Report from July 2002 through July 2003

Title: Competitive Exclusion of *E. coli* O157 using non-pathogenic bacteriocins producing *Escherichia coli* strains.

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***Summary*:** In this investigation, we screened 496 *E. coli* strains isolated from humans, horses, pigs and sheep for the production of colicin that would inhibit the growth of pathogenic Shiga toxin producing *E. coli* (STEC) from cows. The STEC strains tested carried virulent attributes such as shiga toxins, hemolysin and intimin (eae). We established three different screening procedures for detecting colicin production among the isolates. The inhibitory effect of colicin was determined by the zone of inhibition produced around the bacteria when overlaid with STEC culture. The results of the experiments reflected 12% strains from humans, 15% strains from porcine, 2.5% strains from horses and 6% strains from sheep that were screened, exhibited colicin activity out of which three strains (2 from humans and 1 from pig) were very effective colicin producers. These three strains were further tested against 90 STEC cultures belonging to different serogroups such as O26, O111, O157 and O145. The two human isolates inhibited growth of all STEC strains to the extent of 92-95%. Colicin produced by two strain inhibited growth of *E. coli* O157:H7 to the extent of 95-100% in 2 hours. Colicin produced by two isolates when combined and tested against the growth of *E. coli* O157 showed 100% inhibition in 2 hours. We also established that the colicin produced was proteinaceous in nature and the inhibitory effect was not due to the presence of bacteriophages. The bacteria producing colicins can be potentially used as an alternate approach to control *E. coli* O157:H7 proliferation and colonization of dairy premises and intestinal tract of beef and market cows by using them as competitive inhibitors.

1. Screening for colicin producers: From the large collection of *E. coli* strains at the Gastroenteric Disease Center, isolates obtained from humans, horses, pigs and sheep were screened for bacteriocin (colicin) production that would inhibit growth of pathogenic *E. coli* O157 isolated from cows carrying shiga toxin (*stx1* and *stx2)* genes that encode for shiga-toxin 1 and 2, *eae* (attaching and effacing) and hemolysin genes. The putative bacteriocin producers were grown in LB media in 24-well titer plates. At mid-log phase of growth, mitomycin C (0.25µg/ml) was added to induce the cultures for colicin production and grown for another 3 hours at 37^{o}C to a final concentration of 2x10^{5} CFU/ml. These were transferred on to LB plates using a sterile stainless steel 24 prongs replicator and grown overnight at 37^{o}C. The putative producer strains were killed with chloroform dispensed on filter paper discs. The plates were overlaid with 10 ml soft Luria agar seeded with *E. coli* O157 at about 10^{8} CFU/ml concentrations. The other method used was to grow the bacteria to be screened on LB agar media in petri dishes overnight. The agar was flipped and further overlaid with *E. coli* O157. This method avoided use of chloroform. Twenty colicin producing standards from National Collection

Type Cultures, were used as controls. Clear or hazy zones (Figure 1) were measured after incubating the plates overnight at 37°C. As depicted in Table 1, eleven out of ninety two (12%) cultures obtained from humans were found to inhibit growth of *E. coli* O157 strains, out of which two cultures (2.0457 and 2.0569) produced larger zones of inhibition. One hundred and ninety five cultures from pigs were tested for colicin production. Thirty strains (15%) inhibited growth of *E. coli* O157, out of which one culture (99.1986) exhibited very strong inhibitory activity. Seventy nine cultures from horses and 129 cultures from sheep were also tested for inhibitory activity against *E. coli* O157. Two cultures (2.5%) from horses and 8 (6%) cultures from sheep inhibited growth of *E. coli* O157. There were a total of three colicin producing strains that were highly effective in inhibiting the growth of *E. coli* O157.

2. Effectiveness of colicin against STEC strains: Two strains (2.0457 and 2.0469) isolated from humans and a porcine isolate (99.1986) were found to be highly effective colicin producers. The inhibitory effect of colicins produced by these three strains were further tested against a large number of shiga-toxin producing *E. coli* (STEC) strains belonging to different serogroups. Twenty three strains belonging to serogroup O26, 8 strains belonging to serogroup O103, 19 strains belonging to serogroup O111, 3 strains belonging to serogroup O145 and 37 strains belonging to O157 serogroup were tested for growth inhibition as shown in Table 2. While 2.0457 and 2.0469 inhibited growth of 92.4% and 95.6% of STEC strains respectively, strain 99.1986 inhibited growth of 65.6% STEC strains. However, 99.1986 strain was very effective (indicated by double positive (++) signs) in inhibiting growth of O157 as compared to other STEC strains such as O26 or O103.

3. Inhibitory effect of colicin: Inhibitory effect of colicins produced by strain 99.1986 was tested against the growth of *E. coli* O157. Colicin producing culture 99.1986 was grown overnight at 37°C. Next morning the culture was diluted to an OD_{600} unit of 1.1 and half of the culture was induced by adding mitomycin (25µg/ml) for colicin production and the other half served as control (non-induced) where same amount of water was added instead of mitomycin. The cultures were grown for 2 hrs at 37°C. Since colicins are elaborated by the bacteria in the media, the bacteria were removed by centrifugation at 10,000xg for 10 min. The pellet containing the bacteria was discarded and the supernatants from induced and non-induced cultures were further filtered through 0.2 µm filters to remove all bacteria. *E. coli* O157 grown overnight at 37°C was diluted such that the cell concentrations were between 100 CFU (colony forming units) to 10^4 CFU/ml. Diluted cultures of *E. coli* O157 bacteria were incubated with supernatants from induced and non-induced cultures. The number of cells was counted by plating 0.1 ml of cultures on Luria Bertani agar media at 0 hr, 2 hrs and 4 hrs from both induced and non-induced sets. The results, as depicted in Figures 2 and 3, show that the growth of *E. coli* O157:H7 culture was inhibited to an extent of 95-100% with colicin produced by 99.1986 and 2.0469 strains when incubated for 2 hours and 4 hours at 37°C. When colicins produced by both the strains were combined and growth inhibition of *E. coli* O157:H7 was 100% (Figure 4) in 2 hrs.

4. Is it colicin or phage? To determine if the growth inhibition of *E. coli* was due to colicin and not due to the presence of bacteriophages, producer culture 99.1986 was grown overnight at 37°C in LB broth. It was diluted to OD_{600} to 1.1 and the culture was induced with mitomycin (0.25µg/ml) and incubated for 2 hrs at 37°C. The bacterial culture was centrifuged and the supernatant containing inhibitor was divided into two. One set was treated with proteinase K

(200µg/ml) and the other set was left untreated. The inhibitory activity of the supernatant was tested on overlay assays described in section 1. Following proteinase K treatment the supernatant containing colicin, did not show any inhibitory activity that reflected that colicins are proteinaceous in nature and are elaborated by the culture in the medium. To confirm this finding phage testing was also performed by standard procedures. Agar stab from the zone of inhibition (see Figure 1) was scooped out and mixed with overlay cultures as described in section 1, and grown on petri plates. There were no plaques observed confirming that the cultures were producing proteinaceous colicins and the inhibition was not due to the presence of phages.

Benefits of the investigation: The investigation will provide an alternate approach to controlling *E. coli* O157:H7 proliferation and colonization of dairy premises and intestinal tract of beef and market cows. The use of non-pathogenic colicin producing strains of *E. coli* will be cost effective and will not have any adverse effect on environment, animal or human health. The research may potentially benefit the beef industry as an alternate approach to controlling *E. coli* O157:H7 in beef and dairy cattle by competitive exclusion of pathogenic strains.

Figure 1. Colicin producing *E. coli* cultures showing inhibitory clear zones on overlay assays against *E. coli* O157.

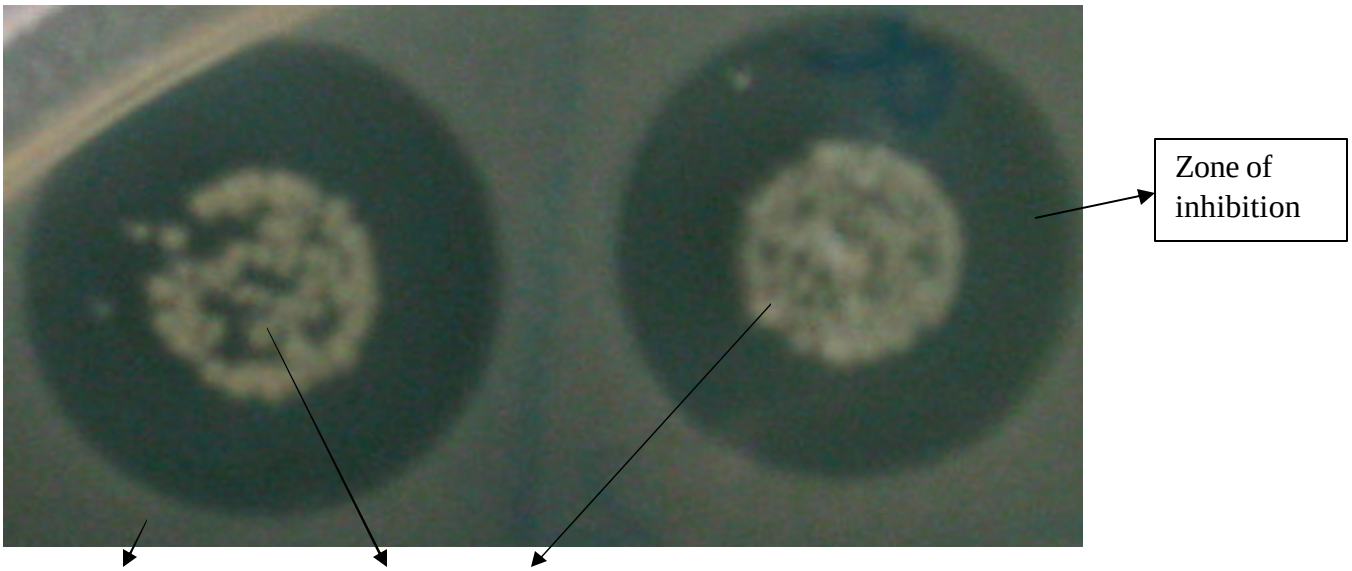


Table 1. *E. coli* isolates screened for colicin production that inhibits growth of *E. coli* O157

Host species	# Cultures screened	# Producing colicin	# Highly effective producer	% colicin producer
Humans	92	11	2	12%
Pigs	195	30	1	15%
Horses	79	2	0	2.5%
Sheep	130	8	0	6 %
Total	496	51	3	10%

Table 2. Inhibitory effects of Colicin producing strains on STEC cultures

				Colicin producers-→								Colicin producers→		
Culture #	Otype	*slt1*	*slt2*	2.0457	2.0469	99.1986	Culture #	Otype	*slt1*	*slt2*	2.0457	2.0469	99.1986
97.0881	26	+	-	+	+	-	97.0915	111	+	-	+	-	-
97.0882	26	+	-	+	+	-	97.0922	111	+	-	-	-	-
97.0883	26	+	-	+	+	-	97.0928	111	+	-	-	-	-
97.0884	26	+	-	+	++	+	97.1623	111	+	-	+	+	+
97.0885	26	+	-	+	+	+	98.0552	111	+	-	+	+	-
97.0886	26	+	-	+	++	+	95.0187	145	+	-	-	+	-
97.0887	26	+	-	+	+	+	97.1388	145	-	+	+	+	-
97.0888	26	+	-	-	-	-	98.0484	145	-	+	+	+	+
99.0683	26	+	-	+	++	+	99.2054	157	+	-	+	+	+
99.0685	26	+	-	+	++	-	99.2055	157	+	+	++	+	+
99.0694	26	+	-	+	+	+	99.2056	157	-	+	+	+	++
99.0695	26	+	-	+	+	-	99.2057	157	-	-	+	+	++
99.0696	26	+	-	+	+	+	99.2058	157	-	+	-	-	++
99.0699	26	+	-	+	+	+	99.2060	157	-	-	+	+	++
99.0702	26	+	-	-	+	+	99.2061	157	-	-	++	+	+
99.0703	26	+	-	+	++	-	99.2062	157	-	+	+	+	++
99.0704	26	+	-	-	++	-	99.2063	157	+	+	+	+	++
99.0723	26	+	-	+	+	+	99.2064	157	-	-	+	+	-
99.0724	26	+	-	+	+	+	99.2066	157	+	+	+	+	+
99.0849	26	+	-	+	++	+	99.2067	157	-	-	++	+	+
99.0850	26	+	-	+	+	-	99.2068	157	-	+	+	+	++
99.0869	26	+	-	+	+	+	99.2069	157	-	+	+	+	++
99.1761	26	+	-	+	+	-	99.2070	157	-	+	+	+	++
97.0659	103	+	-	+	+	-	99.2071	157	-	+	++	+	++
97.0663	103	+	-	+	+	-	99.2072	157	-	+	+	+	++
97.0664	103	+	-	+	+	-	99.2073	157	+	+	+	+	++
97.0665	103	+	-	+	+	-	99.2074	157	-	+	+	+	++
97.0693	103	+	-	+	+	-	99.2075	157	-	+	+	+	++
97.0694	103	+	-	+	+	-	99.2076	157	+	+	+	+	++
97.0695	103	+	+	+	+	-	99.2077	157	+	+	++	+	++
97.0696	103	+	-	+	+	+	99.2078	157	-	+	++	+	++
0.1932	111	+	+	+	+	-	99.2079	157	+	+	++	+	++
95.0122	111	+	-	+	+	+	99.2081	157	-	+	++	+	++
95.0144	111	+	-	+	+	+	99.2082	157	-	+	++	+	+
95.0174	111	+	-	+	+	-	99.2083	157	+	-	+	+	++
95.0182	111	+	-	+	+	++	99.2085	157	+	+	++	+	+
95.0192	111	+	-	+	+	++	99.2086	157	-	+	+	+	-
95.0206	111	+	-	+	+	-	99.2087	157	+	+	+	+	++
95.0209	111	+	-	+	+	-	99.2088	157	+	+	++	+	+
96.0214	111	+	-	+	+	+	0.0027	157	+	-	+	+	++
96.0217	111	+	-	+	+	+	0.0362	157	+	+	+	+	+
96.0218	111	+	-	+	+	-	0.0372	157	+	+	+	+	+
96.0329	111	+	-	+	+	+	0.0373	157	+	+	+	+	-
97.0168	111	+	-	+	+	+	0.1288	157	+	+	+	+	++
97.0373	111	+	-	-	+	+	0.1292	157	+	+	+	+	+

slt1 and slt2: shiga toxin 1 and 2; +: growth inhibition; ++: very effective growth inhibition; - no inhibition

Figure 2. Inhibition of *E. coli* O157 growth in the presence of colicin produced by culture 99.1986.

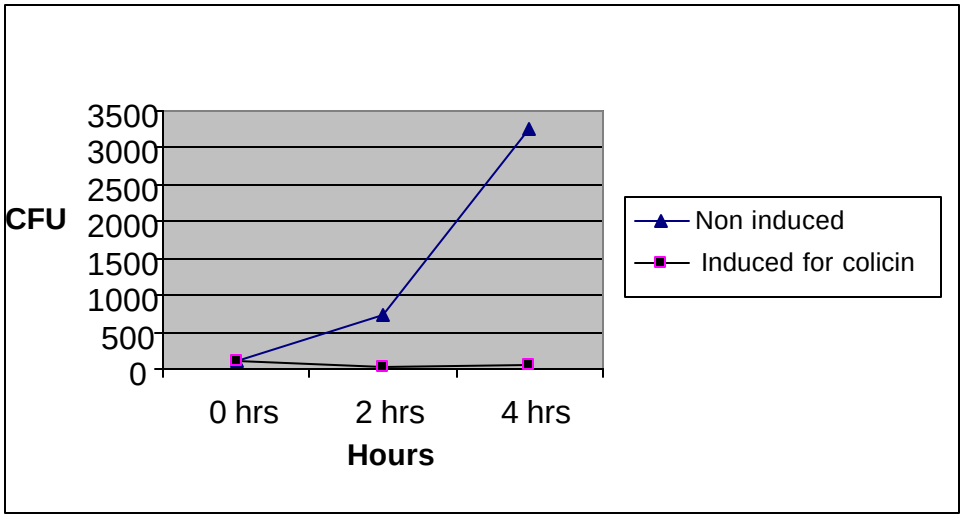


Figure 3. Growth inhibition of *E. coli* O157 in the presence of colicin produced by strain 2.0469 isolated from humans

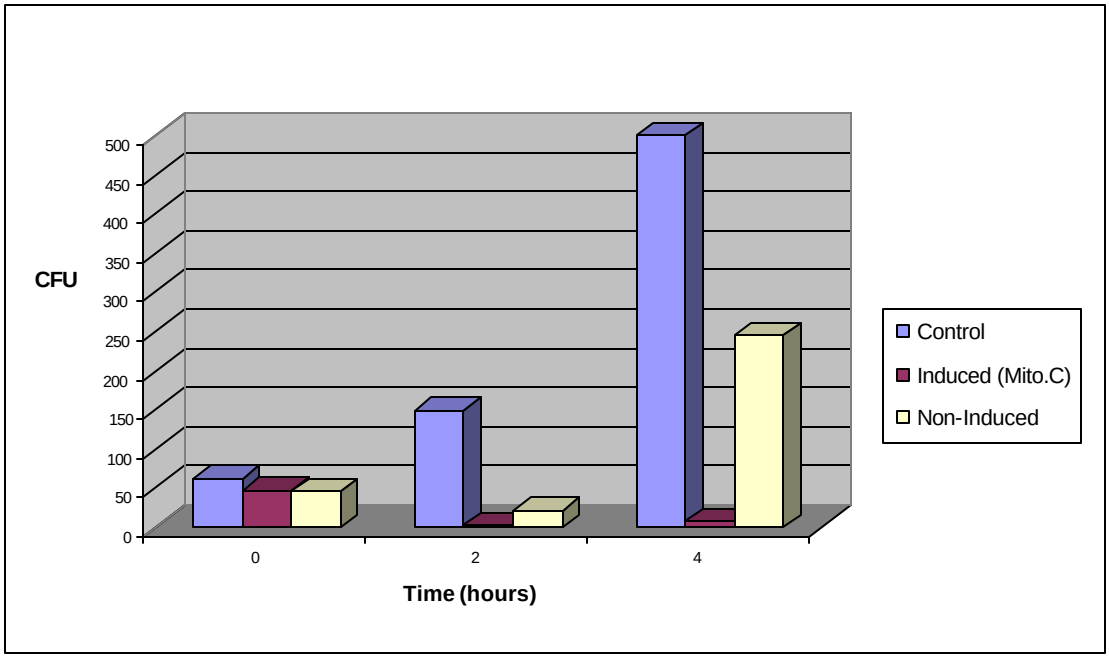
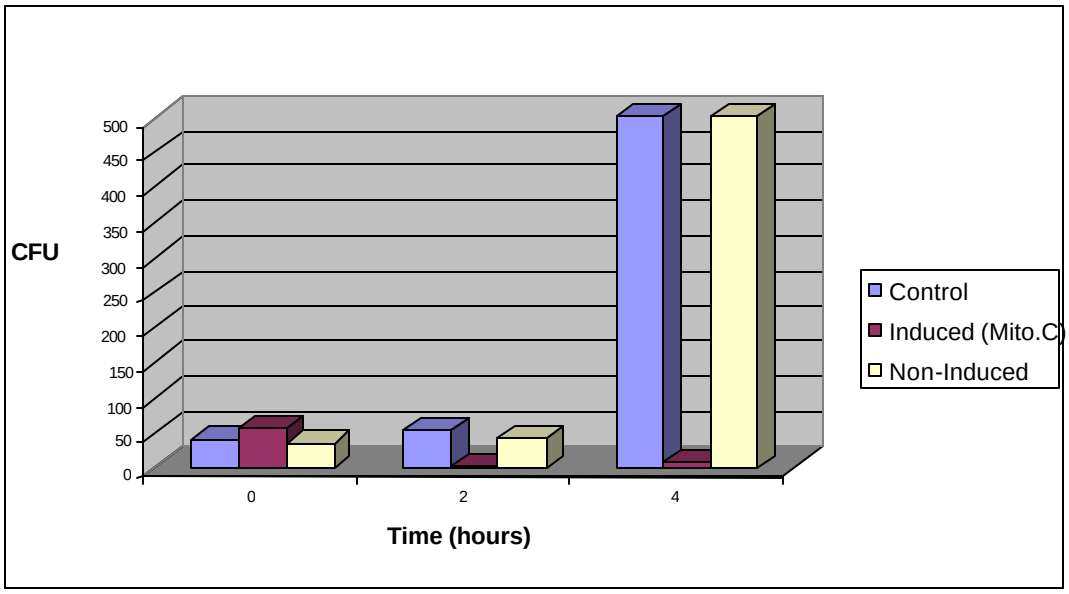


Figure 4. Inhibition of *E. coli* O157:H7 by colicin produced by two strains (99.1986 and 2.0469) combined



Time Line:

July 2002---------- **Dec 2002**----------**July 2003**

Optimizing Methods for overlay	Screening cultures (496)	Examining inhibition against 90 STEC cultures	Inhibitory effect of colicin produced by cultures