Prevalence and characterisation of shigatoxigenic *Escherichia coli* isolated from beef cattle fed with prebiotics

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Abstract

Ten Holstein Friesian calves were divided into two groups of five: one group was given prebiotics in their food, while the other group served as the control group. Every two weeks from birth up to 18 months, samples of feces were taken from the rectal ampulla to determine the concentration of *E. coli*. At each sampling session, three aliquots per sample were collected. The arithmetic mean was calculated and all values (converted into logs) were analysed with GraphPad InStat for analysis of variance, followed by the Tukey-Kramer test. A total of 69 *E. coli* strains were detected, 29 (42.03%) from treated animals and 40 (57.97%) from the control group. The isolates were analysed by PCR for the presence of the *stx-1*, *stx-2*, *hly* and *eae* genes and by the Kirby Bauer test for susceptibility to the most commonly used antimicrobials in cattle breeding. Hierarchical clustering of the isolates was done using Ward's method. Thirty samples were positive for the *stx-1* gene, 18 for *stx-2*, 12 for both *stx-1* and *stx-2*, 8 for *hly*, and 10 for *eae*. 4.3% were resistant to sulfamides, 8.6% to tetracycline, 1.4% to gentamicin, 94.6% to cephalothin, 2.8% to chloramphenicol, 13% to ampicillin, 13% to amoxicillin/clavulanic acid, 7.2% to sulphonamides, 4.3% to ceftriaxone, 5.7% to nalidixic acid, 34.7% to ticarcillin, 88.5% to erythromycin, and 5.7% to streptomycin. The isolates from the samples taken from day 210 to day 300 were grouped into a single cluster. Bacteriological examinations showed a reduction in the concentration of *E. coli* in the feces of the treated animals compared to the control group. The presence of strains with shigatoxigenic *Escherichia coli* virulence profiles and the reduction of these in the treated animal group demonstrated that diet can play an important role in reducing *E. coli* prevalence in cattle.

Introduction

Shigatoxigenic *Escherichia coli* (STEC) are pathogenic bacteria that can cause diarrhea, hemorrhagic colitis and hemolytic-uremic syndrome (HUS) in humans. Although infections caused by enterohemorrhagic *E. coli* are often associated with serotype O157:H7, other serotypes may also be involved and may be highly virulent (Lior, 1994; Brooks *et al*., 2005; Bonardi *et al*., 2007). Cattle are considered the main reservoir for STEC pathogenic for humans (Milnes *et al*., 2006; Jay *et al*., 2009). Most infections are attributed to the ingestion of water or foods contaminated by bovine feces (in particular raw vegetables, raw and undercooked meat and dairy products obtained from unpasteurised milk) (Caprioli *et al*., 2005). The virulence of the STEC strains is linked to the production of certain virulence factors such as intimin (coded by the *eaeA* gene) and hemolysin (coded by the *hlyA* gene), but above all to the production of Shiga toxins (coded by the *stx1* and *stx2* genes) (Cheasty *et al*., 1983; Griffin *et al*., 1995). These are toxins made up of one A subunit, with enzymatic activity, and five identical B subunits, which bind to a specific glycolipid on the membrane of the host cell (globotriaosylceramide, Gb3, which is found in the intestinal villi and renal endothelial cells). Once the A subunit has been internalised, it is split into two molecules, and fragment A1 binds to ribosomal RNA 28S, blocking protein synthesis (Karmali *et al*., 2003). The toxins are produced in the colon (Naylor *et al*., 2003) and are carried by the blood to the arterial endothelial cells of the kidney and the large intestine, which in humans are rich in Gb3 receptors, causing serious damage to those vascular districts (Brigotti *et al*., 2006). In addition, these toxins induce apoptosis in intestinal epithelial cells; the resulting destruction of the intestinal villi causes a decrease in absorption, with a relative increase in fluid secretion (Salminen *et al*., 1998). Shiga toxins also stimulate the production of cytokines, in particular TNF and interleukin-6, which, among other effects, cause an increase in Gb3 expression (Poli *et al*., 2005). To date, most of the efforts to avoid infection in humans have been concentrated on minimising the risk of fecal contamination of carcass at slaughter (Colavita, 2012). To this end, it is equally important to reduce prevalence and excretion of pathogenic *E. coli* in calves by developing alternative methods to the use of antimicrobials, so as to minimise the risk of developing antimicrobial resistance. One of these alternative methods is the addition of prebiotics in the cattle's diet. According to Roberfroid *et al*. (1998), a prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well being and health. They can also induce the growth or activity of beneficial microorganisms that can inhibit other species such as *E. coli* (Gibson *et al*., 1995).

The present study analyses the effect of the administration of prebiotics in the diet of calves on the prevalence of *E. coli* in the feces. The strains with STEC phenotypic traits were tested for the presence of *eaeA*, *hlyA*, *stx1* and *stx2* genes by PCR (Schurman *et al*., 2000; Karama and Gyles, 2013; EFSA, 2017) and the susceptibility profile to the antimicrobials most commonly used in buiatrics was analysed by agar diffusion test.

Materials and Methods

Experimental design

For this study, 10 male Holstein Friesian calves for beef production were selected

using simple random sampling from a list of animal-identification numbers to ensure a representative sample of animals from the farm. The farm, where the entire production cycle took place, was located in central Italy. The animals were randomly divided into two groups: the experimental group consisting of 5 calves was given prebiotics, while the 5 calves of the control group were not given anything besides their normal feed. The major components of the prebiotics used were calcium carbonate, alfalfa meal, wheat middings, extract of *Yucca schidigera* and yeasts. For contractual and privacy reasons the exact composition cannot be revealed.

For the first month, the treated calves group was given 3 g per head a day of prebiotic A, administered orally and in a uniform and homogeneous manner to all the animals. Subsequently, a second prebiotic (prebiotic B) was administered, which was mixed with the unifeed, to maximise the homogeneous distribution of the active ingredient in the feed. The dosage of the second prebiotic was 4 g per head a day. Double dosage was used for the first month according to the producer's guidelines.

Fecal sampling and microbiological analysis

Fecal samples were taken at two-week intervals directly from the rectal ampulla with sterile gloves, transferred to sterile bags and sent to the laboratory in a refrigerated container.

In order to detect the coliform presence each sample of feces was diluted 1:10 in peptonated water (PW Difco, Detroit, MI, USA) and homogenised in Stomacher 400 (PBI International, Milan, Italy). Subsequently, additional 1:10 dilutions were made using Maximum Recovery Diluent (MRD, Oxoid, Basingstoke, Hampshire, UK) to obtain 10-fold diluitions. Dilutions were inoculated in triplicate on Violet Red Bile Lactose Agar (VRBL, Oxoid) using the spread plate technique and incubated at 35-37°C for 18-24 hours. Colonies were then counted on all the plates, using a colony count viewer (Petri light, PBI, Milan) and colony counter pen (Colony Count, PBI, Milan) (Sechi *et al.*, 2012). All values were converted into logs and the arithmetic mean was calculated for each sampling. All values were analysed with Graph Pad In Stat, version 3.0b for Mac OS X; the graphs were obtained with Graph Pad Prism version 6.0d for Mac OS X (Feng *et al.*, 2007).

Some lactose fermenting colonies were harvested from the VRBL and seeded on MacConkey Sorbitol Agar (MCS, Oxoid), incubated at 37°C for 18 hours. All sorbitol-negative colonies were seeded on TBX (Tryptone Bile X-glucuronide Medium, Oxoid) at 37°C for 24 hours to evaluate the presence of beta-glucuronidase, in BGBB (Brilliant Green Bile Broth 2%, Oxoid) with a Durham tube at 44°C for 24 hours, and in peptonate water at 44°C for 24 h for confirmation of *E. coli* (growth at 44°C with gas and indole production). All the colonies of presumed sorbitol-negative *E. coli* on the basis of the tests described, for a total of 69 bacterial strains, were transferred to TSB (Tryptone Soya Broth, Oxoid) and frozen at -80°C until needed for the subsequent analyses (Cenci Goga *et al.*, 2005; Rosmini *et al.*, 2011).

Genetic characterisation

The strains of *Escherichia coli* were thawed and aerobically cultivated in Nutrient Broth (NB, Oxoid) at 37°C for 24 hours. Extraction of the DNA was then carried out by boiling, a protocol defined in previous studies by Cenci Goga *et al.* (2004): in short, samples of the colonies were diluted in 1 ml of PBS (phosphate buffered saline). The suspension was centrifuged at 13,000 rpm for 5-7 min. and the supernatant was eliminated. The pellet containing the bacteria was then resuspended in 100 μL of sterile distilled water and boiled for 10 minutes. The DNA was recovered after centrifugation at 13,000 rpm for 5-7 min., then stored at -20°C until amplification.

DNA amplification was conducted on a volume of 25 μL using: 12.5 μL of RED Taq (10 mM of Tris HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.001% of gelatin, 0.2 mM of each deoxyribonucleoside triphosphate), 0.5 microL (1 microM) of each primer, 5 μL of extracted DNA, 6.5 μL H2O. The presence of the *stx1*, *stx2*, *eaeA* and *hly* genes was investigated. The primers and the amplification conditions used are given in Table 1. The PCR reaction was carried out in a Thermocycler Gene Amp, PCR System, 9700 Gold (Applied Biosystem, Foster City, CA, USA). The amplifications were analysed by electrophoretic run on 1.5% agarose gel containing ethidium bromide (0.5μg / ml); 10 μL of each PCR sample was loaded with 2 μL of 6X loading buffer (Fermentas-VWR-Italy) and 5 μL of marker-PCR as reference DNA (Fermentas- VWR-Italy); the run was carried out at a voltage of 100V for about 1 hour in TBE 10X (Trizma base, boric acid, EDTA 0.5M pH 8). At the end of the run the bands were viewed with the UV transilluminator (Fotodine 3-3102 Celbio, Milan, Italy) (Paton *et al.*, 1998).

Antimicrobial resistance

The strains were thawed and cultured in Brain Heart Infusion Broth (BHI, Oxoid) at 35-37°C for 24 hours. Part of the culture broth was inoculated into 6 ml of 0.9% sterile physiological saline solution until reaching a turbidity of 2 McFarland. Using a sterile swab the solution thus obtained was seeded on Muller-Hinton Agar (Oxoid). Disks containing antimicrobials (Oxoid) were placed over it, and the plates were incubated at 35-37°C for 18-24 hours in aerobiosis. The antimicrobials tested were: compound sulphonamides 300 mg, sulphamethoxazole/trimethoprim 25 mg, cyprofloxacin 5 mg, nalixidic acid 30 mg, enrofloxacin 5 mg, chloramphenicol 30 mg,

Table 1. Primers and amplification conditions used.

Target gene	Nucleotidic sequence (5'-3')	Amplification (bp)	Mix conditions	Amplification programme
STX1	ACACTGGATGATCTCAGTGG CTGAATCCCCCTCCATTATG	614 bp	Volume: 25 μL; Dna: 5 μL; water: 6.5 μL; Primer F-R: 0.5 μL; MixTaq: 12.5 μL	95°C×3'; 95°C×20''; 58°C×40''; ×35 cycles72°C×1,5'; 72°C×5'
STX2	CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCAGCACTTTG	779 bp	Volume: 25 μL; Dna:5 μL; water: 6.5 μL; Primer F-R: 0.5 μL; MixTaq: 12.5 μL	95°C×3'; 95°C×20''; 58°C×40''; ×35 cycles 72°C×1.5'; 72°C×5'
EAEA	GTGGCGAATACTGGCGAGACT CCCCATTCTTTTTCACCGTCG	890 bp	Volume: 25 μL; Dna: 5 μL; water: 6.5 μL; Primer F-R: 0.5 μL; MixTa: 12.5 μL	95°C×3'; 95°C×20''; 58°C×40''; ×35 cycles 72°C×1.5'; 72°C×5'
HLY	GCATCATCAAGCGTACGTTCC AATGAGCCAAGCTGGTTAAGCT	534 bp	Volume: 25 μL; Dna: 5 μL; water: 6.5 μL; Primer F-R: 0.5 μL; MixTaq: 12.5 μL	95°C×3'; 95°C×1'; 58°C×1'; ×35 cycles 72°C×1'; 72°C×10'

amoxycillin/clavulanic acid 30 (20+10) mg, ampicillin 10 mg, cefotaxime 30 mg, ceftriaxone 30 mg, cephalothin 30 mg, ticarcillin 75 mg, tetracycline 30 mg, amikacyn 30 mg, gentamicin 10 mg, kanamycin 30 mg, neomycin30 mg, streptomycin 10 mg, erythromycin 15 mg, colistin 10 mg. At the end of the incubation, the diameters of the growth inhibitory zones were measured, and these were interpreted according to specific tables (CLSI, 2011), where the bacterium is classified as sensitive, intermediate or resistant. Hierarchical clustering of the isolates was performed using Ward's method by means of Hierarchical Clustering – Software.

Results and Discussion

The excretion of coliforms progressively decreased over time in both groups, ranging from 8 log cfu/g to about 6 log cfu/g, stabilising at around four months of age. The decrease was related to the normal development of the calf intestinal microbiota. From Figure 1 it can be seen how the treated group had lower average values than the control group, although the difference was not statistically significant. The excretion of sorbitol-negative bacteria also followed a similar trend; at T0 both groups had a value of about 7 log cfu/g, which dropped to 5.3 log cfu/g in the control group and 4.7 log cfu/g in the treated group (Figure 2). Twenty-nine (42%) of the 69 *E. coli* strains were isolated from the treated group, while 40 (58%) were isolated from the control group. The molecular characterisation of the virulence profile obtained by PCR showed that out of the 29 strains isolated from the treated animals, 11 (37.93%) were positive for the *stx1* gene, 8 (27.59%) for *stx2*, 4 (13.79%) for *hlyA*, 5 (17.24%) for *eaeA* and 6 (20.69%) for both *stx1* and *stx2*. Of the 40 strains isolated from the control group, 19 (47.5%) were positive for *stx1*, 10 (25%) for *stx2*, 8 (20%) for *hlyA*, 8 (20%) for *eaeA* and 6 (15%) for both *stx1* and *stx2*. A lesser prevalence can be found of *E. coli* with phenotypic characteristics attributable to serotypes pathogenic for humans in cattle treated with prebiotics. The difference between the two groups was not statistically significant. The prevalence of *stx1*, *hlyA* and *eaeA* was also lower in treated calves, while *stx2* was similar in both groups. The antibiogram data are shown in Table 2. All the strains were sensitive to ciprofloxacin, cefotaxime, kanamycin and amikacin. In the case of erythromycin, no strains were found to be sensitive. This confirms the intrinsic resistance of *E. coli* to this antimicrobial, linked to the mutation of the L4 and L22
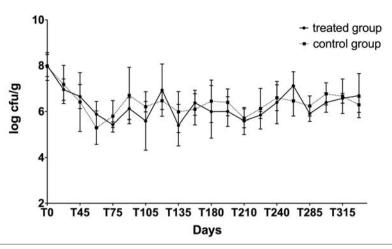

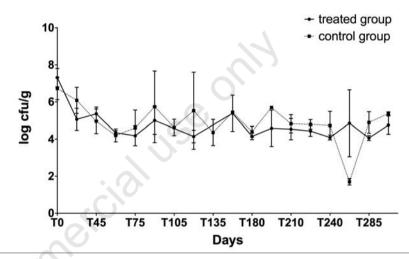**Figure 1. Trend of the excretion of coliforms in the stools.**

Figure 2. Trend of the excretion of sorbitol-negative *Escherichia coli*.

Table 2. Antibiogram data, expressed as percentage.

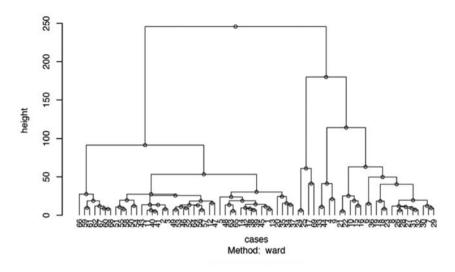| Antimicrobial	Resistant (%)	Intermediate (%)	Sensitive (%)
Compound sulphonamides (S3)	7.2	0	92.8
Sulphamethoxazole/trimethoprim (SXT)	4.3	0	95.7
Cyprofloxacin (CIP)	0	0	100
Nalixidic acid	5.7	0	94.3
Enrofloxacin (ENR)	0	4	96
Chloramphenicol (C)	2.8	0	97.2
Amoxycillin/Clavulanic Acid (AMC)	13	4.3	82.7
Ampicillin (AMP)	13	21.7	65.3
Cefotaxime (CTX)	0	0	100
Ceftriaxone (CRO)	4.3	8.6	87.1
Cephalothin (KF)	94.6	4	1.4
Ticarcillin (TIC)	34.7	1.4	63.9
Tetracycline (TET)	8.6	15.9	75.5
Amikacyn (AK)	0	0	100
Gentamicin (CN)	1.4	0	98.6
Kanamycin (K)	0	0	100
Neomycin (N)	0	18.8	81.2
Streptomycin (S)	5.7	28.9	65.4
Erythromycin (E)	88.5	11.5	0
Colistin	0	10.1	89.9

Figure 3. Hierarchical clustering of the isolates.

proteins of the ribosomal subunits (Wittman and Stöffler, 1973).

E. coli strains showed a lower resistance to tetracycline (8.6%) than that for ampicillin (13%), unlike other studies (Mainda *et al.*, 2015).

The hierarchical clustering shows that there is a clear distinction between the strains isolated after 210 days of life of the calves, which are grouped into a single cluster, compared to all the others (Figure 3). This fact may be related to the physiological change occurring in the calf immune system at about six months of age, with the end of maternal immunity and an increase in the immunocompetence of animals, where the intestinal microbiota could change. However, no distinctions in clusters were observed among the strains isolated from the treated calves compared to those isolated from the control group.

Conclusions

In recent years, human infection caused by *E. coli* has become frequent. An important challenge will be to more definitely identify and characterise the strains, which will allow a more thorough surveillance of the prevalence of the bacteria in animal populations, assessment of the importance of these bacterial species in humans' infections and development of effective on-farm control strategies. In this context, the prebiotics use can be a great strategy to reduce the shedding and consequently the presence of these pathogens on the farm reducing thus the occurrence of infections in humans.
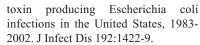
The data obtained show how the administration of prebiotics in the diet of calves may decrease the excretion of coliforms, and more specifically the prevalence of shigatoxigenic *E. coli*. The development of formulas aimed at maximising this effect is of the greatest importance to minimise the risk of transmitting food-borne diseases for the final consumer. Moreover, prebiotics, like other groups of food additives that can modify the composition of the intestinal flora of cattle, are very important tools also because they can be used instead of antimicrobials. The misuse of antimicrobials has in fact contributed to the development of resistance in very dangerous pathogenic bacteria involved in episodes of illnesses affecting the consumer.

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