healthy, human, adult volunteer (both sexes). None of the volunteers suffered from any type of intestinal problem. All donors lived in the city of Brno or its close surroundings (Šmarda *et al.* 2002).

Escherichia coli isolates to be screened for activity bactericidal for or inhibitory to *E. coli* O157:H7 were isolated from cattle feces or cattle gastrointestinal tissue (intestine and colon). Fecal samples were collected from cattle that were confirmed to be negative for *E. coli* O157: H7 by fecal testing. Fifty-five fecal samples were serially diluted (1:10) in 0.1M phosphate buffer (phosphate-buffered saline [PBS]; pH 7.2), 0.1 ml of each dilution was plated onto sorbitol MacConkey agar (SMA), and the plates were incubated for 16 h at 37°C. Up to 10 colonies were randomly selected, and each one was transferred to a test tube containing 10 ml of Trypticase soy broth (TSB). Cultures were incubated for 16 h at 37°C. Sixty-eight tissue samples (1 g each in 9 ml of PBS) were homogenized individually (Ultra-Turrax T25 homogenizer; Janke & Kunkel IKA Labortechnik, Staufen, Germany) at 9,500 rpm for 1 min, and 0.1-ml portions were plated onto the surfaces of SMA plates. The plates were incubated for 16 h at 37°C. Up to 10 colonies were each transferred to test tubes containing 10 ml of TSB, and the tubes were incubated for 16 h at 37°C. *E.coli* isolates showing anti *E.coli* K 88 activity was isolated from cattle rumen, cattle feces, swine feces, and soil (Setia *et.al*, 2008).

2.4 Genotypic identification of *E.coli* isolates:

Escherichia coli has been the appropriate focus for monitoring of potential enteric pathogens in water and foods. Although, several methods have been used for the detection or enumeration of E. coli cells in water and foods, the time and accuracy limitations of these methods suggest the need of a rapid and specific method. By comparison of the gene sequences coding for malic acid dehydrogenase (mdh) of *E. coli* and non-*E. coli* strains, two oligonucleotides were designed and their possible use as E. coli-specific PCR primers was tested. All of the 110 *E. coli* strains tested, including non-pathogenic and various pathogenic strains, generated the expected PCR products with Mw equal to 392 bp. On the other hand, only 97 of these 110 *E. coli* strains were detectable using the BAM gas production method. With the exception of Shigella strains, non-*E. coli* strains, including strains of the family of Enterobacteriaceae, did not generate any false positive PCR results. When this PCR system was used for the monitoring of E. coli cells inoculated into water and milk samples, as low as 10(0) cfu per 100 ml of water or per ml of milk sample could be detected if an 8 h preculture step was performed prior to the PCR. Including the preculture step, the whole PCR detection process may be completed within 12 h (Hsu *et.al*, 2001).

2.5 Probiotic properties of *E. coli* isolates:

2.5.1 Acid tolerance of *E. coli* isolates:

Acid tolerance assay was performed at different pH-1.0, 2.0 and 3.0 for the eight antibiotic sensitive strains. Isolates *E. coli* strains 10, 20 and 16 showed higher acid tolerance whereas *E. coli* strains 3, 44, 45, 14 and 17 showed poor acid tolerance at pH 2.0. But at pH 3.0, all the isolates showed good acid tolerance up to 6 h. However, none of the isolates showed acid tolerance at pH 1.0(Kumar *et.al*, 2009).

Table 2.1: Acid tolerance of *E. coli* isolates

% Survival after incubation							
Strain	p.H. 1.0	p.H. 2.0			p.H. 3.0		
	2h	2h	4h	6h	2h	4h	6h
E.coli 17	0.00±0.00	0.740±0.15	0.090±0.03	0.01±0.00	71.95±2.47	61.50±2.12	39.40±3.68

E.coli 14	0.00±0.00	2.260±0.21	0.600±0.14	0.00±0.00	35.50±4.95	19.80±3.11	11.95±0.92
E.coli 44	0.00±0.00	0.220±0.01	0.150±0.01	0.06±0.01	46.50±6.36	38.65±1.06	35.55±0.21
E.coli 45	0.00±0.00	2.100±0.14	1.180±0.03	0.18±0.01	67.00±5.66	58.15±3.04	14.80±1.13
E.coli 3	0.00±0.00	0.415±0.01	0.350±0.00	0.09±0.01	68.25±2.52	56.00±8.49	43.13±1.28
E.coli 10	0.00±0.00	8.350±0.35	7.350±0.21	6.80±0.28	74.50±4.95	25.15±0.49	21.40±1.41
E.coli 20	0.00±0.00	0.215±0.01	0.130±0.04	0.00±0.00	41.75±2.76	35.53±0.75	31.15±1.63
E.coli 16	0.00±0.00	41.35±0.78	15.70±2.40	0.06±0.01	76.20±1.13	74.10±1.84	45.20±0.85

Acid tolerance of *E. coli* isolates (Kumar *et.al*, 2009)

E. coli strains are known to possess good acid adaptation and acid resistance (Lin *et.al*., 1996). There are three distinct low-pH-induced acid survival mechanisms in *E. coli* (Lin *et.al*., 1995). The first system is expressed in oxidatively metabolizing bacteria grown in complex media but also protects cells in minimal medium at pH. 2.5. This system is not apparent in fermentatively metabolizing cells. The other two systems also protects against pH. 2.5 in minimal medium , only if the medium is supplemented by arginine (i.e. arginine-dependent acid resistance; encoded by *adi)* or glutamate (i.e. glutamate-dependent acid resistance) (Lin *et.al*., 1996). The regulatory gene *rpo*S, is also involved in acid resistance. RpoS is an alternate sigma factor involved in regulating a variety of stress response genes (Lange *et.al*., 1991). Survival via glutamate system for commensal *E. coli* is between 80-100% whereas survival attributed to the glutamate system was between 10-50%. The oxidative system varies according to the strain (Lin *et.al*., 1996).

2.5.2 Antipathogenic properties of probiotic *E.coli* isolates:

2.5.2.1.Antimicrobial properties of probiotic *E.coli* isolates:

In primary screening the antimicrobial activity of pure isolates were determined by agar spot method against *E. coli* (DH5α and BL21) on Luria agar (LA). A total of 47 of 288 isolates of *E. coli* produced zone of inhibition against *E. coli* DH5α and BL21 strains. Secondary screening was performed by induction of 47 isolates with mitomycin C. Antimicrobial activity of the culture filtrates against the test organisms *Escherichia coli*, *Enterobacter asburiae*, *Klebisella* sp., *Staphylococcus aureus*, *Salmonella typhi* and *Salmonella abony.* Culture filtrates which showed antimicrobial activity towards pathogens were treated with proteinase K and protease which resulted in loss of antimicrobial effect thus suggesting that the antimicrobial compound is a protein. Sixteen out of 47isolates showed better inhibitory activity against different pathogens (Kumar *et.al*, 2009).

Table 2.2: Antimicrobial activity of *E. coli* against Enteropathogens

E. coli culture No:	Enteropathogens
10, 14, 14P	*Enterobacter asburiae*
9, 10, 14, 17, 20, 14P	*Klebsiella pneumoniae*
3, 9, 14, 35, 44, 2P, 8P	*Salmonella typhi*
10, 14, 16, 14P	*Staphylococcus aureus*
9, 14, 2P, 8P	*Salmonella abony*

Antimicrobial activity of *E. coli* against Enteropathogens (Kumar *et.al*, 2009).

A total of 216 (18 test strains × 12 *E. coli* K88 indicator strains) individual plate assays were performed. Zones of clearing (no growth) around the test strains were measured with an image analysis device and digital calipers (Alpha Innotech, San Leandro, CA). The most sensitive indicator strain was 2-12 and was subsequently used for screening of the 463 environmental isolates. All 463 environmental isolates were tested using the procedure described above against *E. coli* K88 strain 2-12 in duplicate,

and positive assays showing inhibition were repeated. Approximately 25% of the 463 environmental isolates produced inhibitory activity against the K88 indicator strain (2-12), but none were from the rumen.

Table 2.3:Sources of environmental *E. coli* isolates and distribution of colicin like activity

Source of isolates	Number of isolates	Strains inhibiting K88	Noninhibiting
Cattle feces	358	96	262
Rumen fluid	33	0	33
Swine feces	35	9	26
Manure amended soil	37	16	21
Total	463	121	342

Sources of environmental *E.coli* isolates and distribution of colicin like activity (Setia *et.al*, 2008).

Schamberger and Diez – Gonzalez (2002) screened isolates of *E. coli* from the feces of cats, cattle, chickens, deer, dogs, ducks, horses, humans, pigs, and sheep. They found colicinogenic strains from all sources, but the greatest number was from cats and sheep. No rumen isolates evaluated were effective at inhibiting K88, but isolates from cattle feces were effective. This is rather interesting because it suggests that even though all isolates were phenotypically *E. coli*, there are subtle differences between those inhabiting the rumen compared to those in the hind-gut (Setia *et.al*, 2008).

Colonies isolated from the colon samples were picked with a sterile, sharp-ended toothpick and stab-inoculated into trypticase soy broth–1.2% agar plates and incubated overnight at 37^0C. Then, the bacteria were lysed by exposing to chloroform vapour for 1 h into an airtight container. Subsequently, chloroform was allowed to evaporate for another hour. Next, the plates were overlaid with 5 ml of LB–0.4% agar containing 10^6 E. coli O157:H7 CFU ml)$^{-1}$ and incubated overnight at 37^0C. Bacteria around which were observed zones of growth inhibition of the indicator strain wider than 3 mm were selected as potential probiotic strains (Fredericq 1965; Jordi *et al*. 2001). These bacteria were screened for the presence of virulence factors: stx1, stx2, ehxA, eae, st1a and lt1 by PCR as described previously (Woodward *et al*. 1992; Fratamico *et al*. 1995; Parma *et al*. 1996; Paton *et al*. 1996). Thirteen strains demonstrated the ability to inhibit the

growth of the indicator strain, BP 166. For further studies we selected only seven of them, which corresponded to the strains producing the largest and clearest growth inhibition zones of the indicator strain. All of them were identified as *E. coli* by biochemical tests (Cowan and Steel 1993). The absence of stx1, stx2, eae, ehxA, stIa and ltI virulence genes was demonstrated in all the selected strains when screened by PCR. Additionally, 11 O157:H7 isolates (stx2+, eaeO157+, ehxA+) from grazing cattle (three isolates), grain-fed cattle (three isolates) and humans (HUS, three isolates; bloody diarrhoea, two isolates) were also inhibited by the potential probiotic *E. coli* strains.

53 strains were tested both as a possible producer and as a possible sensitive strain to any of the four inhibitory factors followed. For this testing, a modified Fredericq's (1946) agar stab test for colicinogeny was applied in a 53 by 57 matrix. Agar plates were inoculated by needle stab with fresh broth cultures and incubated for 20 h at 37°C. The plated bacteria were then killed by exposure to chloroform vapor and each plate was overlaid with a thin layer of soft agar containing 107 cells per mL of a fresh indicator strain broth culture. After solidification, the plates were incubated at 37 °C overnight. All 53 strains were cross-tested – as possible producers and as possible indicators – and further tested against 4 additional indicator strains. Each pair of strains was tested repeatedly. The presence of LMW colicin appeared as a growth-inhibition zone that formed around the macrocolony of the producer strain; no LMW bacteriocin zone appeared on agar that contained trypsin. The presence of lysogenic producers appeared as phage plaques (mostly tiny ones) that were scattered around the macrocolonies. The phages were auto-reproducible in subcultures. The presence of HMW colicin appeared as a growth-inhibition zone that was conspicuously narrow, compared to LMW colicin zones and, unlike LMW colicin, it was not sensitive to trypsin. The presence of siderophore appeared as a growth inhibition zone that was usually very wide, only partially hampered growth, was not delineated by a distinct (sharp) edge, and was not sensitive to trypsin. However, the zone was much more distinct on iron-limited agar, *i.e*. on nutrient agar containing the iron-chelating compounds (*see* nutrient media). The siderophore strain specificity was in some cases very low (Smarda *et.al*, 2006).

2.5.2.2 Immunomodulatory action of probiotic *E.coli* isolates:

Inducible epithelial human beta-defensins (hBD) play an important role in intestinal barrier function. *In vitro* studies showed that clinically effective probiotics induce antimicrobial hBD-2. Here, the study was aimed to assess the *in vivo* effect in healthy volunteers and also addressed how defensins affect probiotic survival. Symbioflor 2 containing one strain of several viable genotypes of *Escherichia coli* was administered to 23 healthy individuals. After 3 weeks, fecal hBD-2 peptide was increased in 78 % (mean 3.7-fold; $P < 0.0001$). Interestingly, the fecal hBD-2 peptide was still elevated 9 weeks after treatment ($P = 0.008$). *In vitro* studies revealed that this effect was mediated by only one out of three tested *E. coli* genotypes and comparable to probiotic *E. coli* Nissle 1917 (10- to 15-fold). Functional assays showed that all tested bacteria were similarly killed by defensins allowing to speculate about a suicidal character of this effect. Defensin induction seems to be a common and important mechanism of probiotic treatment (Mondel *et.al*, 2008).

2.5.2.3 Competitive exclusion by probiotic *E.coli* isolates:

Different *Escherichia coli* strains generally have the same metabolic capacity for growth on sugars in vitro, but they appear to use different sugars in the streptomycin-treated mouse intestine (Fabich *et al*.,2008). Here, mice were precolonized with any of three human commensal strains (*E. coli* MG1655, *E. coli* HS, or *E. coli* Nissle 1917) and 10 days later were fed 10^5 CFU of the same strains. While each precolonized strain nearly eliminated its isogenic strain, confirming that colonization resistance can be modeled in mice, each allowed growth of the other commensal strains to higher numbers, consistent with different commensal *E. coli* strains using different nutrients in the intestine. Mice were also precolonized with any of five commensal *E. coli* strains for 10 days and then were fed 105 CFU of *E. coli* EDL933, an O157:H7 pathogen. *E. coli* Nissle 1917 and *E. coli* EFC1 limited growth of *E. coli* EDL933 in the intestine (10^3 to 10^4 CFU/gram of feces), whereas *E. coli* MG1655, *E. coli* HS, and *E. coli* EFC2 allowed growth to higher numbers(10^6 to 10^7 CFU/gram of feces). Importantly, when *E. coli*

EDL933 was fed to mice previously co-colonized with three *E. coli* strains (MG1655, HS, and Nissle 1917), it was eliminated from the intestine (<10 CFU/gram of feces). These results confirm that commensal *E. coli* strains can provide a barrier to infection and suggest that it may be possible to construct *E. coli* probiotic strains that prevent growth of pathogenic *E. coli* strains in the intestine.

Two *E. coli* strains, EMO, isolated from human feaces and JM105 K-12 were tested for their ability to prevent in vivo and in vitro infection by *Salmonella typhimurium* C5.

Inhibition of C5 cell invasion by *E.coli* was investigated in vitro using Caco-2/TC7 cells. The protective effect of *E.coli* was examined in vivo in germ free mice or conventional C3H/He/Oujco mice orally infected by the lethal strain C5.

In vitro the two strains did not prevent the growth of C5 by secreted microcins or modified cell invasion of C5. In vivo establishment of EM0 or JM105 in the gut of germfree mice resulted in significant increase in the number of surviving mice. 11 out of 12 mice and 9 out of 12 mice survived respectively, at 58 days after infection (2×10^6/ mouse) versus 0/12 in control germfree group at 13 days after infection. Colonisation level and translocation rate of C5 were significantly reduced during the three days after infection. So establishment of *E.coli* strains, which do not display antimicrobial activity, protects germfree mice against infection and delays the establishment of C5 in the gut.

2.6 Safety evaluation of probiotic *E.coli* isolates:

2.6.1 Antibiotic Resistance of the *E.coli* isolates:

E. coli strains were examined for resistance to amikacin (30 µg), amoxyclav (30 µg), ampicillin (10 µg), ceftazidime (30 µg), cephotaxime (30 µg), co-trimoxazole (25 µg), ciprofloxacin (5 µg), cefuroxime (30 µg), furazolidone (100 µg), gentamicin (10 µg), nitrofurantoin (300 µg), nalidixic acid (30 µg), netilin (30 µg), ofloxacin (10 µg) and tetracycline (30 µg) and tobramycin (10 µg) using commercial discs (HiMedia Laboratories). *E. coli* strain BL21 sensitive to all the antibiotics was included for quality control. Characterization of strains as susceptible, resistant or having reduced susceptibility was done in accordance with the manufacturer's instructions on sizes of inhibition zones around each disc, which matched the interpretive criteria recommended

by the Clinical and Laboratory Standards Institute (CLSI). Most isolates did not show multi-drug resistance and the isolates which showed low multiple resistance were eliminated in initial screening with commercial antibiotics. Sixteen isolates were finally screened by using commercial antibiotics discs(Kumar *et.al*, 2009).

Table 2.4: Antibiotic susceptibility pattern of *E.coli* isolates

St. No.	A 10	G 10	Ac 30	Tb 10	Co 25	Ce 30	Na 30	Nt 30	Nf 300	Ak 30	Cf 5	Ca 30	Of 5	T 25	Cu 30
3	S	S	I	S	S	S	S	S	S	S	S	S	S	S	S
6	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
9	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
10	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
14	S	S	I	S	S	S	S	S	S	S	S	S	S	S	S
16	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
17	S	S	R	S	S	S	S	S	I	S	S	S	S	S	S
19	R	S	I	S	S	S	S	S	S	S	S	S	S	S	S
20	S	S	I	S	S	S	S	S	I	S	S	S	S	S	S
21	S	S	S	S	S	S	S	S	I	S	S	S	S	S	S
35	I	S	I	S	S	S	S	S	S	S	S	S	S	S	S
44	S	S	I	S	S	S	S	S	R	S	S	S	S	S	S
45	S	S	S	S	S	S	S	S	I	S	S	S	S	S	S
2P	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
8P	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
14P	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

Antibiotic susceptibility pattern of *E.coli* isolates(Conc. Of antibiotic in µg/ml)(Kumar *et.al*, 2009).

The antibiotic sensibility of the probiotic *E.coli* isolates determined by Etcheverrı´a *et.al*, 2005 following the Bauer– Kirby procedure (Chengappa 1990). The amount of each antibiotic per disc was: ampicillin (10 micro g), cephalothin (30 micro g), cephotaxime

(30 micro g) ciprofloxacin (5 micro g), chloramphenicol (30 micro g), gentamicin (10 micro g), imipenem (10 micro g), trimethoprim/sulfamethoxazole (TMS) (25 micro g).

Table 2.5: Antibiotic susceptibility of bacteriocin-producing bacteria

Antibiotics	**Bacteriocin-producing bacteria**						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Ampicillin	S	S	S	S	S	S	S
Cephalothin	S	R	R	I	S	R	R
Cephotaxime	S	S	S	S	S	S	S
Ciprofloxacin	S	S	S	S	S	S	S
Chloramphenicol	S	S	S	S	S	S	S
Gentamicin	S	S	R	I	S	S	S
Imipenem	S	S	S	S	S	S	S
TMS	S	S	S	S	S	S	S

Antibiotic susceptibility of bacteriocin-producing bacteria (Etcheverrı´a *et al.* 2005)

2.6.2. Multiplex PCR Assay for Identification of Human Diarrheagenic *E.coli* :

. *Escherichia coli* is the most important etiologic agent of childhood diarrhea and represents a major public health problem in developing countries. Identification of diarrheagenic *E. coli* strains requires that these organisms be differentiated from nonpathogenic members of the normal flora. Serogrouping of O antigen is not sufficient to identify a strain as diarrheagenic, because it does not correlate, in most cases, with the presence of virulence factors. Thus, identification of diarrheagenic *E. coli* strains needs to detect factors that determine the virulence of these organisms. With the advent of PCR, it has become possible to detect pathogenic genes in bacterial isolates, allowing the rapid diagnosis of diarrheagenic *E. coli*. PCR methods using single primer sets have been reported elsewhere, but screening of bacterial isolates requires a large number of individual PCRs if single primer sets are used in separate reactions. To reduce the number of tests needed for diagnosis of diarrheagenic *E. coli*, several multiplex PCR systems have been reported previously. However, usually more than one multiplex PCR is required for identification of a diarrheagenic *E. coli* strain. Pass *et*

al.2000 reported a multiplex PCR to detect 11 virulence genes, but it has not been fully evaluated against a large panel of isolates. This study attempted to develop a multiplex PCR for identification of enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), and Shiga toxin producing *E. coli* (STEC). Thirteen *E. coli* control strains were used in this study . EPEC, STEC, and EIEC strains were characterized in previous studies and confirmed to have the relevant gene by single PCRs and phenotypic assays. For ETEC strains, the production of heat-labile enterotoxin was determined by a reversed passive latex agglutination test (Denka Seiken, Co., Ltd., Tokyo, Japan), and the production of heat-stable enterotoxin(ST) was determined by an enzyme immunoassay kit (Denka Seiken Co., Ltd.). For EAEC strains, the HEp-2 cell adherence assay was performed as described by Cravioto *et al*, 1979. The targets selected for each category were *eae* for EPEC, *stx* for STEC, *elt* and *est* for ETEC, and *ipaH* for EIEC. The primers to detect the *bfpA* (bundle-forming pilus) gene, which is present in typical EPEC, were not included in this multiplex PCR since the presence of *eae* is sufficient to define EPEC. On the other hand, atypical EPEC strains which do not possess *bfpA* and a high rate of spontaneous cure of the EAF plasmid have been reported previously by Nataro *et al*, 1998. For each of the target genes, different pairs of primers were selected from the literature and tested in a single CR. Universal primers were selected when different alleles could be present to reduce the number of primer sets. Therefore, primer set SK1-SK2 , which can detect all the intimin variants, was used for detection of *eae*; primer set VTcom-u–VTcom-d, which allows amplification of *stx*1, *stx*2, and its variants , was selected for *stx*; and the primer set AL65-AL125 , which reacts with the two ST-I toxin genes (ST-Ia and ST-Ib), was used for detection of the *est* gene. Primers sets LTL-LTR and ipaIII-ipaIV were selected to detect *elt* and *ipaH*, respectively, so that PCR products were sufficiently different in size to be distinguishable by agarose gel electrophoresis. This study identified EPEC, STEC, EIEC, and ETEC strains because the virulence markers for these four categories are well defined. To include the identification of EAEC strains in the multiplex PCR, we selected the primer set aggRks1-aggRkas2, which gave the best result when combined with the other five sets of primers. Some *aggR*-negative strains with aggregative adherence will not be detected by this assay. However,

considering the difficulty of performing phenotypic assays in some laboratories, the multiplex PCR presented in this studyis a practical and rapid diagnostic tool for identification of diarrheagenic *E*. *coli* in a single reaction tube (Toma *et.al*,2003).

CHAPTER 3

Materials and Methods

MATERIALS AND METHODS

3.1. Reagents and media used:

- Mac Conkey Broth
- Purple E.M.B Agar
- Nutrient Agar
- Luria Bertani or Lysogeny Broth
- Normal Saline solution
- PUM Buffer
- n-Hexadecane $C_{16}H_{34}$
- PBS
- Antibiotic discs

3.2. Methods:

3.2.1. Isolation of *Escherichia coli* from human and bovine fecal materials:

Equipments used:

- Olympus CH20i Microscope: Model CH20iBIMF (India).

Method:

Faecal samples were collected from five human and four bovine subjects. Samples were dissolved in 10 mL of 0.85% NaCl and agitated vigorously. Serial dilutions of the resulting suspensions were spread on E.M.B agar (Himedia Laboratories) and incubated for 24 h at 37°C. Approximately 3 to 4 of the characteristic green metallic colonies from each plate were inoculated in MacConkey's lactose broth and incubated overnight for 24 hrs at 37°C. IMViC tests were then performed on the isolates. The isolates were observed under microscope after staining with nigrosin stain. Then Gram's staining was done. Isolated strains confirmed by above tests as *E. coli* were used to screen for their probiotic ability.

3.2.2. Probiotic attributes of the obtained *E.coli* isolates:

3.2.2.1. Antimicrobial activity of the obtained *E.coli* isolates:

Equipments used:

- Sigma 3-18 K centrifuge **(**Germany**).**

Method: Agar well method was followed for evaluation of the antimicrobial activity (Anand *et.al*., 1984). Nutrient agar containing 0.1% of Tween 80 was seeded with 200 µL of overnight grown culture of indicator organisms *E.coli* O157: H7, *Salmonella typhi* and *Shigella dysenteriae* and poured into the plates and were allowed to solidify. Wells (8 mm diameter) were cut and 50 µL cell free culture filtrate of the 24 hr LB broth culture of the *E.coli* isolates was poured into the respective well and incubated at 37°C overnight and the diameter (mm) of inhibition zone around the well was measured.

3.2.2.2. Acid tolerance of the isolated *E.coli* isolates:

Equipments used:

- Sigma 3-18 K centrifuge (Germany).
- p.H. Meter**:** Thermo, Scientific.

Method: *E. coli* strains were grown in Luria broth at 37°C overnight. The cultures were centrifuged at 5000g for 10 min. The pellets were washed in sterile Phosphate-Buffered Saline (PBS) pH 7 and resuspended in PBS. Initial count was determined with appropriate dilution. Each strain was diluted 1/100 in PBS at pH 2.0. Incubation times were 1, 3 and 5 h. Bacterial cultures were then transferred to Luria agar plates and incubated at 37°C overnight. The number of colonies was counted to obtain the viable counts at each time interval. Survival percentage of strains to different pH values was then calculated as percentage survival = (viable counts after acid exposure/initial viable counts) x 100.

3.2.2.3. Bile tolerance of the isolated *E.coli* isolates:

Equipments used:

- Sigma 3-18 K centrifuge (Germany).
- p.H. Meter: Thermo, Scientific.

Method: *E. coli* strains were grown in Luria broth at 37°C overnight. The cultures were centrifuged at 5000g for 10 min. The pellets were washed in sterile Phosphate-Buffered Saline (PBS) pH 7 and resuspended in PBS. Initial count was determined with appropriate dilution. Each strain was diluted 1/100 in PBS at 0.5% and 1.0% ox gall. Incubation times were 1, 3 and 5 h. Bacterial cultures were then transferred to Luria agar plates and incubated at 37°C overnight. The number of colonies was counted to obtain the viable counts at each time interval. Survival percentage of strains to different pH values was then calculated as percentage survival = (viable counts after bile exposure/initial viable counts) x 100.

3.2.2.4. Cell Surface Hydrophobicity of the isolated *E.coli* isolates:

Equipments used:

- Sigma 3-18 K centrifuge (Germany).
- Jenway Spectrophotometer; Model Genova **(**United Kingdom).

Method: Adhesion to n-Hexadecane, a hydrophobic marker was carried out to assess the cell surface hydrophobicity of the lactobacilli cultures. The cell surface hydrophobicity of lactobacilli to adhere to the n-Hexadecane was carried out as per the method described by Rosenberg *et al.* (1980) with required modification as follows. The *E. coli* cultures were grown in LB broth (16-18 hrs at 37°C) and cells were harvested by centrifuging at 2000g for 10 minutes at 4°C, followed by washing (twice) in PUM buffer and finally, suspended in the buffer. Absorbance (A_O) was adjusted to 0.7 OD at 580 nm. The cell suspension (3 ml) and n-Hexadecane (1 ml), taken in clean falcon tubes, incubated at 37°C, 1 h for separation of phases. The lower aqueous phase was taken out and the absorbance was measured (A). The percent cell surface hydrophobicity (H%) was calculated as follows:

$$H\% = \{(A_0 - A)/ A_0\} \times 100 .$$

3.2.3. Safety assessment of the *E.coli* isolates:

3.2.3.1. Antibiotic resistance of *E.coli* isolates:

Method: *E. coli* strains were examined for resistance to amikacin (30 µg), amoxyclav (30 µg), ampicillin (10 µg), ceftazidime (30 µg), cephotaxime (30 µg), ciprofloxacin (5

μg), cefuroxime (30 μg), gentamicin (10 μg), nitrofurantoin (300 μg), netilin (30 μg), tobramycin (10μg) and tetracycline (30 μg) and using commercial discs (HiMedia Laboratories). Characterization of strains as susceptible, resistant or having reduced susceptibility was done in accordance with the manufacturer's instructions on sizes of inhibition zones around each disc, which matched the interpretive criteria recommended by the Clinical and Laboratory Standards Institute (CLSI).

3.2.3.2. Detection of pathogenic strains by PCR of indicator genes:

Equipments used:

- Sigma 3-18 K centrifuge (Germany).
- Genepro Thermal Cycler: Model TC-E-96G (United States of America).
- Cell Biosciences Alpha Manager EP

3.2.3.2.1. Isolation and quantification of Genomic DNA from *E.coli* isolates:

Isolation of genomic DNA was performed as described by Rajeshwari and Sonti (2000).

1. The *E.coli* isolates were cultured in 5 ml LB medium for overnight at 37°C in incubator.
2. 2ml of the culture grown overnight was taken in eppendorf tube.
3. Cells were pelleted by centrifugation at 8000 rpm for 6 min.
4. The pellets were dissolved in 3 ml of solution-I (50 mM glucose + 25 mM TrisCl (pH8) + 10 mM EDTA (pH 8)) and resuspended gently.
5. 600 μl of lysozyme (10 mg/ml stock)was added and it was incubated at room temperature for 20 min.
6. 160 μl of 10% SDS solution was added and incubated again at 50˚C for 10 min. 200μl of RNase A (10mg/ml stock solution) was added to it and it was incubated at 37˚C for 90 min.
7. 170 μl of 0.5M EDTA (pH8) was added and incubated at 50˚C for 10 minutes.
8. The sample was brought to room temp and 200μl Proteinase K (5mg/ml stock solution) was added and incubated for overnight at 37˚C.
9. The samples were removed to corex tubes and 2 volume of phenol saturated with 0.1M Tris-HCl (pH8) was added and mixed thoroughly (for 10 -15 min).
10. It was then centrifuged at 10000 rpm for 15min and the phenol step was repeated for 2 times.

11. The aqueous phase was taken in the corex tube and 2 vol Phenol: CIA (1:1) was added. The mixture was spun at 10000rpm for 15min.
12. The aqueous phase was transferred again into another corex tube and equal volume CIA (24:1)was added and spun at 10000rpm for 15 minutes.
13. The DNA was pooled with a tip and washed with 70% ethanol then spun at 10000rpm for 15min. The pellets were dried and dissolved in 50µl MQ water by keeping at 65°C for 10 min.
14. The quality of DNA was checked by running a 0.7% Agarose gel at 80 volts.

3.2.3.4.2. PCR detection of stx toxin gene: *stx* is shiga toxin gene which is the primary toxin gene found in *E. coli* O157:H7 which belongs to the class STEC. For this the primers VTcom-U and VTcom-D were used (Toma et.al, 2003). The sequence of the primers were:

- VTcom-U: GAGCGAAATAATTTATATGTG
- VTcom-D: TGATGATGGCAATTCAGTAT

. These primers allow the amplification of *stx*1, *stx*2 and its variants. The amplicon size is of 518 bp. The standardized PCR mix was prepared in the following way.

Table 3.1: PCR mix for detection of *stx* gene

Ingredients	Composition (µl)
Nuclease free H_2O	320.40
Taq Buffer	45
DNTP	18
VTcom-U	11.6
VTcom-d	11.6
Taq polymerase	7.38
DNA	2.0

The PCR program was 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min, for 30 cycles, and 72°C for 10 min. PCR products were then electrophoresed on a 2.5% agarose gel, stained with ethidium bromide, and visualized by UV transillumination. The buffer in the electrophoresis chamber and in the agarose gel was 0.5xTris-borate-EDTA.

3.2.3.3. Haemolysis: Bovine blood was collected in a vacutainer. Nutrient agar was prepared and sterilized. It was then cooled to 45 to 50°C, add 5% (vol/vol) sterile blood that has been warmed to room temperature. Swirl the flask to mix thoroughly, avoiding the formation of bubbles, and dispense into sterile plates, continuing to avoid bubbles and froth on the surface. The plates were then kept in a refrigerator for 30 mins and taken out. The cultures were streaked on it and was incubated for 37^0C for 24 hours.

3.2.3.4. Gelatin liquefaction test: Gelatinases are proteolytic enzymes that hydrolyze gelatin into polypeptides and individual amino acids. These enzymes destroy the structure of gelatin and gelatin becomes liquid 5% gelatin was added to nutrient broth to prepare the nutrient gelatin medium. 5 ml of the nutrient gelatin medium was filled in each of the test tubes. A loopful of culture was then inoculated in each of the test tubes**.** The tubes were incubated overnight at 37^0C. The tubes were then taken out from the incubator and kept in a refrigerator for 30 minutes. The tubes were then taken out and kept at 37^0C for 15 minutes. The tubes were then checked for liquification of gelatin. The tubes in which gelatin is still solid should be considered to be gelatinase negative and those that have become liquid should be considered gelatinase positive.

CHAPTER 4

Results and Discussion

RESULTS AND DISCUSSION

4.1 Isolation and identification of the *E. coli* isolates from fecal sources:

A total of 45 isolates of *E. coli* were obtained, out of which 18 isolates came from bovine fecal samples and the rest 27 from human fecal samples. All the isolates were Gram –ve, short and slender rods (Fig 4.1a & b) that produced acid and gas in Mac Conkey's broth, and conformed to the IMVIC test by Indole production, acid production (Methyl red test), negative VP test and no citrate utilisation. The isolates were subcultured in Mac Conkey's broth at regular intervals and were plated on EMB agar. The isolates gave characteristic colonies with green metallic sheen on EMB agar. These 45 isolates were studied further.

4.2 Probiotic attributes of the *E. coli* isolates

4.2.1. Antimicrobial activity shown against different gram negative pathogens:

All the 45 isolates were checked for antimicrobial activity against three different pathogens i.e. *E.coli* O157:H7, *Salmonella typhi NCTC 6017*and *Shigella dysenteriae.* Among these 15 isolates designated from E1 to E15 were selected on the basis of formation of zones around test organisms (Table 4.1). The isolates E1, E2, E4, E6, E8, E9, E10, E12 and E14 were isolated from human fecal samples whereas the isolates E3, E5, E7, E11, E13 and E15 were isolated from bovine fecal samples. Among these isolated E1 and E7 showed antimicrobial properties against all the three pathogens tested. The other isolates showed antimicrobial property against at least one pathogen. In a similar study, Kumar *et.al.* (2009) screened 288 isolates of *E. coli and found 47 isolates that* produced zone of inhibition against *E. coli* DH5α and BL21 strains. Secondary screening performed by using colicin inducer mitomycin C. displayed antimicrobial activity against *Escherichia coli*, *Enterobacter asburiae*, *Klebisella* sp., *Staphylococcus aureus*, *Salmonella typhi* and *Salmonella abony*. Sixteen isolates showed better inhibitory activity against different pathogens.

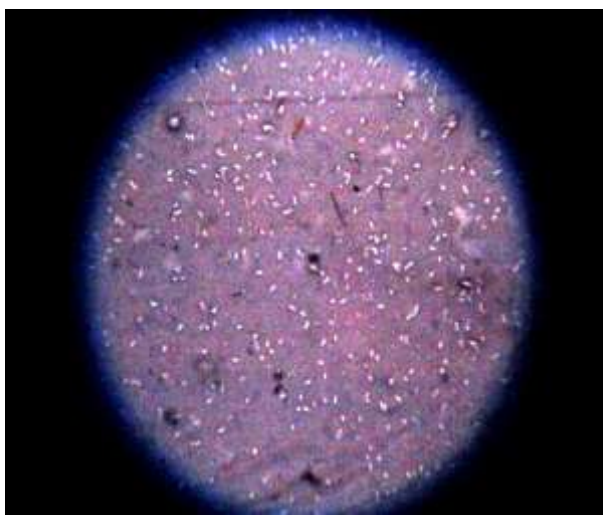

Fig 4.1a. Morphology of the E. coli isolates as seen by negative staining

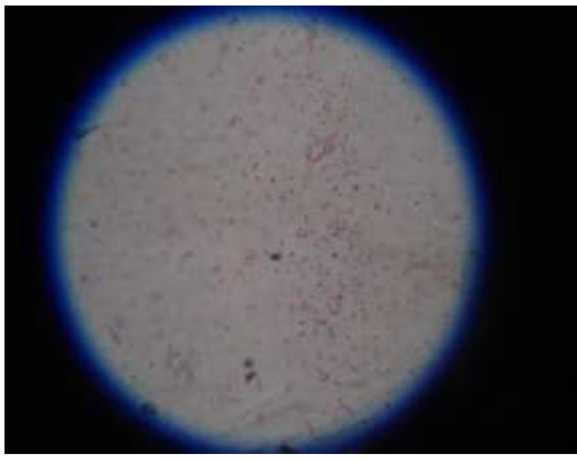

Fig 4.1b. Morphology of the E. coli isolates as seen by Gram's staining

Table 4.1: Antimicrobial property of the selected *E. coli* isolates against enteric pathogens

Sl no.	Strain Designation	Source	*Zone of clearance diameter (cm), including the well diameter of 0.6 cm*		
			***E. coli*O157:H7**	***S. typhi***	***S. dysenteriae***
1.	E2	human	1.6	_	_
2.	E4	human	1.0	_	_
3.	E9	human	1.3	_	_
4.	E1	human	1.0	1.3	1.2
5.	E6	human	_	1.3	_
6.	E8	human	_	_	1.1
7.	E14	human	_	_	1.4
8.	E10	human	_	1.1	_
9.	E12	human	1.2	_	1.3
10.	E3	bovine	_	0.9	_
11.	E5	bovine	_	1.5	_
12.	E11	bovine	1.1	_	_
13.	E7	bovine	1.3	1.2	1.4
14.	E13	bovine	_	1.3	_
15.	E15	bovine	_	_	1.2

Fig 4.3. Antimicrobial activity of the isolates against E. coli O157:H7

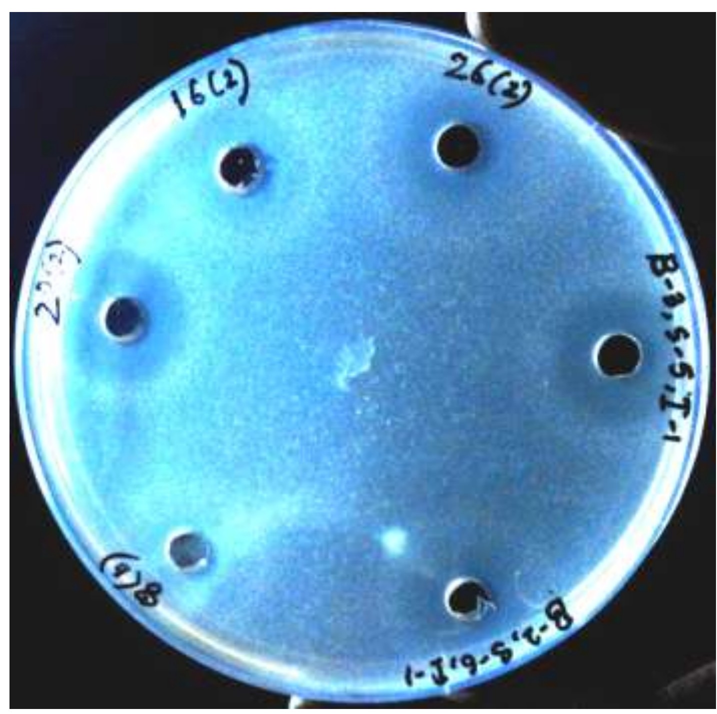

Fig 4.4. Antimicrobial activity of the isolates against S.typhi

The isolates showing antimicrobial property against at least one pathogen were further checked for probiotic attributes including safety aspect. Figure 4.3 shows antimicrobial activity by the isolates E1 and E2 against the pathogenic *E.coli* O157:H7. Figure 4.4 shows the antimicrobial activity by the isolates E1, E6, E10, E5, E7 and E13 against test organism *Salmonella typhi*.

4.2.2. Acid tolerance of the *E.coli* isolates:

The isolates E1 to E15 showed good acid tolerance at pH 2.0. The isolates E1, E4, E9, E13 and E15 showed good acid tolerance even after 5 hours of acid treatment. *E. coli* strains are known to possess good acid adaptation and acid resistance (Lin *et.al.,* 1996). There are three distinct low-pH-induced acid survival mechanisms in *E. coli* (Lin *et. al*, 1995). The first system is expressed in oxidatively metabolizing bacteria grown in complex media but also protects cells in minimal medium at pH 2.5. This system is not apparent in fermentatively metabolizing cells. The other two systems are arginine or glutamate dependent as they protectect against pH. 2.5 (in minimal medium) only if the medium is supplemented with arginine (encoded by *adi)* or glutamate (i.e. glutamate-dependent acid resistance) (Lin et.al, 1996). The regulatory gene *rpo*S, is also involved in acid resistance. RpoS is an alternate sigma factor involved in regulating a variety of stress response genes. Survival via arginine system for commensal *E. coli* is between 80-100% whereas survival attributed to the glutamate system was between 10-50%. The oxidative system varies according to the strain (Lin *et.al*, 1996). The acid tolerance of the *E. coli* isolates was determined at pH 2.0 which is close to the pH of human stomach. The acid tolerance of the isolates is given in Table 4.2.

4.2.3. Bile tolerance of the *E.coli* isolates:

All the coliforma bacteria are known for bile tolerance and bile salts are included in selective and differential media to selectively inhibit Gram +ve bacteria, for the isolation of coliforms or E. coli, All the 15 isolates (E1 to E15) showed bile tolerance in general, however, isolates E1, E7, E8, E10, E13 and E15 showed comparatively higher bile tolerance even after 5 hours of bile treatment (Table 4.3.). The bile treatment in E. coli s led to decrease in counts by not more than one logarithmic cycle, whereas in case of lactobacillus most pf the reports show bile tolerance only

upto 0.3% (Mirlohi, et. al., 2009). The bile tolerance of the *E. coli* isolates upto bile concentration of 1.0% which is close to the bile concentratin of human small intestine. Thanassi et.al (1997) stated that outer membrane barrier of gram-ve bacteria plays an important role in this resistance by retarding the influx of bile salts.

Table 4.2: Acid tolerance of the selected *E. coli* isolates

	Logarithmic count after incubation at pH 2.0			
St. no.	**0 h**	**1h**	**3h**	**5h**
E1	8.10	8.02	7.79	7.73
E2	8.28	8.18	7.90	7.66
E3	8.20	8.10	7.77	7.69
E4	8.29	8.20	8.01	7.86
E5	8.36	8.28	7.91	7.79
E6	8.37	8.21	8.00	7.86
E7	8.29	8.20	7.99	7.93
E8	8.32	8.10	7.80	7.73
E9	8.29	8.12	8.04	7.98
E10	8.30	8.15	7.94	7.79
E11	8.37	8.30	7.98	7.86
E12	8.31	8.20	7.95	7.79
E13	8.18	8.15	8.01	7.86
E14	8.24	8.10	7.98	7.81
E15	8.14	8.07	7.91	7.73

Table 4.3: Bile tolerance of the selected *E. coli* isolates

	Logarithmic count after incubation at 1.0%bile			
St. no.	**0h**	**1h**	**3h**	**5h**
E1	8.45	8.28	8.17	7.96
E2	8.26	8.15	7.96	7.68
E3	8.36	8.21	8.08	7.79
E4	8.31	8.09	8.02	7.91
E5	8.18	8.02	7.87	7.72
E6	8.33	8.13	7.97	7.79
E7	8.37	8.26	8.18	8.02
E8	8.32	8.17	8.06	7.92
E9	8.21	8.12	7.97	7.75
E10	8.32	8.28	8.20	8.10
E11	8.19	8.07	7.99	7.63
E12	8.28	8.21	8.08	7.87
E13	8.45	8.38	8.29	8.21
E14	8.11	8.00	7.86	7.70
E15	8.17	8.07	8.01	7.91

4.2.4. Cell surface hydrophobicity of the *E. coli* isolates:

Bacterial cell surface hydrophobicity is one of the most important factors that influence bacterial adhesion to hydrocarbons (BATH). The attachment of the bacterial cells to the hydrophobic marker after 1 hour of incubation denotes the cell surface hydrophobicity. The higher the % cell surface hydrophobicity, the more is the adhesion ability of the probiotic cells to the intestinal epithelium. Cell surface hydrophobicity assay was carried out using n- hexadecane as hydrophobic marker. The cell surface hydrophobicity values ranged between 32.44% to 51.05% (Table 4.4). .The highest cell surface hydrophobicity (51%) was observed for the isolate E7. The isolates E1, E8, E9, E10, E12 and E15 also showed good cell surface hydrophobicity (42.44 to 48.54). The cell surface hydrophobicity observed in present study is much higher than that reported earlier for *Lactobacillus plantarum* LP9 (35.73%) by Duary et.al, (2010). So *E.coli* may show better adhering ability and colonization in the gut as compared to *Lactobacillus* sp.

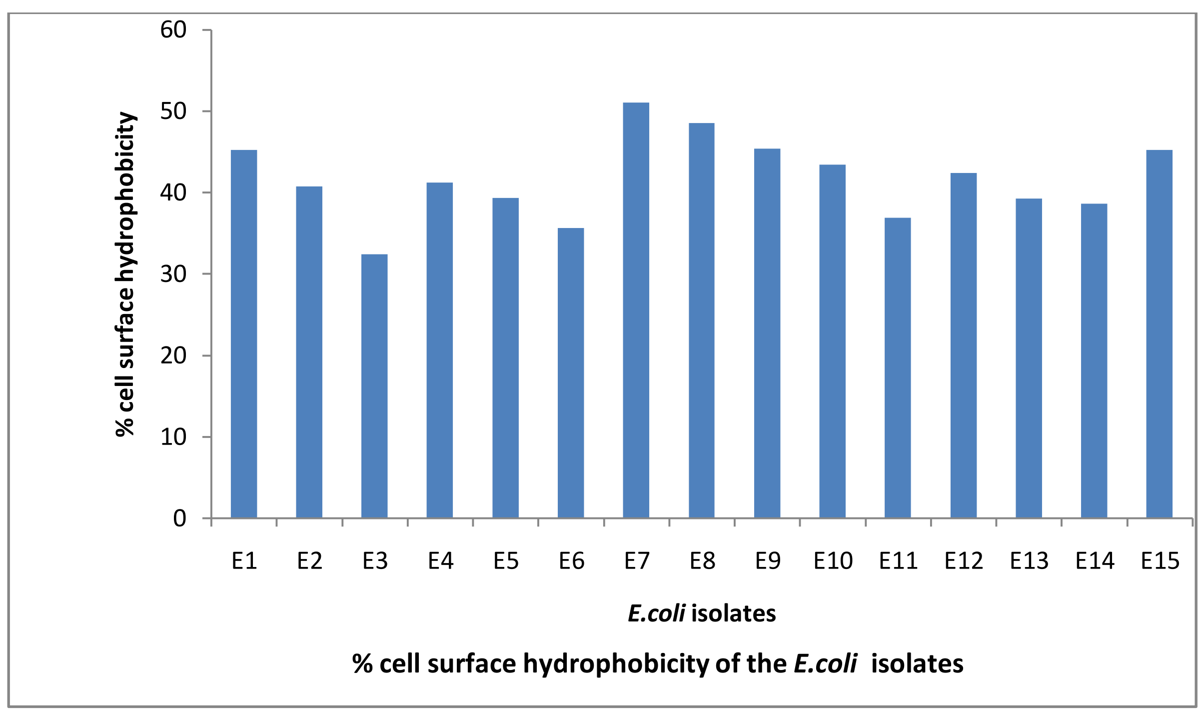

Fig 4.5. Graphical representation of cell surface hydrophobicity of the isolates.

Table 4.4: Cell surface hydrophobicity of the *E. coli* isolates

Sl no	Strain number	% Cell surface hydrophobicity
1	E1	45.23
2	E2	40.73
3	E3	32.44
4	E4	41.23
5	E5	39.35
6	E6	35.63
7	E7	51.05
8	E8	48.54
9	E9	45.41
10	E10	43.42
11	E11	36.95
12	E12	42.44
13	E13	39.23
14	E14	38.63
15	E15	45.27

4.3. Safety evaluation of the selected *E. coli* isolates:

4.3.1. Antibiotic resistance pattern:

The selected isolates were checked for antibiotic resistance against 12 antibiotics namely (ciprofloxacin, cefuroxime, amikacin, cephotaxime, tobramycin, ceftazidime, gentamycin, tetracycline, nitrofurantoin, amoxyclav and netillin). Isolates showing clearance zones of greater than 1.0 cm in diameter were considered to be susceptible and below 0.5 cm were considered to be resistant, and zones with diameters greater than 0.5 cm but lesser than 1.0 cm were considered to be intermediate (CLSI, 2006).

Table 4.5. Antibiotic resistance pattern of the *E. coli* isolates

Sl no	Isolate	*The zone of inhibition is in cm (R denotes resistant, diameter below 0.5 cm, I denotes intermediate resistance)*											
		Cip	Cef	Ami	Cep	Amp	Tob	Ceft	Gen	Tet	Nit	Amo	Net
1.	E1	1.0	2.1	1.3	3.0	2.0	1.1	2.3	1.3	1.2	1.7	2.5	1.4
2.	E2	4.0	1.8	1.5	2.2	2.0	1.5	2.6	1.3	2.0	2.0	2.3	1.6
3.	E3	2.3	1.3	1.5	1.6	R	1.6	2.6	1.5	1.6	1.5	1.0	1.7
4.	E4	2.0	1.3	2.0	1.5	R	2.0	1.7	1.7	1.0	1.6	0.8	2.2
5.	E5	1.7	1.0	2.0	1.9	R	1.3	1.5	1.0	1.0	1.4	1.3	1.0
6.	E6	3.5	2.5	2.3	3.0	2.1	1.8	3.0	2.2	2.3	2.2	2.4	2.4
7.	E7	3.0	R	1.5	1.0	1.0	1.3	1.2	1.3	2.2	2.3	1.0	1.5
8.	E8	2.0	2.2	1.5	3.0	2.6	1.4	2.7	1.3	1.2	1.7	2.3	1.3
9.	E9	2.5	2.2	1.5	2.7	2.2	1.4	2.8	2.6	2.2	2.3	2.6	1.6
10.	E10	2.7	R	1.6	1.0	1.0	1.7	1.8	1.7	2.0	2.4	1.0	1.8
11.	E11	1.8	2.3	1.6	3.5	R	1.4	2.7	1.1	1.5	2.0	1.2	1.7
12.	E12	1.5	1.7	1.2	2.4	0.7 (I)	1.7	2.2	1.4	R	2.0	1.8	1.6
13.	E13	2.5	1.5	2.0	2.0	1.7	1.7	2.0	2.0	2.3	1.6	2.0	2.1
14.	E14	1.5	2.2	1.3	3.3	R	1.2	2.5	1.3	1.5	1.9	1.6	1.4
15.	E15	2.4	R	1.7	1.6	R	1.7	1.8	1.9	2.4	2.3	1.9	2.2

Most of the isolates were found to be sensitive to all the antibiotics, whereas three isolates showed resiatance against ciprofloxacin, six against ampicillin and one strain against tetracycline. Isolate E15 showed show resistance to more than 1 antibiotics i.e. ciprofloxacin and ampicillin (Table 4.5). The isolates E1, E2, E6, E8, E9 and E13 did not show resistance against any of the antibiotics tested. However the isolate E15 showed resistance to two antibiotics cefuroxime and ampicillin.

However, In a study, by Akond *et. al*. (2009), 52-88% of *E.coli* isolated from poultry sources were found to be resistant to the antibiotics penicillin, ciprofloxacin, riphampicin, kanamycin, streptomycin, cefixine, erythromycin, ampicillin and tetracycline, 20% strains showed resistance to both chloramphenicol and neomycin. No strains showed resistance to norfloxacin and gentamycin. Sensitivity was recorded in case of 60-86% strains to norfloxacin, gentamycin, chloramphenicol and neomycin; and 26-36% strains against tetracycline, streptomycin and ampicillin. Intermediate resistance to various antibiotics were observed for 12-36% *E.coli* strains. Multiple drug resistance was found in case of 6-10 antibiotics for all strains tested. Antibiotic resistance may be higher as the overall usage of antibiotics in the poultry sector is higher.

4.3.2. Detection of Shiga toxin producing (stx) gene:

The isolates were tested for the presence of *stx* gene by PCR using the primers VTcom-U and VTcom-D which amplifies the stx1, stx2 and its other varients.. Six of the isolates E3, E5, E6, E7, E10 and E11 showed presence of *stx* genes and thus may be considered as potential pathogens. Whereas, nine of the isolates (E1, E2, E4, E8, E9, E12, E13, E14 and E15) were found to be negative for the presence of *stx* gene and may be considered as potent candidates for probiotic applications. However, presence of other toxin genes also needs to be ruled out as Kumar et al. (2009) screened the *E. coli* isolates against 8 sets of primers for presence of different toxins. Toma *et al* (2003) developed a multiplex PCR for detection of toxigenic strains of *E. coli*. The present work needs to be extended further to investigate and rule out the presence of all the reported toxin genes found in *E. coli.*

4.3.3. Haemolysis assay of the *E.coli* isolates:

The selected nine isolates, E1, E2, E4, E8, E9, E12, E13, E14 and E15 showed the absence of any kind of zone formation around the colonies i.e. neither green zones of α haemolysis nor yellow transparent zones indicating β haemolysis (Fig. 4.8). These isolates thus showed γ haemolysis or no haemolysis and hence can be considered safe from the haemolytic point of view. The commensal *E. coli* strains do not exhibit hemolysis.

4.3.4. Gelatin liquefaction assay of the *E.coli* isolates:

All the selected isolates E1, E2, E4, E8, E9, E12, E13, E14 and E15 showed negative for gelatinase production on gelatin liquifaction assay.

In the present study 9 isolates have been found to posess antimicrobial activity against Gram-ve pathogens i.e. *E. coli* O175 H7, *Salmonella typhi* NCTC6017 and *Shigella dysenteriae*. Isolate E-1 and E-7 showed inhibitory effect on all three pathogenic strains. However, E7 was found to be STX positive. Isolates E1, E2, E4, E8, E9, E12, E13, E14 and E15 showed good acid and bile resistance, cell surface hydrophobicity, antagonoism against selected pathogens, absence of shiga like toxin genes and did not display gelatin liquefaction or production of hemolysins. These isolates also showed very negligible incidence of antibiotic resistance and thus appear to be potet candidates for further studies to evaluate and develop a potent Gram-ve probiotic strain which may have the higher potential to combat gut infections caused by Gram –ve pathogens.

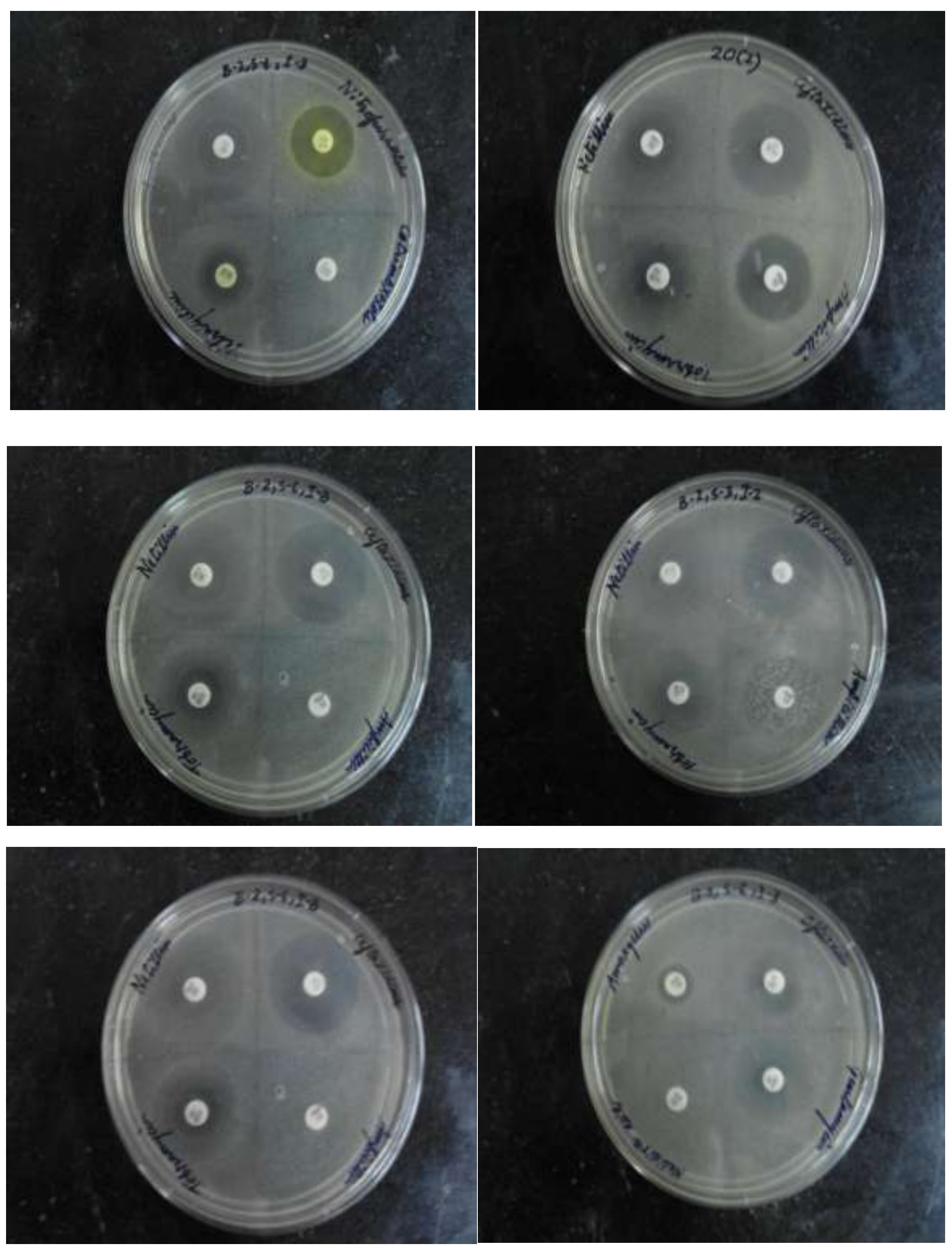

Fig 4.6. Antibiotic resistance pattern of the E. coli isolates E1, E11 and E14 against various antibiotic discs

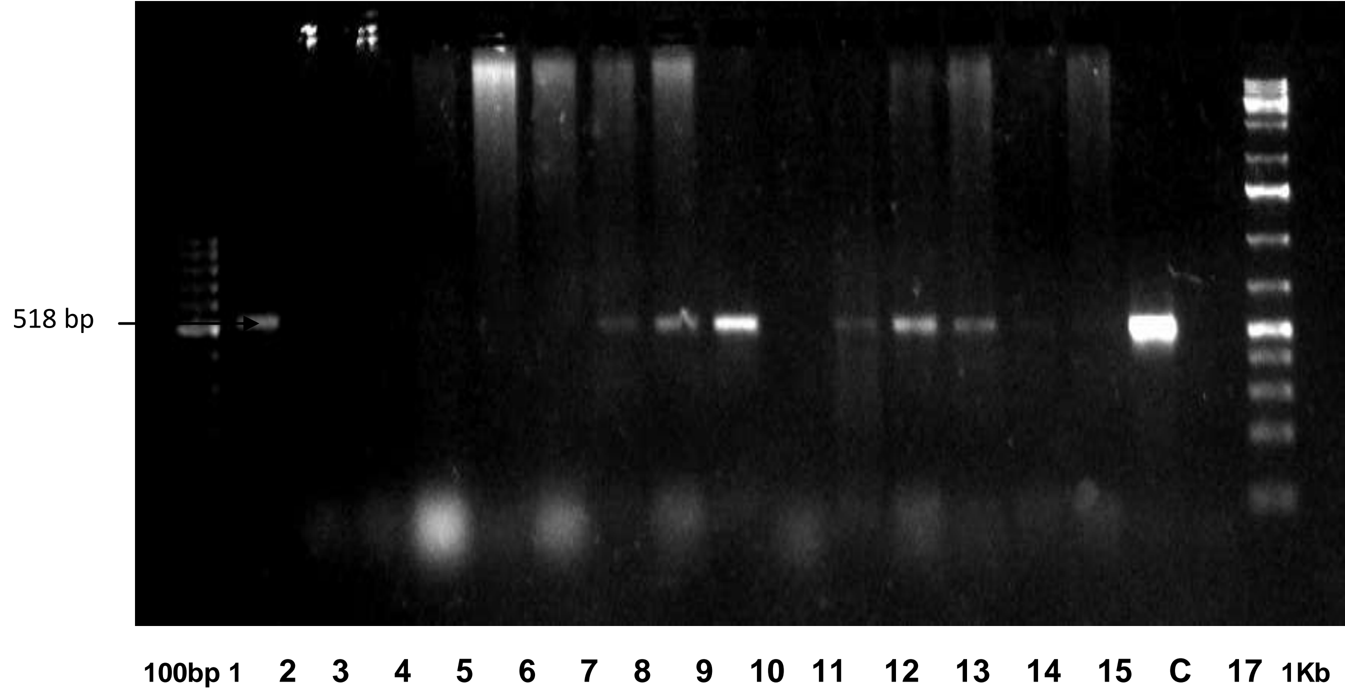

Fig 4.7. The isolates in the lanes from left to right are E10, E2, E1, E4, E9,E8, E5, E11,E3,E13, E14, E7, E6, negative control and positive control. The isolates E10, E5, E11, E3, E7 and E6 showed the presence of stx gene.

Fig 4.8. Plates showing negative haemolysis or γ haemolysis

CHAPTER 5

Summary and Conclusion

SUMMARY & CONCLUSION

1. A total of 45 strains of *E. coli* were isolated from six human and 4 bovine fecal samples.
2. The isolates were confirmed to be *E. coli* as they produced acid and gas from lactose and showed Indole production, methyl red positive and negative for citrate utilization and VP tests.
3. The isolates were further screened for their antimicrobial activity against *E. coli* O157:H7, *Salmonella typhi* and *Shigella dysenteriae.* Fifteen of the isolates named E1 to E15 displayed good inhibitory action against the se pathogens. The isolates E1, E2, E4, E6, E8, E9, E10, E12 and E14 were isolated from human fecal samples whereas the isolates E3, E5, E7, E11, E13 and E15 were isolated from bovine fecal samples. E1 and E7 showed antimicrobial property against all the three pathogens tested.
4. The isolates were then checked for their acid tolerance at pH 2.0 and counts were taken after 1, 3 and 5 hours and their bile tolerance was checked at 1.0% bile concentration and counts were taken after 1, 3 and 5 hours. All the isolates showed good acid and bile tolerance. Normally lactobacilli are tested upto 0.3% bile tolerance and pH 3.0. The acid and bile resistance of the E. coli isolates is much higher than the lactic acid bacteria.
5. The cell surface hydrophobicity (%) was tested based on their attachment ability to the hydrocarbon n-hexadecane. The isolates exhibited cell surface hydrophobicity values ranging from 32% to 51%. The isolate E7 exhibited the highest percentage cell surface hydrophobicity. The hydrophobicity was much higher than previously reported for the probiotic strains of *Lactobacillus.*
6. The isolates were then evaluated for their safety aspects. The antibiotic resistance of the isolates was found out following the Bauer-Kirby procedure. Using ciprofloxacin, cefuroxime, amikacin, tobramycin, ceftazidime, gentamycin, tetracycline, nitrofurantoin, amoxyclav and netillin. Most of the isolates were found to be susceptible to all the antibiotics i.e. the isolates did not show any multiple drug resistance.

7. The isolates were then screened for the presence of shiga toxin producing stx gene by using PCR primers. Six isolates E3, E5, E6, E7, E10 and E11 were found to possess the stx gene and thus did not fulfill the safety requirement. Other nine strains tested negative for the shiga toxin genes.
8. The rest of the isolates (9) were checked for haemolysis and gelatin liquefaction assays. All the isolates tested negative for both gelatin liquefaction and haemolysis.
9. Isolates E1, E2, E4, E8, E9, E12, E13, E14 and E15 appear to be potet candidates for further studies to evaluate and develop a potent Gram-ve probiotic strain which may have the higher potential to combat gut infections caused by Gram –ve pathogens.
10. Further studies may be conducted to characterize the antimicrobial potency, nature of the antimicrobial agent, and presence of other toxin genes. The potent E. coli strains may prove more useful in combating the Gram-ve pathogens as they have displayed remarkable acid & bile resistance, hydrophobicity and antagonistic action against different Gram-ve pathogens.

Bibliography

BIBLIOGRAPHY

Altenhoefer, A., Oswlad, S., Sonnenborn, U., Enders, C., Schulze, J., Hacker, J. and Oelschlaeger, T.A. (2004). The probiotic Escherichia coli strain Nissle 1917 interferes with invasion of human intestinal epithelial cells by different enteroinvasive bacterial pathogens. FEMS Immunol Med Microbiol 40, 223–229.

Akond, M A, Alam, S, Hassan, S M R and Shirin, M (2009) Antibiotic resistance of Escherichia coli isolated from poultry and poultry environment of Bangladesh. Internet Journal of Food Safety 11 19-23.

Autieri, S. M., J. J. Lins, M. P. Leatham, D. C. Laux, T. Conway, and P. S. Cohen**.** 2007. L-Fucose stimulates utilization of D-ribose by *Escherichia coli* MG1655 _*fucAO* and *E. coli* Nissle 1917 _*fucAO* mutants in the mouse intestine and in M9 minimal medium. Infect. Immun. **75:**5465–5475.

Bhandari, S. K., C. M. Nyachoti, and D. O. Krause. 2008. Raw potato starch in weaned pig diets and its influence on post-weaning scours and the molecular microbial ecology of the digestive tract. J. Anim. Sci. Oct. 24.

Blattner, F.R., Plunkett III, G., Bloch, C.A., Perna, N.T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J.D., Rode, C.K., Mayhew, G.F., Gregor, J., Davis, N.W., Kirkpatrick, H.A., Goeden, M.A., Rose, D.J., Mau, B., Shao, Y., 1997. The complete genome sequence of Escherichia coli K-12. Science 277, 1453–1474.

Bollman R., Seeburg A., Parschau J., : Genotypic and phenotypic determination of five virulence markers in clinical isolates of *Escherichia coli*. *FEMS Immunol.Med.Microbiol.* **17**,263–271 (1997).

Bosi, P., L. Casini, A. Finamore, C. Cremokolini, G. Merialdi, P. Trevisi, F. Nobili, and E. Mengheri. 2004. Spray-dried plasma improves growth performance and reduces inflammatory status of weaned pigs challenged with enterotoxigenic *Escherichia coli* K88. J. Anim. Sci. 82:1764-1772.

Bradley, D.E., Howard, S.P. and Lior, H. (1991) Colicinogeny of O157:H7 enterohemorragic Escherichia coli and their O-antigenic side chains. Can J Microbiol 37, 97–104.

Braun, V., Gross, R., Koster, W. and Zimmermann, I. (1983) Plasmid and chromosomal mutants in the iron(III)-aerobactin transport system of Escherichia coli. Use of streptonigrin for selection. Mol Gen Genet 192, 131–139.

Buenau, R., L. Jaekel, E. Schubotz, S. Schwarz, T. Stroff and M. Krueger, 2005. *Escherichia coli* strain Nissle 1917: Significant reduction of neonatal calf diarrhea. J. Dairy Sci., 88: 317-323.

Bures J., Horak V., Buresova E., Fixa B., Komarkova O.: Colicinogeny in nonspecific intestinal inflammation – I. Colicinogeny in patients with ulcerous colitis, Crohn's disease and in healthy subjects. (In Czech) *eskosl.Gastroenterol.Výž.* 37, 81–86(1983).

Butler, D.G. and Clarke, R.C. (1994) Diarrhoea and dysentery in calves. In Escherichia coli in domestic animals and humans ed. Gyles, C.L. pp. 91–116. Wallingford: CAB International.

Cascales, E., S.K. Buchanan, D. Duché, C. Kleanthous, R. Lloubès, K. Postle,Riley, S. Slatin and D. Cavard, 2007. Colicin biology. Microbiol. Mol Biol. Rev., 71: 158-229.

Chang, D. E., D. J. Smalley, D. L. Tucker, M. P. Leatham, W. E. Norris, S. J.Stevenson, A. B. Anderson, J. E. Grissom, D. C. Laux, P. S. Cohen, and T. Conway**.** 2004. Carbon nutrition of *Escherichia coli* in the mouse intestine. Proc. Natl. Acad. Sci. USA **101:**7427–7432.

Chinen, I., M. Rivas, M. I. Caffer, R. O. Cinto, and N. Binsztein. 1993. Diagnosis of enteroinvasive *Escherichia coli* associated with diarrhea. Rev. Argent. Microbiol. 25:27–35.

Cleveland, J., Montvile, T.J., Nes, I.F and Chikindas, M.L. (2001) Bacteriocins: safe natural antimicrobial for food preservation. Int J Food Microbiol 2, 21–29.
coli: infectious disease management, p. 357–363. *In* J. B. Kaper and A. D. O'Brien (ed.), *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* strains. ASM Press, Washington, DC.

Conway, T., and P. S. Cohen. 2007. *Escherichia coli* at the intestinal mucosal surface, p. 175–196. *In* K. A. Brogden, F. C. Minion, N. Cornick, T. B. Stanton, Q. Zhang, L. K. Nolan, and M. J. Wannemuehler (ed.), Virulence mechanisms of bacterial pathogens, 4th ed. ASM Press, Washington, DC.

Cukrowska, B., Lodinova´-Zˇ a´dnı´kova´, R., Enders, C., Sonnenborn, U., Schulze, J. and Tlaskalova´-Hogenova´, H. (2002) Specific proliferative and antibody responses of premature infants to intestinal colonization with nonpathogenic probiotic E. coli strain Nissle 1917. Scand J Immunol 55, 204–209.

Cursino, L., D. Šmajs, J. Šmarda, R.M.D. Nardi, J.R. Nicoli, E. Chartone-Souza and A.M.A. Nascimento, 2006. Exoproducts of the *Escherichia coli* strain H22 inhibiting some enteric pathogens both *in vitro* and *in vivo*. Applied Environ. Microbiol., 100: 821-829.

Dunne, C., L. O'Mahony, L. Murphy, G. Thornton, D. Morrissey, S. O'Halloran, M. Feeney, S. Flynn, G. Fitzgerald, C.D.B. Kiely, G.C. O'Sullivan,F. Shanahan and J.K. Collins, 2001. *In vitro* selection criteria for probiotic bacteria of human origin: Correlation with *in vivo* findings. Am. J.Clin. Nutr., 73: 386-392.

Fairbrother, J. M., E. Nadeau, and C. L. Gyles. 2005. *Escherichia coli* in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. Anim. Health Res. Rev. 6:17-39.

Freter, R. 1983. Mechanisms that control the microflora in the large intestine p. 33–54. *In* D. J. Hentges (ed.), Human intestinal microflora in health and disease. Academic Press, Inc., New York, NY.

Gillor, O., A. Etzion and M.A. Riley, 2008. The dual role of bacteriocins as anti- and probiotics. Applied Microbial. Biotechnol., 81: 591-606.

Gillor, O., A. Etzion and M.A. Riley, 2008. The dual role of bacteriocins as anti- and probiotics. Applied Microbial. Biotechnol., 81: 591-606.

Gordon, D.M., O'Brien, C.L., 2006. Bacteriocin diversity and the frequency of multiple bacteriocin production in Escherichia coli. Microbiology 152, 3239–3244.

Gostner1, A., M. Blaut, V. Scha¨ffer1, G. Kozianowski and S. Theis *et al*., 2006. Effect of isomalt consumption on faecal microflora and colonic metabolism in healthy volunteers. Br. J. Nutr., 95: 40-50.

Grauke, L.J., Kudva, I.T., Yoon, J.W., Hunt, C.W., Williams, Ch.J. and Hovde, C.J. (2002) Gastrointestinal tract location of Escherichia coli O157:H7 in ruminants. Appl Environ Microbiol 68, 2269–2277.

Griffin, P. M., L. C. Olmstead, and R. E. Petras**.** 1990. *Escherichia coli* O157:H7-associated colitis. A clinical and histological study of 11 cases. Gastroenterology 99**:**142–149.

Grozdanov, L., U. Za¨hringer, G. Blum-Oehler, L. Brade, A. Henne, Y. A. Knirel, U. Schombel, J. Schulze, U. Sonnenborn, G. Gottschalk, J. Hacker, E. T. Rietschel, and U. Dobrindt. 2002. A single nucleotide exchange in the *wzy* gene is responsible for the semirough O6 lipopolysaccharide phenotype and serum sensitivity of *Escherichia coli* strain Nissle 1917. J. Bacteriol.184:5912–5925.

Hansen, L.H., Johannesen, E., Burmolle, M., Sorensen, A.H., Sorensen, S.J., 2004. Plasmid-encoded multidrug efflux pump conferring resistance to olaquindox in Escherichia coli. Antimicrob. Agents Chemother. 48, 3332–3337.

Hayashi, T., Makino, K., Ohnishi, M., Kurokawa, K., Ishii, K., Yokoyama, K., Han, C.-G., Ohtsubo, E., Nakayama, K., Murata, T., Tanaka, M., Tobe, T., Iida, T., Takami, H., Honda, T., Sasakawa, C., Ogasawara, N., Yasunaga, T., Kuhara, S., Shiba, T., Hattori, M., Shinagawa, H., 2001. Complete genome sequence of enterohemorrhagic Escherichia coli O157:H7 and genomic comparison with a laboratory strain K-12. DNA Res. 8, 11–22.

Hentges, D. J. 1983. Role of the intestinal microflora in host defense against infection, p. 311–331. *In* D. J. Hentges (ed.), Human intestinal microflora in health and disease. Academic Press, Inc., New York, NY.

Hora´k, V. (1994) Seventy colicin types of Shigella sonnei and an indicator system for their determination. Zentralbl Bakteriol 281, 24–29.

Hudault, S., Guignot, J. and Servin, A.L. (2001) Escherichia coli strains colonising the gastrointestinal tract protect germfree mice against Salmonella typhimurium. Gut 49, 47–55.

Inoue, R., T. Tsukahara, N. Nakanishi and K. Ushida1, 2005. Development of the intestinal microbiota in the piglet. J. Gen. Applied Microbiol., 51: 257-265.http://www.ncbi.nlm.nih.gov/pubmed/16205033.

Jansen, G.J., Wildeboer-Veloo, A.C., der Waaij, D. and Degener, J.E. (1998) Escherichia coli as a probiotic? Infection 26, 232–233.

Jernberg, C., A. Sullivan, C. Edlund and K. Janet, 2005. Monitoring of antibiotic-induced alterations in the human intestinal microflora and detection of probiotic strains by use of terminal restriction fragment length polymorphism. Applied Environ. Microbiol., 71: 501-506. DOI: 10.1128/AEM.71.1.501-506.2005.

Jernberg, C., A. Sullivan, C. Edlund and K. Janet, 2005. Monitoring of antibiotic-induced alterations in the human intestinal microflora and detection of probiotic strains by use of terminal restriction fragment length polymorphism. Applied Environ. Microbiol., 71: 501-506. DOI: 10.1128/AEM.71.1.501-506.2005

Jin, Q., Yuan, Z., Xu, J., Wang, Y., Shen, Y., Lu, W., Wang, J., Liu, H., Yang, J., Yang, F., Zhang, X., Zhang, J., Yang, G., Wu, H., Qu, D., Dong, J., Sun, L., Xue, Y., Zhao, A., Gao, Y., Zhu, J., Kan, B., Ding, K., Chen, S., Cheng, H., Yao, Z., He, B., Chen, R., Ma, D., Qiang, B., Wen, Y., Hou, Y., Yu, J., 2002. Genome sequence of Shigella flexneri 2a: insights into pathogenicity through comparison with genomes of Escherichia coli K12 and O157. Nucleic Acids Res. 30, 4432–4441.

Klaenhammer, T.R. and M.J. Kullen, 1999. Selection and design of probiotics. Int. J. Food Microbiol., 15: 45-57.

Kleessen, B., E. Bezirtzoglou and J. Mättö, 2000. Culture-based knowledge on biodiversity,development and stability of human gastrointestinal microflora. Microbial Ecol. Health Dis., 12: 53-63.

Kotlowski, R., C. N. Bernstein, S. Sepehri, and D. O. Krause. 2007. High prevalence of *Escherichia coli* belonging to the B2+D phylogenetic group in inflammatory bowel disease. Gut. 56:669-675.

Krause, D. O., S. K. Bhandari, J. D. House, and C. M. Nyachoti. 2008. Response of nursery pigs to a synbiotic based on starch (prebiotic) and an anti-*Escherichia coli* K88 probiotic.

Kruis, W., Fric, P., Pokrotnieks, J., Lukas, M., Fixa, B., Kascak, M., Kamm, M.A., Weismueller, J. et al. (2004) Maintaining remission of ulcerative colitis with the probiotic Escherichia coli Nissle 1917 is as effective as with standard mesalazine. Gut 53, 1617–1623.

Kruis, W., Schutz, E., Fric, P., Fixa, B., Judmaier, G. and Stolte, M. (1997) Double-bind comparison of an oral Escherichia coli preparation and mesalazine in maintaining remission of ulcerative colitis. Aliment Pharmacol Ther 11, 853–858.

Kumar, et. al. (2009). Isolation and Characterization of Potiential Probiotic E coli strains from Rat Fecal Samples. *American Journal of Infectious Diseases , 5* (2), 119-124.

Levine, M. M., E. J. Bergquist, D. R. Nalin, D. H. Waterman, R. B. Hornick, C. R. Young, and S. Sotman.(1978). *Escherichia coli* strains that cause diarrhea but do not produce heat-labile or heat-stable enterotoxins and are non-invasive. Lancet 1:1119–1122.

Lima-Filho, J.V.M., Vieira, L.Q., Arantes, R.M.E. and Nicoli, J.R. (2004). Effect of the Escherichia coli EMO on experimental infection by Salmonella enterica subsp. enterica serovar Typhimurium in gnotobiotic mice. Braz J Med Biol Res 37, 1005–1013.

Lin, J, Smith, M P, Chapin, K C, Baik, H S, Bennett, G N and Foster, J W (1996) Mechanisms of acid resistance in enterohemorrhagic Escherichia coli. Applied and environmental microbiology 62 3094-3100.

Lodinova´-Zˇa´dnı´kova´ , R., Sonnenborn, U. and Tlaskalova´, H. (1998) Probiotics and E. coli infections in man. Vet Q 20(Suppl 3), S78–S81.

Mirlohi, M, Soleimanian-Zad, S, Dokhani, S, Sheikh-Zeinodin, M and Abghary, A (2009) Investigation of acid and bile tolerance of native lactobacilli isolated from fecal samples and commercial probiotics by growth and survival studies. Iranian Journal of Biotechnology 7 233-240.

Mourad, K. and K. Nour-Eddine, (2006). *In vitro* preselection criteria for probiotic *Lactobacillus plantarum* strains of fermented olives origin. Int. J. Probiot. Prebiot., 1:27-32.

Mubita, C., M. S. Lima, C. Chisenga, M. Munyeme and M. Bwalya *et al*., (2008). Antibiograms of faecal *Escherichia coli* and *Enterococci* species isolated from pastoralist cattle in the interface areas of the kafue basin in Zambia. Vet. Arh., 78: 179-185.

Murinda, S.E., Roberts, R.F. and Wilson, R.A. (1996) Evaluation of colicins for inhibitory actvity against diarrheagenic Escherichia coli strains, including serotype O157:H7. Appl Environ Microbiol 62, 3196–3202.

Nascimento, A.M.A., Campos, C.E., Campos, E.P., Azevedo, J.L., Chartone-Souza, E.,(1999). Re-evaluation of antibiotic and mercury resistance in Escherichia coli populations isolated in 1978 from Amazonian rubber tree tappers and Indians. Res. Microbiol. 150, 407–411.

NCCLS. 2003. National Committee for Clinical Laboratory Standards (6th ed. M7-A6), Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard Vol. 23, No. 2, NCCLS, Wayne, PA (2003).

Neill, M. A. **(**1998). Treatment of disease due to Shiga toxin-producing *Escherichia*

Otte, J.M. and D.K. Podolsky, (2004). Functional modulation of enterocytes by gram-positive and gram-negative microorganisms. Am. J. Physiol. Gastrointest. Liver Physiol., 286: 613-626.

Owusu-Asiedu, A., C. M. Nyachoti, and R. R. Marquardt. (2003). Response of early weaned pigs to an enteropathogenic *Escherichia coli* (K88) challenge when fed diets containing spray dried porcine plasma or pea protein isolate plus egg yolk antibody, zinc oxide, fumaric acid or antibiotic. J. Anim. Sci. 81:1790-1798.

Padola, N.L., Sanz, M.E., Blanco, J.E., Blanco, M., Blanco, J., Etcheverrı´a, A.I., Arroyo, G.H., Usera, M.A. et al. (2004) Serotypes and virulence genes of bovine Shigatoxigenic Escherichia coli (STEC) isolated from a feedlot in Argentina. Vet Microbiol 100, 3–9.

Parma, A.E., Sanz, M.E., Blanco, J.E., Blanco, J., Vin˜as, M.R., Blanco, M., Padola, N.L. and Etcheverrı´a, A.I. (2000) Virulence genotypes and serotypes of verotoxigenic Escherichia coli isolated from cattle and foods in Argentina. Importance in public health. Eur J Epidemiol 16, 757–762.

Parma, A.E., Vin˜as, M.R. and Sanz, M.E. (1996) Improvement of the polymerase chain reaction to detect Escherichia coli Shiga-like toxin II gene from clinical isolates. J Microbiol Methods 26, 81–85.

Patzer, S.I., Baquero, M.R., Bravo, D., Moreno, F., Hantke, K., (2003). The colicin G, H and X determinants encode microcins M and H47, which might utilize the catecholate siderophore receptors FepA, Cir, Fiu and IroN. Microbiology 149, 2557–2570.

Peterson, D. A., D. N. Frank, N. R. Pace, and J. I. Gordon**. (**2008). Metagenomic approaches for defining the pathogenesis of inflammatory bowel diseases. Cell Host Microbe **6:**417–427.

Rang, C. U., T. R. Licht, T. Midtvedt, P. L. Conway, L. Chao, K. A. Krogfelt, P. S. Cohen, and S. Molin. **(**1999). Estimation of growth rates of *Escherichia coli* BJ4 in streptomycin-treated and previously germfree mice by in situ rRNA hybridization. Clin. Diagn. Lab. Immunol. **6:**434–436.

Rideout, T. C., Q. Liu, Wood P, and M. Z. Fan. (2008). Nutrient utilisation and intestinal fermentation are differentially affected by the consumption of resistant starch varieties and conventional fibres in pigs. Br J Nutr. 99:984-992.

Riley, M.A. and W.E. John, (2002). Bacteriocins: Evolution, ecology and application. Annu. Rev. Microbiol., 56: 117-137.

Sˇ majs, D., Weinstock, G.M., (2001). The iron- and temperature regulated cjr BC genes of Shigella and enteroinvasive Escherichia coli strains code for colicin Js uptake. J.Bacteriol. 183, 3958–3966.

Sˇ marda, J., Sˇ majs, D., Horynova´, S., (2006). Incidence of lysogenic, colicinogenic and siderophore-producing strains among human non-pathogenic Escherichia coli. Folia Microbiol. 387–391.

Sˇmajs, D., Karpathy, S.E., Sˇ marda, J., Weinstock, G.M., (2002a).Colicins produced by the Escherichia fergusonii strains closely resemble colicins encoded by Escherichia coli. FEMS Microbiol. Lett. 208, 259–262.

Sˇmarda, J., Obdrzˇa´lek, V., (2001). Incidence of colicinogenic strains among human Escherichia coli. J. Basic Microbiol. 41, 367–374.

Savage, D.C., (1977). Microbial ecology of the gastrointestinal tract. Annu. Rev. Microbial., 31: 107-133.

Schamberger, G. P., and F. Diez-Gonzalez. (2002). Selection of recently isolated colicinogenic *Escherichia coli* strains inhibitory to *Escherichia coli* O157:H7. J. Food Prot. 65:1381-1387.

Schamberger, G. P., and F. Diez-Gonzalez. 2004. Characterization of colicinogenic *Escherichia coli* strains inhibitory to enterohemorrhagic *Escherichia coli*. J. Food Prot. 67:486-492.

Schamberger, G. P., R. L. Phillips, J. L. Jacobs, and F. Diez-Gonzalez. 2004. Reduction of *Escherichia coli* O157:H7 populations in cattle by addition of colicin E7-producing *E. coli* to feed. Appl. Environ. Microbiol. 70:6053-6060.

Schierack, e. a. (2011). E. coli Nissle 1917 affects Salmonella adhesion to porcine epithelial cells. *Plos ONE , 6* (2), e14712 doi:10.1371/journal.pone.0014712.

Schierack, P., A.J. Jores, H. Kaspar, S. Guenther, M. Filter, J. Eichberg and L.H.Wieler, 2009. Isolation and characterization of intestinal *Escherichia coli* clones from wild boars in Germany. Applied Environ. Microbiol., 75: 695-702.DOI: 10.1128/AEM.01650-08.

Schultz, F., and N. A. Strockbine. 2005. Genus I. *Escherichia*, Castellani and Chalmers 1919,941TAL in Bergey's Manual of Systemic Bacteriology- 2nd edition, volume II part Bed.Garitti G M, Springer, Michigan USA pp. 607-623.

Setia, A. and D.O. Krause, 2008. *E. coli*: Probiotic for Piglet Diarrhoea: *Escherichia coli* K88+Post Weaning Diarrhoea. VDM Verlag Publishers, ISBN: 10: 3639026276, pp: 116.

Smajs, D., McKevitt, M., Wang, L., Howell, J.K., Norris, S.J., Palzkill, T., Weinstock, G.M., 2002b. BAC library of T. pallidum DNA in E. coli. Genome Res. 12, 515–522.

Šmarda J., Šmjas D., Lhotova H.: Three recently acknowledged *Escherichia* species strikingly differ in the incidence of bacteriocinogenic and lysogenic strains. *J.Basic Microbiol.* **42**, 429–433 (2002).

Stahl, C. H., T. R. Callaway, L. M. Lincoln, S. M. Lonergan, and K. J. Genovese. 2004. Inhibitory activities of colicins against *Escherichia coli* strains responsible for postweaning diarrhoea and edema disease in swine. Antimicrob. Agents Chemother. 48:3119-3121.

Sweeney, N. J., P. Klemm, B. A. McCormick, E. Moller-Nielsen, M. Utley, M. A. Schembri, D. C. Laux, and P. S. Cohen. 1996. The *Escherichia coli* K-12 *gntP* gene allows *E. coli* F-18 to occupy a distinct nutritional niche in the streptomycin-treated mouse large intestine. Infect. Immun. **64:**3497–3503.

Thanassi, D G, Cheng, L W and Nikaido, H (1997). Active efflux of bile salts by *Escherichia coli*.Journal of bacteriology 179 2512-2518.

Toma, C., Y. Lu, N. Higa, N. Nakasone, I. Chinen, A. Baschkier, M. Rivas and M. Iwanaga1, 2003.Multiplex PCR assay for identification of human diarrheagenic *Escherichia coli*. J. Clin. Microbiol., 41: 2669-2671.

Tuohy, K. M., G. C. Rouzaud, W. M. Bruck, and G. R. Gibson. 2005. Modulation of the human gut microflora towards improved health using prebiotics-assessment of efficacy. Curr.Pharm. Des. 11:75-90.

Woodward, M.J., Carroll, P.J. and Wray, C. (1992) Detection of entero-and verocytotoxin genes in Escherichia coli from diarrhoeal disease in animals using the polymerase chain reaction. Vet Microbiol 31, 251–261.

Zhao, T., M. P. Doyle, B. G. Harmon, C. A. Brown, P. O. Mueller, and A. H. Parks. 1998. Reduction of carriage of enterohemorrhagic Escherichia coli O157:H7 in cattle by inoculation with probiotic bacteria. J. Clin. Microbiol. 36:641-647.

Zhao, T., Tkalcic, S., Doyle, M.P., Harmon, B.G., Brown, C.A. and Zhao, P. (2003) Pathogenicicty of enterohemorrhagic Escherichiacoli in neonatal calves and evaluation of fecal shedding by treatment with probiotic Escherichiacoli. J Food Protect 66, 924–930.

Appendix-I

1. **Mc Conkey's broth:** 40.00 gm of Mac Conkey's broth was added to 1 litre of water. It was mixed properly and sterilized at 121^{0}C for 15 minutes.

Components	Quantity
Peptone	20.00gms/lit
Lactose	10.00 gms/lit
NaCl	5.00 gms/lit
Na-taurocholate	5.00 gms/lit
Bromo cresol purple	0.01gms/lit

2. **EMB Agar:** 37.46 gm of Mac Conkey's broth was added to 1 litre of water. It was mixed properly and sterilized at 121^{0}C for 15 minutes.

Components	Quantity
Peptone	10.00gms/lit
Di potassium phosphate	2.00gms/lit
Lactose	10.00gms/lit
Eosin-Y	0.40 gms/lit
Methylene blue	0.065 gms/lit
Agar	15.00gms/lit

3. **Nutrient Agar:** The components were mixed in 1 litre of water , mixed properly and autoclaved at 121^0C for 15 minutes.

Components	Quantity
Peptone	5.00gm/lit
Beef extract	1.50gm/lit
Yeast extract	1.50gm/lit
Agar	15.00gm/lit

4. **Luria Bertani broth:** The components were mixed in 1 litre of water , mixed properly and autoclaved at 121^0C for 15 minutes.

Components	Quantity
Tryptone	10.00gms/lit
Yeast extract	5.00gms/lit
NaCl	10.00gms/lit

5. **Phosphate Buffered Saline:** The components were mixed in 1 litre of water , mixed properly and autoclaved at 121^0C for 15 minutes.

Components	Quantity
NaCl	8.01
KCl	0.20
$Na_2HPO_4 \cdot 2 H_2O$	1.78
KH_2PO_4	0.27
pH	7.4

6. **PUM Buffer:** The components were mixed in 1 litre of water, mixed properly and kept in the refrigerator.

Components	Quantity
K_2HPO_4	22.2 gms/lit
KH_2PO_4	7.26 gms/lit
Urea	1.8gms/lit
$MgSO_4.7H_2O$	0.2gms/lit
pH	7.1

Appendix-II

1. REAGENTS FOR ISOLATION OF GENOMIC DNA

1.1 SET BUFFER(Solution I)

6.7% Sucrose	6.70g
50mM Tris	0.606 g
1mM EDTA (Di sodium EDTA)	0.0372 g
Deionized Water	100 ml

All these components were dissolved in 80 ml of water and pH was adjusted to 8.0 with 6N NaOH and volume was made to 100 ml. Buffer was autoclaved (121^0C/15 min) after preparation.

1.2 SDS Solution (10%)

SDS	10 g
Deionized Water	100 ml

Solution was autoclaved before use.

1.3 5M NaCl

NaCl	29.22 g
Deionized Water	100 ml

1.4 Chloroform: Isoamyl alcohol (24:1)

Isoamyl Alcohol	4.0 ml
Chloroform	96.0 ml

1.5 TE Buffer

Tris (10mM)	0.1212 g
Di-Sodium EDTA (1mM)	0.0372 g
Deionized Water	100 ml

All the components were dissolved in 80 ml of water and pH was adjusted to 8.0 with 6 N NaOH and volume was made upto 100 ml. Buffer was autoclaved (121^{0}C/15) min after preparation.

2. REAGENTS FOR AGAROSE GEL ELECTROPHORESIS OF DNA

2.1 TAE Buffer (50X) (Stock Solution)

Tris	24.20 g
50 M Di-Sodium EDTA (pH 8.0)	10.00 ml
Glacial Acetic Acid	5.71 ml
Deionized Water	100 ml

pH of this solution was not adjusted.

2.1.1 Working Solution (1X)

Working solution was prepared by diluting 1 ml of stock solution to 50 ml with distilled water .

2. TBE Buffer (10X)

Tris	54 g
Di-sodium EDTA	3.722 g
Boric Acid	27.5 g
pH	8.3

The volume was made up to 500 ml, filtered and autoclaved at 121^{0}C/ 15 minutes.

2.2.1. Working Solution (1X)

Working solution was prepared by diluting 100 ml of stock solution to 900 ml with distilled water.

2.3 Gel loading solution

Bromophenol Blue	0.05 g
Sucrose	40.00 g

Di-Sodium EDTA	3.72 g
SDS	0.50 g
Deionized Water	100 ml.

2.4 Staining Solution

2.4.1 Stock Solution (10 mg/ml):

Ethidium bromide was prepared as a stock solution of 10 mg/ml in water, stored at room temperature in screw cap tubes wrapped in aluminium foil.

2.4.2 Working Solution (0.5 µg/ml):

The working solution was prepared by adding 50 µl of stock solution to 10 ml of distilled water.