CHAPTER 2

Isolation and Characterization of Potential Probiotic Escherichia coli Strains from Rat Faecal Samples

2.1 Introduction

The GI tract is a biologically diverse and complicated system which contains around 10^{14} bacterial cells and up to 1000 species (Backhand *et al.*, 2005). The dominant microflora in faecal samples is obligatory anaerobic (*Bacteroides* spp., *Eubacterium* spp., *Bifidobacterium* spp., *Lactobacilli*) and anaerobic (*Cocci* and *Clostridium* spp.). In addition, facultative anaerobic organisms such as *E. coli*, *Enterococci* and *Streptococci* are also present (Kleessen *et al.*, 2000). 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. Probiotic bacteria are friendly to the host and protect the host against infection by secreting antimicrobial substance (Klaenhammer *et al.*, 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 (Cascale *et al.*, 2007). According to Klaenhammer, 99% of all bacteria may make at least one bacteriocin (Klaenhammer *et al.*, 1999; Riley *et al.*, 2002). 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 (Otte *et al.*, 2004; Gillor *et al.*, 2008). Consortium of organisms might be more effective than the application of a single strain because the protein released by gram positive and gram negative bacteria modulate the intestinal epithelial of the host by different mechanism including the competition of whole organisms for contact with the epithelial surface as well as stabilization of the cytoskeleton barrier function and the induction of mucin expression. The use of probiotic microbes to improve health is becoming well established. Most of the bacteria in commercial preparations are Gram-positive, however some enteric *E. coli* isolates, the normal GI microflora were also proved to be a useful as probiotic. A non-pathogenic *E. coli* strain Nissle 1917 has been well established in human medicine since 1917 (Buenau *et al.*, 2005).

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, reduce intestinal inflammation, strengthen the intestinal barrier against pathogen and boost up the host immune function (Buenau *et al.*, 2005; Gronbach *et al.*, 2010). 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. *E. coli* strain M-17 (EC-M17) is also a Probiotic agent with beneficial effects on the GI tract. EC-M17 is believed to be a direct descendant of the M17 strain first identified by the Russian bacteriologist L. G. Peretz in 1933 (Alder, 2006). This strain has been used extensively in humans as a therapy for GI diseases and infections. The Russian literature documents the use of EC-M17 in thousands of individuals, mostly for the treatment of dysentery.

E. coli H22 produces bacteriophage particles, aerobactin, colicins E1 and Ib, and microcin C7 type of antimicrobial compounds with inhibitory capabilities against pathogenic *Enterobacter* spp. and inhibited the GI enteric infections (Cursino *et al.*, 2006). Bacteriocins inhibit pathogens within the closely related species such as *Salmonella, Shigella, Klebsiella, Enterobacter* and pathogenic *E. coli*, which are the most common cause of GI disorders.

This chapter deals with the isolation and characterization of potential probiotic of *E. coli* strains from rat faecal matter which could eliminate gram negative pathogens. For this purpose, *E. coli* strains were isolated and tested for probiotic properties such as antimicrobial activity against enteropathogens, antibiotic susceptibility and resistance to low pH, absence of virulence traits, susceptibility to proteolytic activity and detection of colicins type.

2.2 Materials and Methods

2.2.1 Isolation of *E. coli* from rats faecal samples:

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 (HiMedia) 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). Isolated strains confirmed by above tests as *E. coli* were used to screen for their probiotic ability.

2.2.2 Testing for antimicrobial activity:

Detection of antimicrobial activity, agar spot tests were performed (Schillinger *et al.*, 1989). Indicator strains were grown in Luria broth at 37°C and approximately $5x10^7$ cells were inoculated into 4 mL of soft agar containing 0.7% agar) and poured over the plate on which the producer was spotted. The plates were incubated at 37°C for 24 h. Inhibitory activity was demonstrated by a clear zone around the spots.

2.2.3 Testing for resistance to antibiotics:

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).

2.2.4 Tolerance to acidic pH values:

E. coli strains were grown in Luria broth (HiMedia) at 37°C overnight, subcultured into fresh Luria broth and incubated till the culture was grown up to 0.6 OD at 550nm. The cultures were centrifuged at 5000 g for 10 min. The pellets were washed in sterile Phosphate-Buffered Saline (PBS) pH 7.0 and resuspended in PBS. Initial count was determined with appropriate dilution. Each strain was diluted 1/100 in PBS at pH 1.0, 2.0 and 3.0. Incubation times were 2, 4 and 6 h. Bacterial cultures were then transferred to Luria agar plates and incubated at 37°C overnight (Dunne *et al.*, 2001; Mourad *et al.*, 2006). 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 (Schillinger *et al.*, 1989; Dunne *et al.*, 2001).

2.2.5. Detection of pathogenic strains:

PCR reactions were in a 25 µL volume and comprised 1 µL of $MgCl_2$ (15 mM), 0.5 µL of 2.5 mM dNTPs, 2.5 µL of *Taq* buffer (10X), 0.5 µL of each primer (25 pM each), 1 µL of *Taq* DNA polymerase (Bangalore Genei Pvt Ltd.), and 2 µL of the DNA sample. Polymerase chain reaction was done with appropriate temperature profile according to the melting temperature (Tm) of the primers as reported (Toma *et al.*, 2003). Positive controls for 8 target genes were generated from DNA extracted from activated sludge and were identified on the basis of amplicon length (**Table 2.1**).

Table 2.1: Size of Amplicon target for identification of different *E. coli* pathogen

Primer	Amplicon (Size bp)	Target Gene	Strain
SK	881	*eae*	EPEC
VT	518	*stx*	STEC
AL	147	*est*	ETEC
LT	322	*elt*	ETEC
Ipa	619	*ipaH*	EIEC
Agg	254	*aggR*	EAEC
Eagg	194	*CVD432*	EAEC
Asp	282	*aspU*	EAEC

The PCR reaction mixture was incubated in a thermal cycler (Biorad) under the following cycling regime.

Lid temp – 105 °C

Step -1 (Initial denaturation): - 96 °C - 3 min

1) Cycle: 1
- Step - 2 (Denaturation):- 94 °C – 1 min
- Step -3 (Annealing): - 52 °C - 1 min
- Step – 4 (Extension): - 72 °C - 1 min

Steps 2, 3, 4 were repeated for 30 cycles

Step -5 (Final extension): - 72 °C - 10 min.

2) For Enteropathogen (eae) 70 ^{0}C annealing temperature was used.

Cycle: 1
- Step - 2 (Denaturation):- 94 °C – 1 min
- Step -3 (Annealing): - 70 °C - 1 min
- Step – 4 (Extension): - 72 °C - 1 min

Steps 2, 3, 4 were repeated for 30 cycles

Step -5 (Final extension): - 72 °C - 10 min.

2.2.6. Characterization of the antimicrobial agent:

Samples of culture supernatants, pellets and crude extract were examined for susceptibility to proteolytic enzymes. The following enzymes were used: Proteinase K (5 mg mL^{-1}) and Trypsin (2 mg mL^{-1}) (Mourad *et al.*, 2006). The crude extract was treated with enzymatic solutions and incubated at 37°C for 1h, after which the remaining activity was determined by the agar spot technique (Schillinger *et al.*, 1989).

2.2.7. Detection and identification of Colicins:

Culture was grown overnight and freshly inoculated 50 µL in 5 mL in Minimal medium containing 0.6% casamino acid and 1% glucose and grown till 0.6 OD. This culture was split into two parts and one part was induced with Mitomycin C (200 ng mL-1). After 4 h of incubation at 37°C (for induced culture), the samples

were centrifuged at 15000 g/10 min/4°C. Supernatant was filtered with cellulose acetate filter (0.2 µm filter) and 5 µL was spotted on the indicator organism i.e., *Salmonella* sp, *Klebisella pneumoniae*, *Staphylococcus aureus* and *Enterobacter asburiae*. The pellet was suspended in 0.05 M Phosphate buffer pH 7.0 and sonicated 30-60 sec and centrifuged at 15000 g/10 min/4°C, lysis culture was filtered with cellulose acetate filter (0.2 µm filter) and 5 µL was spotted on the indicator organism. PCR based method was used for identification of 18 different colicins using 9 primers (Setia *et al.*, 2009) (**Table 2.2**).

Table 2.2: Primers used for amplification of colicin genes

Target gene	Primer Name	Primer sequences (5' to 3)	PCR Product Size, bp	Annealing temperature, °C
Colicin A, N, S4	NS4f NS4r	CGTAGCTATAATGAAGCAATGGCTTCA ACCTCCAACAGGAGAGGTCCCCAGTT	225	57
Colicin M	Mf Mr	CCAGCAACCCTCTCACATTGCAG CCAGAAAACATCGCCCCGAGCC	556	68
Colicin V	Vf Vr	CACGCCCTGAAGCACCACCA CCGTTTTCCAAGCGGACCCC	400	68
Colicin 1a 1b	1abf 1abr	GCACAACAGGCCCGTCTGCTC CACCTTCCACATCCTCTGTCACC	385	68
Colicin E2, E3, E3a, E4, E5, E6, E7, E8, E9	Mixf Mixr	CGACAGGCTAAAGCTGTTCAGGT TGCAGCAGCATCAAATGCAGCCT	219	60
Colicin U, Y	UYf UYr	GTGAACGGACAGAAACCCGCC CAATCTGTCTGACAGCCTCTCCC	243	68
ColicinB, D, D157	BDf BDr	TCGCTCCATCCATGCCTCCG CCATCCCGACCAGTCTCCCTC	138	68
Colicin E1	E1f E1r	ACGGGAGTGGCTCTGGCGG CTCTTTACGTCGTTGTTCTGCTTCCTG	389	68
Colicin 5, 10, K	510Kf 510Kr	AAAGCTGAACTGGCGAAGGC CAACTCATCATCCCCTATGTAAGAAG	803	60

2.3 Results

2.3.1 Isolation of *E. coli* strains from rat faecal matter:

On the Hichrome coliform agar plates, rat faecal samples contained *E. coli* (4.79±1.204) $\times 10^8$ (Cfu gm-1 wet faecal samples) after weaning and (2.32±1.021)$\times 10^6$ (Cfu gm-1 wet faecal samples) at adults stage.

2.3.2 Screening of *E. coli* for antimicrobial activity:

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* spp., *Staphylococcusaureus*, *Salmonella typhi* and *Salmonella abony* is shown in **Table 2.3** and **Fig. 2.1**. Culture filtrates which showed antimicrobial activity towards pathogens were treated with Proteinase K or Protease (trypsin) which resulted in lost of antimicrobial effect thus suggesting that the antimicrobial compound is a protein Fig. **2.2**. 16 out of 47 isolates showed better inhibitory activity against different pathogens.

Fig. 2.1: Antimicrobial activity in *E. coli* isolates the presence of Mitomycin C induction

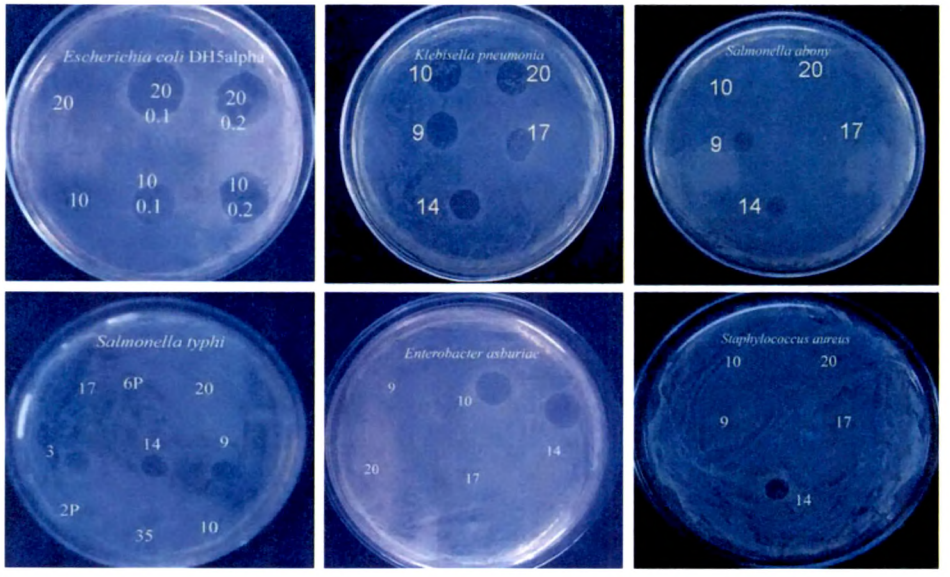
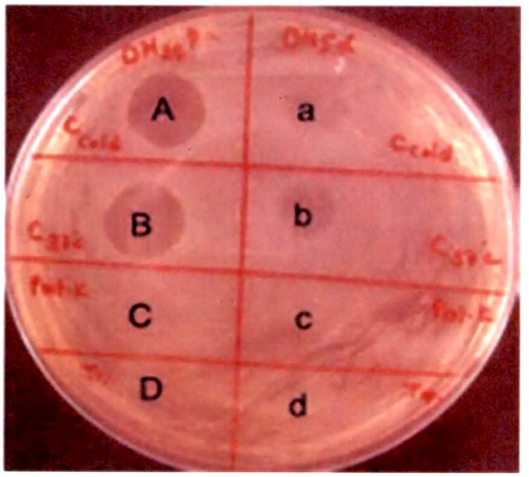


Table 2.3: Antimicrobial activity of *E. coli* against enteropathogens

***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*

Fig 2.2 Loss of Antimicrobial activity upon treatment of Trypsin and ProteinaseK



A. Induced supernatent at cold condition
B. Induced supernatent incubated at 37°C
C. Proteinase K treatment
D. Trypsin treatment
a. Uninduced supernatant at cold condition
b. Uninduced supernatant at 37°C
c. Proteinase K treatment
d. Trypsin treatment

2.3.3 Antibiotic susceptibility of rat faecal *E. coli* isolates:

Most isolates did not show multi-drug resistance and the isolates which showed low multiple resistances were eliminated in initial screening with commercial antibiotics (data not shown). 16 isolates were finally screened by using commercial antibiotics discs (HiMedia) **Table 2.4**.

Table 2.4: Antibiotic susceptibility pattern of *E. coli* isolates (Concentration of antibiotic in µg/ml)

Culture No	*A*	*G*	*Ac*	*Tb*	*Co*	*Ce*	*Na*	*Nt*	*Nf*	*Ak*	*Cf*	*Ca*	*Of*	*T*	*Cu*
	10	**10**	**30**	**10**	**25**	**30**	**30**	**30**	**300**	**30**	**5**	**30**	**5**	**25**	**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

Survival percentage (% + SD) after incubation

2.3.4. Acid tolerance assay:

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 (**Table 2.5**).

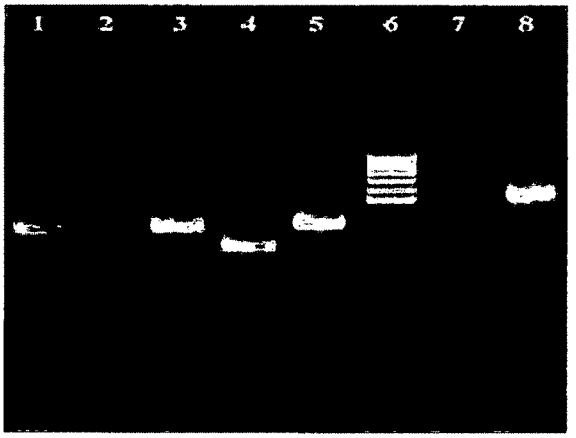
Table. 2.5: Acid tolerance tests for *E. coli* isolates

	pH 1.0	pH 2.0			pH 3.0		
STRAIN	**2h**	**2h**	**4h**	**6h**	**2h**	**4h**	**6h**
E. coli 17	0.00±.00	0.74 ± 0.15	0.09 ± 0.03	0.01 ± 0.00	71.95 ± 2.47	61.50 ± 2.12	39.40 ± 3.68
E. coli 14	0.00±.00	2.26 ± 0.21	0.60 ± 0.14	0.00 ± 0.00	35.50 ± 4.95	19.80 ± 3.11	11.95 ± 0.92
E. coli 45	0.00±.00	0.22 ± 0.01	0.15 ± 0.01	0.06 ± 0.01	46.50 ± 6.36	38.65 ± 1.06	35.55 ± 0.21
E. coli 44	0.00±.00	2.1 ± 0.14	1.18 ± 0.03	0.18 ± 0.01	67.00 ± 5.66	58.15 ± 3.04	14.80 ± 1.13
E. coli 3	0.00±.00	0.415 ± 0.01	0.35 ± 0.00	0.09 ± 0.01	68.25 ± 2.52	56.00 ± 8.49	43.13 ± 1.28
E. coli 10	0.00±.00	8.35 ± 0.35	7.35 ± 0.21	6.80 ± 0.28	74.50 ± 4.95	25.15 ± 0.49	21.40 ± 1.41
E. coli 20	0.00±.00	0.215 ± 0.01	0.13 ± 0.04	0.00 ± 0.00	41.75 ± 2.76	35.53 ± 0.75	31.15 ± 1.63
E. coli 16	0.00±.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

2.3.5. Detection of pathogenic strains by Multiplex PCR of indicator genes.

For 7 indicator genes: *aggR, est, aspU, CVD432, elt, eae* and *stx*, positive control were obtained. (**Fig. 2.3**). The sizes of the amplicons were similar to the reported pathogenic genes. Hence, the primers were used to determine the presence of the pathogenic genes in the *E. coli* isolates. No amplicons were obtained from the genomic DNA of all the *E. coli* isolates.

Fig. 2.3: Pathogenic PCR of activated sludge samples

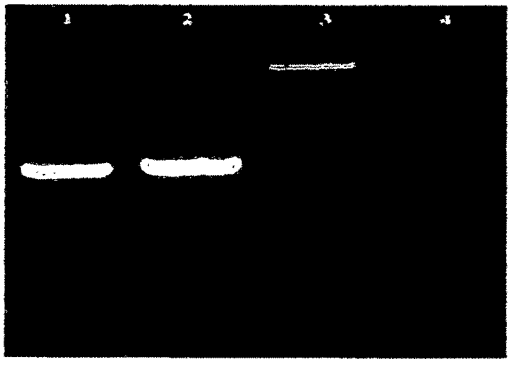


Lane 1-aggR
Lane 2-AL
Lane 3-aspU
Lane 4-Eagg
Lane 5-LT
Lane 6 – 100bp ladder
Lane 7 – SK amplicon
Lane 8 – VT

2.3.6. Polymerase chain reaction (PCR) for Identification of colicin gene in *E. coli* isolatesIsolates

17 contained only E1 type; isolates 3 9, 10,35, 44, 8P and 14P contained E1 and 1a/1b type showed in **Fig. 2.4**; isolates 16 and 2P contain E1, 1a/1b and Emix type; and isolates 14 and 20 contained E1, 1a/1b and B/D type colicins.

Fig. 2.4 PCR amplification of colicin genes



Lane 1:. 1a/1b colicin 385bp
Lane 2: E1 colicin 389 bp,
Lane 3: E1 and 1a colicin 1.1kb
Lane 4: 100bp DNA ladder

2.4 DISCUSSION

E. coli count in the rat faecal matter decreased from weaning to the adult stage by about hundred fold. Variation in the abundance and diversity of microflora has been found to occur during the development of animals. In the initial stages the microflora is found to be more diverse and decrease during the transition to the adult stage (Inoue *et al.*, 2005). In addition, the composition of the intestinal microflora is influenced by nature of the diet, antibiotic treatment and infection of exogenous opportunistic pathogens (Jernberg *et al.*, 2005; Gostner *et al.*, 2006). The results of the present study demonstrated that some of the *E. coli* strains (isolated of faecal samples) showed antimicrobial activity against the members of *Enterobacteriaceae* family *viz. Enterobacter asburiae*, *Klebisella* spp., *Staphylococcus aureus*, *Salmonella typhi*, and *Salmonella abony*. These isolates were susceptible to the most of antibiotics tested and low resistance was observed in some cases which were eliminated in the screening. In feaces, population of antibiotic resistant and sensitive microorganisms varies. (Mubita *et al.*, 2008; Schierack *et al.*, 2009). Multiplex PCR studies demonstrated that these *E. coli* strains do not contain any pathogenic traits. Antibiotic sensitivity and non-pathogenic traits are essential for probiotic organisms.

Antimicrobial activity of these *E. coli* strains was enhanced by Mitomycin C induction. Mitomycin C is known to induce the production of colicins (Cascale *et al.*, 2007). Antimicrobial activity of *E. coli* strains 10, 14, and 14P demonstrated broad host range. Majority of the *E. coli* strains contained colicins E1 and 1a/1b, in contrast to the combination of Microcin H47 with Microcin M was reported (Gordon at el., 2006) These colicins belong to pore forming type. Interestingly, 4 *E. coli* strains along with these pore forming colicins produced other colicins. Isolates 16 and 2P possessed E mix which belong to nuclease type, whereas isolates 14 and 20 had B/D types, which are translation blockers.

Before reaching the intestinal tract, probiotic bacteria must first survive transit through the stomach. Preliminary experiments showed that these colicin producing *E. coli* strains also had good but variable acid tolerance at pH 3.0. *E. coli* can survive at a

pH as low as 2.0 upon induction of acid resistant genes (Hersh *et al.*, 1996; Lin *et al.*, 1996). Thus, these *E. coli* strains have great potential as probiotic bacteria against GI diseases caused by enteropathogens.