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ORIGINAL ARTICLE

Escherichia coli with anti-O157:H7 activity isolated from bovine colon

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Abstract

Aims: To isolate bacteria from bovine gastrointestinal tract and investigate their inhibitory effect on *Escherichia coli* O157:H7 *in vitro*.

Methods and Results: A total of 2400 bacterial colonies were isolated from cattle colonic mucous membrane. Thirteen strains demonstrated the ability to inhibit the growth of *E. coli* O157:H7. From these, seven were screened for the presence of virulence factors as: stx_1, stx_2, *ehxA*, *eae*, *st1a* and *lt1* by polymerase chain reaction. The selected bacteriocin-producing bacteria showed susceptibility to most of the antibiotics used.

Conclusions: The strains of *E. coli* isolated, which exhibit inhibitory activity on *E. coli* O157:H7 growth by the production of inhibitory substances, may be useful in the control of this pathogen in reservoirs. An important characteristic of these strains was the absence of any of the virulence factors assayed and the susceptibility to most of the antibiotics used for Gram-negative bacteria.

Significance and Impact of the Study: These microorganisms might be used as probiotic bacteria to reduce the carriage of *E. coli* O157:H7 in cattle, thus limiting the contamination of carcasses at slaughter and subsequently the contamination of foods and the transfer of this pathogen to man.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are important food-borne pathogens that cause severe diseases in humans such as haemorrhagic colitis (HC) and haemolytic–uraemic syndrome (HUS). The most often reported STEC serotype associated with the disease in humans is O157:H7 although other non-O157 serotypes are isolated worldwide from humans with HC and HUS (López *et al.* 1998; Parma *et al.* 2000; Bettelheim 2001; Elliot *et al.* 2001). The primary feature of STEC is its ability to produce potent cytotoxins called Shiga toxins (Stx_1 and Stx_2). It also has a number of other virulence factors such as a megaplasmid (Mp), and a protein called intimin encoded by the *eae* gene, which enhances its pathogenicity (Tesh and O'Brien 1991; Robbins-Brown 2000).

In Argentina, HUS is the most frequent cause of acute renal damage and the second most frequent cause of chronic renal injury and kidney transplantation in children (Voyer 1996; Voyer and Rivas 1997). Argentina has the highest frequency of HUS in the world, with an estimated number of 420 cases per year in Buenos Aires city and the surrounding countryside (López *et al.* 1998).

Domestic ruminants, particularly cattle and sheep, are the main reservoirs of STEC (Wells *et al.* 1991). Cattle harbour many of the serogroups isolated from man including O157:H7 although this serotype is not pathogenic for them (Butler and Clarke 1994; Cray and Moon 1995). However, colostrum-deprived neonatal calves inoculated with *E. coli* O157:H7 developed severe, sometimes fatal, diarrhoea (Dean-Nystrom *et al.* 1997). In Argentina, 33% of the bovines belonging to different categories (calves with and without diarrhoea, grazing cattle, grain-fed cattle and cattle at slaughterhouse) are reservoirs of STEC strains, many of which belong to the enterohaemorrhagic *E. coli* group (EHEC) (Sanz *et al.* 1998; Padola *et al.*

2004). Grauke *et al.* (2002) found that *E. coli* O157 persists in the middle to lower gastrointestinal tract (lower ileum, cecum and colon) and that is not harboured for long periods of time in the stomach (rumen, reticulum, omasum and abomasum) or duodenum.

Considering the high prevalence of the pathogen in faeces and the potential contamination of carcasses during processing at slaughterhouse it is necessary to develop procedures to curb its presence in ruminants and thereby reduce the incidence of human infections with this pathogen (Grauke *et al.* 2002; Brashears *et al.* 2003). One of them, based on competitive exclusion strategies, might be the administration of probiotic bacteria (Fuller 1989; Zhao *et al.* 1998). Probiotics are live microbial feed additions that affect the host by improving its intestinal microbial balance, eliminating or reducing harmful microorganisms (Fuller 1992; Perdigón *et al.* 1998). These bacteria attach to enterocytes and thus inhibit the binding of enteric pathogens to the intestinal mucosa by production of inhibitory substances that include bacteriocins, lactic acid and toxic oxygen metabolites (Nemcová 1997; Kopp-Hoolihan 2001).

Opinions are divided about to whether the resistance of some probiotic strains to specific antibiotics represents an advantage. The commercial introduction of probiotic preparations containing antibiotic-resistant strains may also have negative consequences, for example, when resistance is transferred to intestinal pathogens (Curragh and Collins 1992; Charteris *et al.* 1998).

The presence of bacteria that produce metabolic inhibitors to *E. coli* O157:H7 at sites where this strain localizes is another factor that may influence the localization of O157:H7 in the gastrointestinal tract. Some wild strains of *E. coli* are able to produce bacteriocins that inhibit the growth *in vitro* of diarrheagenic *E. coli* strains, including those belonging to the O157:H7 serotype (Bradley *et al.* 1991; Murinda *et al.* 1996).

The aim of this study was to isolate bacteria from the bovine gastrointestinal tract and investigate their inhibitory effect on *E. coli* O157:H7 growth as well as their susceptibility to antibiotics *in vitro*.

Materials and methods

Isolation of intestinal bacteria

The bacteria were isolated from 200 samples of cattle colon from animals whose faeces were negative for Stx_1/Stx_2 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 as indicated below.

Preparation of *E. coli* O157:H7 cultures

One strain of *E. coli* O157:H7 (BP166; stx_2+), isolated from the faeces of a grazing cow, was used as an indicator strain to screen for the inhibitory activity from the intestinal bacteria. The strain was transferred into 10 ml of Luria–Bertani (LB) broth and incubated overnight at 37°C with shaking. The number of cells was estimated from the absorbance at 600 nm. Additionally, 11 O157:H7 isolates (stx_2+, *eae*O157+, *ehxA*+) from grazing cattle (three isolates), grain-fed cattle (three isolates), and humans HUS cases (three isolates) and human bloody diarrhoea cases (two isolates) were also used as indicator strains in assays with the intestinal strains.

O157:H7 inhibition assay

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°C. 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°C. 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: stx_1, stx_2, *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).

Differentiation between bacteriocin and temperate bacteriophage

Bacterial lysogens can produce false-positive indications of bacteriocin production in some tests. The inhibition of indicator cell growth caused by bacteriocins was confirmed by picking a plug of agar from the inhibition zone with a sterile Pasteur pipette. Each plug was resuspended in tubes with 1 ml of sterile LB broth containing 20 μl of chloroform. Each tube was homogenized by vortex-mixing and allowed to stand for 5 min. From each tube, 100 μl was taken and added into 3 ml of LB–0·4% agar containing 10^6 CFU ml^{-1} of the indicator strain and plated out on LB agar layer. The plates were incubated

overnight at 37°C and examined for the presence or absence of plaques (Pugsley and Oudega 1987).

Differentiation between high and low molecular weight bacteriocins

The bacteria selected as potential probiotics were plated as described in the O157:H7 inhibition assay. After killing the bacteria with chloroform vapour, a sterile cellophane sheet was placed covering the entire plate surface and then overlaid with 5 ml of soft LB agar containing 10^6 *E. coli* O157:H7 CFU ml^{-1}. The plates were incubated at 37°C overnight (Pugsley and Oudega 1987).

Antimicrobial susceptibility test

The antibiotic sensibility of the selected bacteria was determined by diffusion methods following the Bauer–Kirby procedure (Chengappa 1990). The amount of each antibiotic per disc was: ampicillin (10 μg), cephalothin (30 μg), cephotaxime (30 μg) ciprofloxacin (5 μg), chloramphenicol (30 μg), gentamicin (10 μg), imipenem (10 μg), trimethoprim/sulfamethoxazole (TMS) (25 μg).

In vitro competition experiment

In this experiment, we prepared three different mixtures with one of the selected strains as potential probiotics and the indicator strain. The first mixture was prepared with 10 μl of an overnight culture of the potential probiotic bacteria and 100 μl of an overnight culture of the indicator strain. Both suspensions were previously adjusted to $1{\cdot}7 \times 10^9$ viable cells per millilitre. The second mixture was done with 10 μl of the potential probiotic bacteria and 1 ml of the indicator strain, and the third mixture consisted of 10 μl of the potential probiotic bacteria and 10 ml of the indicator strain. The three inoculums mixtures were independently seeded into 25 ml LB and incubated at 37°C on an orbital shaker. Samples were taken at time (h) 0, 4, 8, 10, 24 and 48. Each of the mixtures was serially diluted (1:10) in 0·85% NaCl, and 0·1 ml of each dilution was plated onto MacConkey sorbitol plates. Plates were incubated at 37°C overnight for determining *E. coli* O157:H7 and bacteriocin-producing bacterial populations.

Results

Isolation of bacteria

A total of 2400 bacterial colonies were isolated from cattle colonic mucous membrane. No shedding of STEC could be previously demonstrated in those animals by PCR. These microorganisms were also screened for their ability to inhibit the growth of *E. coli* O157:H7 *in vitro*.

Inhibition of *E. coli* O157:H7

Thirteen strains demonstrated the ability to inhibit the growth of the indicator strain, BP 166 (Figure 1). For further studies we selected only seven of them (positions 2, 3, 5, 6, 8, 9 and 10), 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 stx_1, stx_2, *eae*, *ehxA*, *stIa* and *ltI* virulence genes was demonstrated in all the selected strains when screened by PCR.

Additionally, 11 O157:H7 isolates (stx_2+, *eae*O157+, *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.

Production of bacteriocins

All selected bacteria inhibited the growth of *E. coli* O157:H7 BP166 in agar plates. The phenomenon was mediated by the production of antimicrobial substances called bacteriocins, discarding the presence of temperate bacteriophage by the absence of lysis plaques following the steps described before.

Three from seven bacteria produced bacteriocins of low molecular weight (Fig. 1, positions 2, 8 and 10), which were able to diffuse through a cellophane sheet, and the

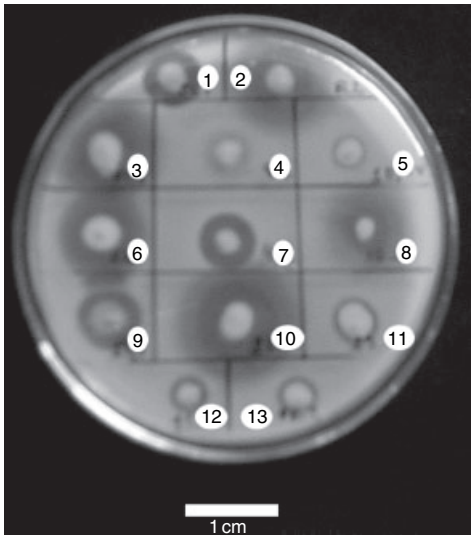

Figure 1 Growth inhibition of the indicator strain. Numbers 1–13 indicate bacteriocin-producing bacteria isolated from colon of different cattle at slaughter.

Table 1 Antibiotic susceptibility of bacteriocin-producing bacteria

	Bacteriocin-producing bacteria						
Antibiotic	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

S, sensitive; R, resistant; I, intermediate.

others, which did not do that, corresponded to bacteriocins of higher molecular weight (Fig. 1, positions 3, 5, 6 and 9).

Antibiotic resistance

The selected bacteriocin-producing bacteria showed susceptibility to most of the antibiotics used in the diffusion method as described before. These data are shown in Table 1.

Competition assay

Plates were observed for determining the colony population belonging to *E. coli* O157:H7 (sorbitol-negative colonies) and bacteriocin-producing bacteria (sorbitol-positive colonies). The results were expressed as percentage of sorbitol-negative colonies. Although the decrease in the percentage of O157:H7 was dependent upon the starting proportions of bacterial strains present, at 48 h incubation, no sorbitol-negative colonies were observed in any of the mixed culture (Fig. 2). Results are the average of three independent experiments.

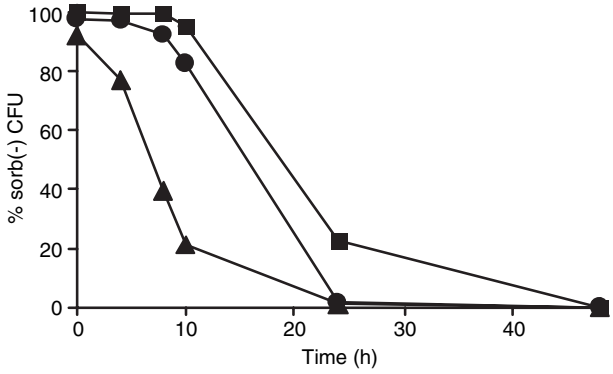

Figure 2 Percentage of sorbitol-negative colonies belonging to *E. coli* O157:H7 serotype versus incubation time in a mixed culture with a bacteriocin-producing *E. coli*. Inoculum A corresponds to Bact+/O157 ratio 1/10; B, 1/100; C, 1/1000. ▲, A; ●, B; ■, C.

Discussion

A possible alternative to avoid infections in humans caused by STEC strains is to try the control or the eradication of this bacterium from the food chain. Several strategies are currently being explored to reduce the prevalence of *E. coli* O157:H7 in cattle (Stevens *et al.* 2002). A few studies are referred to the use of antibiotics to eradicate STEC from cattle (Misawa 2000). This attempt has three main disadvantages. First, the treatment is very expensive; second, the risks of inducing the production of toxins by the antibiotics themselves; and, third, the risk of inducing and spreading antibiotic resistance. Some other interventions include the use of vaccines, feed additives, diet shifts and bacteriophages (Stevens *et al.* 2002).

Another attempt to achieve that purpose might be by reducing bovine faecal shedding. The most promising methods to reduce pathogenic microorganisms in livestock are based upon antagonistic bacteria, competitive exclusion or direct-fed microbial products (Schamberger *et al.* 2004). Several authors have identified bacteria with the potential ability to inhibit or exclude *E. coli* O157:H7 in the gastrointestinal tract of cattle. The first account of the use of probiotics to inhibit *E. coli* O157:H7 in cattle was by Zhao *et al.* (1998) who identified several *E. coli* strains. Three of them were used by Zhao *et al.* (2003) to reduce the faecal shedding of EHEC in neonatal calves. Schamberger and Diez-Gonzalez (2002) also reported the selection of 24 *E. coli* strains with the ability to inhibit O157:H7.

The strains of *E. coli* isolated by us, which exhibit inhibitory activity against *E. coli* O157:H7 growth by the production of bacteriocins, may be useful in the control of this pathogen in reservoirs. It is an important characteristic of these strains that they do not harbour any of the virulence factors assayed and showed susceptibility to most of the antibiotics used for Gram-negative flora.

These microorganisms might be used as probiotic bacteria to reduce the carriage of *E. coli* O157:H7 in cattle and then to limit the contamination of carcasses at slaughter. By this way the contamination of foods would diminish and, consequently, the entry of this pathogen into humans. Future studies will be necessary to evaluate the use of these bacteria as potential probiotics by *in vivo* trials.

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