Effects of a species-specific probiotic formulation on multiresistant *Escherichia coli* isolates from the gut of veal calves

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ABSTRACT: In this study, 254 *Escherichia coli* isolates from faecal samples of veal calves were evaluated for antimicrobial susceptibility using the disk diffusion method. During the experimental period, six mass antibiotic treatments were administered to the animals (about one treatment per month). The active principles used were oxytetracycline, colistin, tylosin, doxycycline, chlortetracycline, and sulphonamides. An extremely high resistance prevalence (> 70%) towards penicillin, sulphonamide, tetracycline, ampicillin, and spyramicin was detected. Sixty *E. coli* isolates could be defined as multiresistant, showing resistance to at least 6 antimicrobial classes. Subsequently, we evaluated the inhibitory effect of a species-specific probiotic against multiresistant *E. coli*, showing its beneficial action with large inhibition halos for 76% of the isolates. This suggests the potentiality of the probiotic, putting in evidence a clear advantage of its use in veal calves nutrition, in particular during the first phases, when the animals are more susceptible to severe enteric infections by *E. coli*.

Keywords: lactic acid bacteria; antibiotic resistance; prevalence; gastrointestinal functionality

The repeated exposure to antimicrobials is considered a critical factor for the observed increase in resistance frequency of the commensal microflora in both animals and humans (van den Boogard and Stobberingh, 2000). Carriage of resistance genes by commensals has been proposed as an indicator of antibiotic resistance in a population, and its decrease should be regarded as a suitable public health goal (van den Boogard, 1997). In fact, intestinal commensal microflora acts as a potential reservoir of resistance genes that may be transferred to pathogenic bacteria inside the host. The diffusion of antimicrobial resistance among pathogens, that has been evidenced since the seventies, has resulted in a compromised efficacy of antimicrobial agents used in the treatment of infectious diseases, and is actually considered an emerging and serious public health concern (van den Boogard and Stobberingh, 2000; Martinez and Baquero, 2002; Ramos et al., 2013). In particular, the possibility of transferring antibiotic-resistant bacteria from food animals to humans through the consumption of meat or other animal products, by the contact with farm wastewater, and other ways must be regarded as a worldwide issue (Sàenz et al., 2004; Marshall and Levy, 2011). It is evident that resistance resulting from the use of antimicrobial agents in food-producing animals may have adverse human health consequences, with a potential increase in morbidity and mortality from bacterial infections. A relationship between the use of antibiotics in food animals and antimicrobial resistance among bacteria isolated from humans is reported for *Salmonella* spp. and *Campylobacter* spp., but it has been described for other bacteria, and represents an item of increasing importance for some potential pathogens such as enterococci, *Staphylococcus aureus*, and especially

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Escherichia coli (Barza, 2002; Swartz, 2002; Lee, 2003; Angulo et al., 2004; Tollefson and Karp, 2004). *E. coli* is a commensal bacterium living in the intestine of humans and animals, including food animals, but it is strongly considered for its potential pathogenicity for consumers. Since the early 1980s some serotypes (especially O157:H7) have caused several foodborne human infections (outbreaks or sporadic cases), in particular by the consumption of minced/comminuted beef (Pennington, 1998; Williams et al., 2000). Cattle are the major reservoir of this pathogen that is carried asymptomatically and excreted from the gastrointestinal tract. During cattle slaughtering, faeces and hides are considered the main sources of *E. coli*; carcass contamination can occur during dressing phases, especially skinning and evisceration (Chapman et al., 2001; Aslam et al., 2003; Carney et al., 2006; Nastasijevic et al., 2009). *E. coli* is able to acquire resistance genes from other microorganisms or from the environment, and can represent a potential reservoir for the horizontal transmission of these genes to different bacterial species occurring in the food chain (Trobos et al., 2008; Ahmed et al., 2009; Marshall and Levy, 2011; EFSA-ECDC, 2012). The resistance in these organisms is considered a good indicator of the selective pressure resulting from antimicrobial use in target populations (van den Boogard and Stobberingh, 2000). Recently, a report issued by EARSS (European Antimicrobial Resistance Surveillance System) dealt with antimicrobial resistance of *E. coli* and *S. aureus* population in the European Union, showing, during the period 2002–2009, a decreasing trend in antibiotic susceptibility especially for *E. coli*, that was associated with a significant increase in human septicemic infections, reaching levels of concern. Infections caused by such antimicrobial-resistant strains are becoming common worldwide and are posing serious health problems for human medicine (EARSS, 2008; ECDC, 2011).

The need for a reduction in antimicrobial use in animal breeding has encouraged, during last years, the interest for alternative methods to improve gut health and animal performances; in this context, the use of probiotics as feed additives seems to be very promising (Timmerman et al., 2005; Bakhshi et al., 2006; Frizzo et al., 2010; Kawakami et al., 2010; Morrison et al., 2010; Riddell et al., 2010). In particular, the administration of multistrain and multispecies probiotics showed to be more effective than that of monostrain probiotics; a better capability of colonizing the gastrointestinal tract was demonstrated, associated with a synergic activity due to the combination of different mechanisms of action (Timmerman et al., 2004; Soto et al., 2010).

In a previous work, functional aspects of lactic acid bacteria isolated from faecal samples of veal calves were evaluated for a potential probiotic use, designing a multispecies multistrain formulate composed of *Lactobacillus animalis-Lactobacillus paracasei* subsp. *paracasei-Bacillus coagulans* (30 : 35 : 35). The same was then successfully tested for *in vitro* antimicrobial activity towards different potentially pathogenic bacteria (Ripamonti et al., 2009; 2011).

The aim of this study was to evaluate the prevalence of antibiotic resistance among *E. coli* isolates from the faeces of veal calves and to investigate the inhibitory effect of our probiotic formulate against the most resistant strains.

MATERIAL AND METHODS

Design of the experiment

Faecal samples were obtained from 24 veal calves, bred in multiple boxes of six animals each on slotted floor and fed a standard milk replacer/concentrate diet. During the trial three different milk replacers (on dry matter basis) were used from the arrival to the farm: Elite Start FE at days 0–14 (22% crude protein, 18% crude fat), Elite 20 at days 15–100 (19% crude protein, 17% crude fat), and Elite 100 at days 101–180 (20% crude protein, 18.5% crude fat) (Zoogamma s.p.a, Ghedi, Italy) with a mean reconstitution rate of 130 g/l and supplied at a temperature of 37°C. Calves were fed an increasing amounts of a commercial concentrate Fibravit Wet Mais (Zoogamma s.p.a, Ghedi, Italy) on dry matter basis (13.20% crude protein, 4.41% crude fat, 4.25% crude fibre, 4.25% ash) starting from 100 g per head/day until 750 g per head/day at the end of the trial.

During the experimental period, six mass antibiotic treatments were administered to the animals (about one treatment each month was administered to veal calves, the first one performed at the arrival to the farm): the active principles used were oxytetracycline (25 mg/kg body weight (BW)), colistin (50 mg/kg BW), tylosin (40 mg/kg BW), doxycycline (10 mg/kg BW), chlortetracycline (25 mg/kg BW), and sulfadimethoxine (25 mg/kg

BW). Monthly, faecal samples were collected from each calf upon rectal stimulation, stored in vials with transport medium (Fecal™ Enteric Plus; Oxoid, Basingstoke, UK), and kept refrigerated at 4°C until the analyses, which were performed the same day. Sampling period started from 15 days of life of veal calves (T0) and lasted for 180 days (T5) in order to monitor the whole breeding cycle. Four faecal pools coming from 6 animals each were subjected to microbiological analysis. 10 g of each pool were diluted with 90 ml of Buffered Peptone Water (Oxoid) and homogenized in a Seward Stomacher 400 Lab Blender Mixed Homogenizer (International PBI, Milano, Italy) for 1 min. Serial 10-fold dilutions were plated onto TBX agar (Oxoid) for the enumeration of *Escherichia coli*, according to ISO 16649-2:2001 method.

Isolation of *Escherichia coli* and evaluation of antibiotic susceptibility

A total of 254 *E. coli* colonies grown on TBX plates from faecal samples were randomly selected and subcultured in Tryptic Soy Broth (TSB) (Oxoid, Basingstoke, UK). The standard CLSI disk diffusion test was performed on each isolate (CLSI, 2007) using Tryptic Soy Agar (TSA) (Oxoid). Eight different classes of antimicrobial agents were chosen for the test: penicillins (penicillin G 10 IU, ampicillin 10 µg), sulphonamide 300 µg, cephalosporins (cephalothin 30 µg), tetracyclines (tetracycline 30 µg), aminoglycosides (neomycin 30 µg, apramycin 15 µg), macrolides (spyramicin 100 µg), lincosamides (lincomycin-spectinomycin 109 µg), and quinolones (nalidixic acid 30 µg, enrofloxacin 5 µg). Negative control was performed using blank disks. The diameters of the inhibition zones were measured and the results (an average of 5 readings) were recorded and compared to the breakpoint value indicated by CLSI (2005), SFM (2011), and EUCAST (2012).

Inhibition of *Escherichia coli* by the probiotic

For the evaluation of the inhibitory effect on multiresistant *E. coli* strains, the species-specific probiotic was inoculated into MRS broth and grown anaerobically at 37°C for 48 h. After incubation, the broth was spread by a sterile swab onto the surface of MRS agar plates, subsequently incubated for 48 h at 30°C in an anaerobic jar (Anaerojar; Oxoid, Basingstoke, UK). Multiresistant *E. coli* isolates were selected by the test described above; the requisite for the selection was the resistance to penicillins, sulphonamide, tetracycline, and macrolides and to two of the other antimicrobial classes tested. These isolates were then subcultured aerobically overnight at 37°C in 10 ml of TSB. For each *E. coli* isolate, 0.2 ml of bacterial suspension (approximately 10^7 UFC/ml) were added to a 5 ml share of semisolid agar (BHI broth (Oxoid) + agar 0.7%), maintained in a water bath (45°C) and then poured over the plates inoculated with the probiotic. After aerobic incubation at 37°C for 24 h, the plates were checked. If *E. coli* growth was inhibited, a clear zone was observed around the probiotic colonies. *Lactobacillus acidophilus* ATCC 4356 was used as a negative control; the size of no-growth zones, when compared with those produced by the control strain, indicated the susceptibility, as reported by Rebucci et al. (2007).

RESULTS AND DISCUSSION

E. coli counts

In the present study the prevalence of antibiotic resistance of *E. coli* isolates from veal calves was evaluated. Among food-producing animals, calves can be considered a good indicator of antimicrobial resistance diffusion, as they are frequently submitted to mass and individual treatments to prevent the effects of bacterial infections. It is widely recognized that resistance due to the use of antimicrobial agents in animal breeding represents an emerging threat for human health. The results of microbial counts obtained from faecal samples are shown in Table 1. *E. coli* number in faecal samples increased during the first period, reaching at T1 a plateau level (6.5–7 Log CFU/g) that was maintained until the end of the experimental time (T5). It has to be noted that the administration of

Table 1. Microbial counts of *Escherichia coli* obtained from faecal samples (mean values of 4 pools of 6 animals each)

	T0	T1	T2	T3	T4	T5
Log CFU/g faeces ± DS	4.16 ± 0.28	6.66 ± 0.28	6.86 ± 0.28	6.86 ± 0.25	6.86 ± 0.25	6.79 ± 0.25

mass antibiotic treatments to calves did not result in any decrease in the concentration of *E. coli*, suggesting the possibility of a high prevalence of antimicrobial resistant strains in gut population.

Antimicrobial susceptibility of *Escherichia coli*

The investigation on *E. coli* isolates confirmed the hypothesis of a high prevalence of antibiotic resistance among bacterial population, evidencing the diffusion of multi-resistant strains. The results of antimicrobial susceptibility tests are reported in Table 2. According to the levels settled by EFSA-ECDC (2012) to describe the antimicrobial resistance, the frequency of resistance of *E. coli* population analyzed could be defined as extremely high (> 70%) towards penicillin, sulphonamide, tetracycline, ampicillin, and spyramicin, but high resistance frequencies (20–50%) were detected also for the other antibiotic classes, such as neomycin, cephalothin, spectinomycin, and nalidixic acid. Our results confirmed the data obtained in previous studies: Bradford et al. (1999) observed high resistance frequencies in *E. coli* strains isolated from calves, in particular towards ampicillin (100%), kanamycin, streptomycin, and tetracycline. In another study performed on 120 strains of *E. coli*, Güler et al. (2008) found a high rate of resistance towards cephalothin (72%), followed by kanamycin (69.3%), tetracycline (69.3%), ampicillin (65.3%), trimethoprim (52%), and gentamicin (24%). Similar rates were detected also by Hariharan et al. (2004) in *E. coli* strains isolated from calves (81% against oxytetracycline and 64% against neomycin) and by Werckenthin et al. (2002), who found resistance rates over 80% for tetracyclines, ampicillin, sulphonamide/trimethoprim combination, and chloramphenicol. A recent report published by EFSA-ECDC (2012) underlined the common presence of resistance in *E. coli* strains isolated from fowl, pigs, cattle, and food to tetracyclines, ampicillin, and sulphonamides, whereas resistance to third-generation cephalosporins and fluoroquinolones remained low. In our trial, a significant rate of multiresistance was detected: 60 *E. coli* isolates could be defined as multiresistant (23.6% of the total tested isolates), showing resistance to at least 6 antimicrobial classes, and 4% of tested strains were resistant to all of the 11 antimicrobials considered, confirming the growing diffusion of this phenomenon in Italy (ECDC, 2011).

Table 2. Antimicrobial susceptibility of *Escherichia coli* isolates

Antimicrobial agents	Limit halo (mm)	Number of resistant/total	(%)
Penicillin[a] 10 IU	< 18	249/254	97.88
Sulphonamide[a] 300 µg	< 12	242/254	95.30
Tetracycline[a] 30 µg	≤ 17	236/254	92.91
Ampicillin[b] 10 µg	≤ 14	230/254	90.70
Spyramicin[a] 100 µg	≤ 19	212/254	83.47
Neomycin[a] 30 µg	≤ 15	123/254	48.33
Cephalotin[a] 30 µg	≤ 18	108/254	42.52
Nalidixic acid[a] 30 µg	≤ 15	89/254	35.18
Lincomycin-Spectinomycin[a] 109 µg	< 20	84/254	33.05
Apramycin[c] 15 µg	< 18	50/254	19.55
Enrofloxacin[a] 5 µg	≤ 30	45/254	17.70

[a]SFM (2011), [b]EUCAST (2012), [c]CLSI (2005)

Inhibition of *Escherichia coli* by the probiotic formulate

The present evaluation of the functionality of the species-specific probiotic formulation showed its ability to inhibit the growth of our multiresistant *E. coli*. The results of the inhibition test against each of the 60 multidrug-resistant *E. coli* are given in Table 3. The probiotic showed an inhibitory effect towards all the isolates tested: 76.7% of the strains showed a clear zone ≥ 20 mm (Figure 1), 20.0% between 10 and 20 mm, and 3.3% a zone < 10 mm.

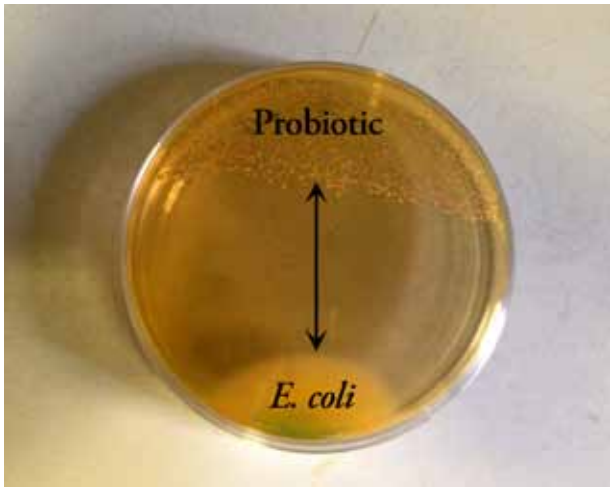

Figure 1. Representative figure of the inhibitory action of the probiotic against a multiresistant *E. coli* isolate

Table 3. Results obtained from inhibition tests on multiresistant *E. coli* isolates

Isolate No.	Inhibition halo (mm)	Isolate No.	Inhibition halo (mm)	Isolate No.	Inhibition halo (mm)	Isolate No.	Inhibition halo (mm)
1	25	16	19	31	28	46	24
2	18	17	23	32	23	47	20
3	23	18	23	33	17	48	27
4	22	19	23	34	24	49	20
5	21	20	18	35	26	50	21
6	20	21	15	36	24	51	24
7	22	22	18	37	25	52	21
8	21	23	17	38	25	53	22
9	24	24	22	39	32	54	17
10	25	25	20	40	22	55	20
11	19	26	20	41	27	56	27
12	21	27	24	42	27	57	24
13	18	28	15	43	22	58	31
14	19	29	9	44	27	59	30
15	25	30	25	45	9	60	25

This activity could be due to different mechanisms, the most likely being the production of organic acids; in fact the production of propionic, acetic, and lactic acids by the strains included in the formulate has been already observed during previous *in vitro* studies (Ripamonti et al., 2011). Further studies are needed to elucidate the behaviour of the probiotic strains in field trials, to confirm data obtained *in vitro* also in the presence of interfering factors such as breeding practices, feeding, and use of antibiotic treatments.

CONCLUSION

The use of probiotics as a feeding strategy to contrast gut infections and to improve the functionality of gastrointestinal tract has been widely applied in different species, especially in pigs and poultry (Ghareeb et al., 2012; Heo et al., 2013), but few products are actually used for veal calves. In this context, our study represents the first step for the perfection of a product that could enlarge the availability of means to contrast the prevalence of severe infections by *E. coli* strains that involve veal calves especially during the first phases of life.

The use of probiotics in animal nutrition represents an alternative and effective approach to antibiotic treatments leading to a reduction of breeding costs and joining current law requirements (Regulation (EC) No. 1831/2003) which focus on the priority of a limitation in the use of antimicrobials for the improvement of public health protection.

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