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Evaluation of protective effect of probiotic in sheep against experimental infection by *Escherichia coli*

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Domestic ruminants, especially sheep and cattle, are the main reservoirs of Shiga toxin-producing *Escherichia coli* (STEC) and may transmit this pathogen to human. Probiotics are "live organisms that, when administered in adequate amounts, confer a health benefit on the host", decreasing the infection and its dissemination. However, these benefits are conferred only after the colonization of probiotic strains in the gut of the animal, which may be impaired by normal microbiota. The aims of this study were to determine whether the inoculation of sheep with probiotic strains decreases the shedding of STEC and to determine whether the age of sheep interferes with this protective effect. Sheep that received oral inoculums at a concentration of 2 × 10^9 cells per mL of viable STEC bacteria, which are carriers of *stx1*, *stx2* and *eae* genes, were compared with other sheep that did not receive inoculums. When probiotic bacteria were inoculated together with the STEC, the number of pathogenic bacteria in the population was similar to the control. The protective effect of probiotic strains was largest in groups with younger animals than with older animals. These findings suggest that the use of probiotic strains in sheep may decrease the intestinal shedding by STEC as well as the fact that the age of the sheep may interfere in the protective effect of probiotics against colonization by STEC.

Key words: Probiotic, protective effect, *Escherichia coli*, sheep.

INTRODUCTION

Healthy ruminants appear to be a main reservoir of Shiga toxin-producing *Escherichia coli* (STEC) (Sanderson et al., 1999). STEC bacteria are food-borne pathogens that may cause human diseases such as diarrhea, hemorrhagic colitis (HC) and, in some cases, hemolytic uremic syndrome (HUS) (World Health Organization, 1998). Some studies have reported the rumen compartment as being a relevant target for intervention strategies to reduce the number of viable STEC cells; one such intervention strategy may be the use of probiotics supplemented in rations (Lema et al., 2001; Zhao et al., 1998; Rattray et al., 2007; Whitley et al., 2009; Chaucheyras-Durant et al., 2010; Maragkoudakis et al., 2010).

Probiotics are live organisms with the capacity to benefit the gastrointestinal tract microflora by promoting health or preventing diseases in the host (Borriello et al., 2003; Rook and Brunet, 2005; Papadimitriou et al., 2007). Probiotics benefit the host by improving microbial balance,

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which includes the elimination or reduction of pathogenic microorganisms that are carried by the host and are harmful to humans (Zhao et al., 1998).

The role of probiotics as microbial bioregulators is to maintain the balance of intestinal and ruminal microbiota an important function to prevent intestinal adhesion and consequently the increase of the number of pathogenic bacteria such as STEC (Avila et al., 2000).

The reduction of human pathogen in live animals is considered by the European Food Safety Authority to be one of the most effective ways of reducing both the contamination of foods and the number of human food poisoning cases (EFSA, 2004).

The levels of STEC shed in the feces can be highly variable, and is influenced by a number of factors including age, season, and diet (Callaway et al., 2009; Sanchez et al., 2010). Cray and Moon (1995) observed a wide variation in the magnitude and duration of fecal excretion of STEC by animals of similar ages. Moreover, there are many differences in the shedding of *E. coli* in ruminants' guts, including individual variation and resident intestinal microbiota (Magnuson et al., 2000).

This study was proposed to verify the reducing of shedding of *Escherichia coli* (STEC) on the sheep´s feces using a mixture of six probiotics strains supplemented in the ration and also to evaluate whether the age of these animals interferes in this reducing.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The STEC non-O157:H7 strain serotype O128 (ERSP), carrying the genes for *stx1*, *stx 2* and *eae,* employed was isolated from healthy cattle in Brazil and had been characterized as described by Possé et al. (2007). The culture was kindly donated by Dr Fernando Antonio de Ávila, Laboratory of bacteriology, Universidade Estadual Paulista (UNESP), Jaboticabal. Working cultures were produced from lyophized stocks, on Luria Bertani with incubation at 37°C for 12 h. Liquid cultures for use as inocula were produced in 40 mL of 0.9% saline solution with incubation without shaking at 37°C for 12 h. Cell numbers were determined spectrophotometrically (O.D. 600nm) and were adjusted to contain $2x10^9$ cfu/ml.

To verify the protective effect of the probiotics strains the animals were distributed at eight Groups containing ten animals each Group. The Group I received only probiotics strains daily supplemented in the ration throughout the experiment. Group II received single dose of (ERSP) oral via the help of a canula directly at the mouth of the animal. Group III received single dose of (ERSP) oral via the help of a canula directly at the mouth plus the probiotics strains supplemented in the ration throughout the experiment, Group IV did not received (ERSP) neither probiotics strains being the control group. The animals in Group V received the same treatment as Group I, Group VI received the same treatment as Group II, Group VII received the same treatment as Group III and Group VIII received the same treatment as Group IV. However, the main difference among the Group (I to IV) and (V to VIII) is that all animals in Group (I to IV) were older than 45 days of age and Group (V to VIII) were younger than 45 day of age.

This study was conducted in accordance with the ethical guidelines for investigations involving laboratory animals and was approved by the Ethics in Animal Research Committee (EARC) of UNESP-Universidade Estadual Paulista. All the animals were treated with feed *ad libitum* and no adverse effects were observed in the animals receiving *E. coli* (STEC) or probiotics during this study.

To prepare the daily doses of the six probiotics strains, individual tubes each containing a single type of lyophilized bacteria, were resuspended in 40 ml of saline to generate a mixture containing *Ruminobacter amylophilus* 3.0×10^8 UFC/g, *Ruminobacter succinogenes* 3.0×10^8 UFC/g, *Succinovibrio dextrinosolvens* 4.4×10^8 UFC/g, *Bacillus cereus* 3.5×10^8 UFC/g, *Lactobacillus acidophilus* 3.5×10^8 UFC/g and *Enterococcus faecium* 3.5×10^8 UFC/g. Each animal received 0.2% probiotic inoculums (2Kg/ton) and all animals received 200 g of rations per animal per day.

Processing of fecal material for the detection of STEC

During the six weeks post inoculation of ERSP fecal samples were collected, always at the same hour in the morning, directly from the rectum of each animal. To screen for the presence of STEC in the test population, one gram (1 g) of feces was homogenized by vigorous vortexing in 10 mL of sterile distilled water, larger particulate material was allowed to settle, and then 1 mL aliquots of fecal suspension were used as inocula for plates of MacConkey agar with incubation at 37°C for 24 h. All lactose positive colonies were subcultured and grown overnight in 9 mL of nutrient broth, without shaking, at 37°C. DNA for use in PCR was generated from bacterial cells, pelleted by centrifugation at 12,000 g for 1 min, resuspended in 200 µL of sterile distilled water, lysed by boiling for 10 min and centrifuged as described above. The primers and PCR cycling conditions reported by Vidal et al. (2005), were employed to detect the presence of *stx1*, *stx2,* and *eae* genes by multiplex PCR, employing 15 µL of cleared cell lysate as template in a final reaction volume of 50 µL. Control reference strains were *E. coli* EDL 933 (O157:H7, positive control for *stx1*, *stx 2*, *eae*) and *E. coli* K12 (negative control). To ensure that the STEC isolates recovered from groups II, III, VI and VIII were in fact the ERSP isolate, all multiplex PCR positive colonies were subjected to fingerprinting by pulsed-field gel electrophoresis (PFGE) of chromosomal DNA. Genomic DNA from suspected ERSP colonies were prepared as previously described by Barret et al. (1994). The agarose-embed-ded DNA was digested with 10U of *XbaI*/plug (Gibco BRL) at 37°C overnight. PFGE was performed in a CHEF-DR II unit (Bio-Rad Laboratories, Hercules, Calif.) using 1% PFGE grade Tris Borate EDTA buffer gels. The DNA was separated by electrophoresis for 20 h at a constant voltage of 200 V (6V/cm) pulse time of 5 to 50 s, an electric field angle of 120°C and a temperature of 15°C before being stained with ethidium bromide. Resulting patterns were analyzed on a DNA Pro Scan, ProRFLP program (DNA Proscan, Inc. Nashville, Tenn), and the size of the DNA fragments was used as the criteria for categorizing distinct patterns.

Statistical analysis

The data on re-isolated STEC were analyzed each week of sample collection and over the entire experimental period (six weeks). The model procedures used were the SAS software package (2001). In all treatments, statistical significance was declared at $P<0.05$. A Duncan´s multiple range test was used to determine the existence of any significant difference in the reduction of STEC caused by probiotic strains in the animals across different treatments and ages.

RESULTS

The animals were inoculated orally, and then, feces samples

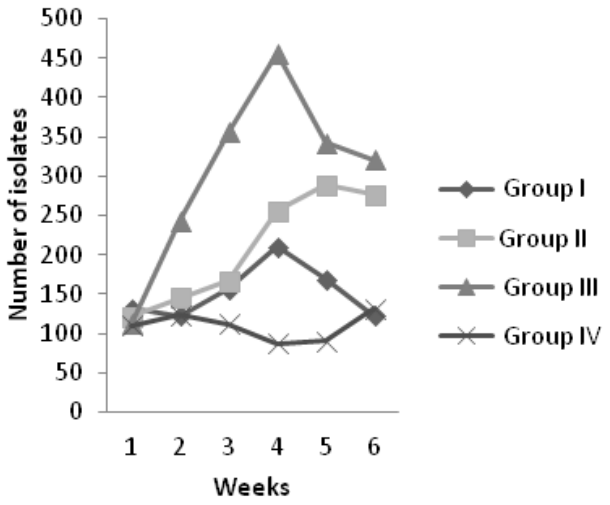

Figure 1. Number of isolates of *Escherichia coli* carrying the *stx1*, *stx2* and *eae* genes (STEC) isolated from feces of sheep older than 45 days in age.

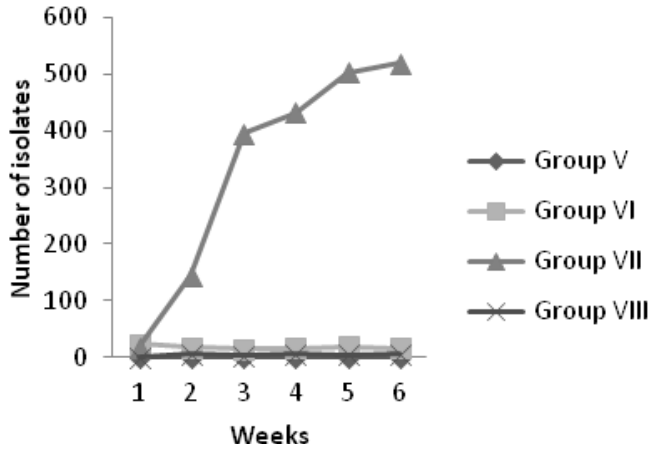

Figure 2. Number of isolates of *Escherichia coli* carrying the *stx1*, *stx2* and *eae* genes (STEC) isolated from feces of sheep younger than 45 days in age.

were collected weekly for bacterial cell count measurements and re-isolation of *E. coli* carrying the *stx1*, *stx2* and *eae* genes. The mean number of STEC strains re-isolated from the feces of animals in each group for each of six weeks were as follows: Group I: 132, 122, 156, 210, 169 and 122; Group II: 121, 145, 167, 256, 289 and 276; Group III: 112, 243, 356, 456, 342 and 321; Group IV: 110, 123, 112, 87, 90 and 132; Group V: no strain carriers of STEC genes were isolated; Group VI: 24, 18, 14, 16, 19 and 16; Group VII: 18, 145, 395, 432, 504 and 520; Group VIII: 0.0, 5, 2, 6, 4 and 5; as observed in Figures 1 and 2.

There were no isolates of *E. coli* carrying the *stx1*, *stx2* and *eae* genes in the control Group V (animals younger than 45 days), whereas in the control Group I (animals older than 45 days), there was a mean of 151 isolates throughout the experiment.

The Group VII presented the largest mean of STEC isolates. This high colonization was probably facilitated by deficiency of normal flora, since the animals of this group had fewer than 45 days old, causing the STEC inoculum did not suffer due to competition from other microorganisms.

The numbers of STEC re-isolated from younger animals were lower than the oldest animals in all groups that received ERSP together with probiotics strains daily supplemented in the ration.

The protective effect from probiotics strains was highest in younger animals than oldest animals, consequently the shedding of ERSP in younger animals was lower than oldest animals.

DISCUSSION

Our results suggest the occurrence of the protective effect from probiotics strains against the shedding of ERSP, since it was observed a lower number of ERSP recovered from groups that received ERSP inoculum together with the probiotics strains daily in comparison to groups received single dose of ERSP without probiotics strains. Likewise the numbers of ERSP recovered from young sheep that received the ERSP were lower than the numbers recovered from older sheep.

The probiotics strains can cause several benefits on the host, one of them is the balancing of the normal flora hampering the establishment of the pathogenic strains (Magnuson et al., 2000), therefore we expected that higher protective effect of this probiotic occurred at older sheep than younger sheep, since that the young sheep do not have normal flora resident established. On the other hand, the exact mechanism of balancing and interference of probiotic strains in the intestinal microbiota in sheep is unknown. More studies are necessary to investigate these relations. Some authors report that a greater percentage of young animals are colonized with *E. coli* O157:H7 than adult animals (Hancock et al., 1994; Wells et al., 1991). However, the comparison of interference of probiotics strains at colonization of *E. coli* O157 and non-O157 between animals young and older is little studied. Usually the studies are made in cattle than sheep (Nielsen et al., 2002) or focusing just the reducing of shedding (Zhao et al., 1998; Lema et al., 2001; Kritas et al., 2006; Maragkoudakis et al., 2010). Strategies using probiotics strains into ruminant´s compartment to reduce the shedding of pathogenic microorganisms on the farm reducing thus the occurrence of diseases have been efficient according to some authors (Chaucheyras-Durant et al., 2010; Lema et al., 2001; Guarner and Malagelada, 2003). Lema et al. (2005) reported that the use of probiotic strains supplemented in rations might be used to reduce fecal shedding of the pathogen by ruminants and also to improve animal meat production.

Intestinal microbiota modulation by probiotic micro-organisms occurs through a mechanism of competitive exclusion (Guarner and Malagelada, 2003), which includes competition for nutrients, adhesion sites in the gut, a decrease in pH and a production of antimicrobial molecules or an interference with quorum-sensing signaling (Millette et al., 2007). However in this study we cannot explain what have occurred on the gut in younger sheep that was different than older sheep. We suggest that the absence of normal flora has facilitated the establishing of the probiotics strains on the gut consequently the establishing of the STEC was hampered. On the other hand, probably the presence of the normal flora or another unknown factor in the older sheep hampered the protective effect of the probiotics strains promoting a greater shedding of ERSP compared with younger sheep.

Our findings suggest that the addition of probiotics strains supplemented in the ration might be used to reduce the shedding of STEC O157 and STEC non-O157 in sheep reducing thus the presence of this STEC on the farm and also the age of these animals might interfere in this dynamic. Therefore more studies are necessary to verify the importance of the animal´s age at the use of probiotics in sheep to reducing the shedding of pathogenic strains.

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