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Effect of probiotic formulation containing *Bacillus* spp. on diarrhoea in dogs

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Abstract
This study was carried out to evaluate the effect of probiotic formulation containing *Bacillus coagulans* Unique IS2 (1 billion) and *Bacillus subtilis* UBBS14 (1 billion) on enteropathogenic *Escherichia coli* (EPEC 4083) induced diarrhoea in dogs. Twenty mongrel dogs (Indian), aged between 1.7 – 2 years (average weight 21.37 ± 3.64 kgs) were kept for one week for acclimatization prior to induction of diarrhoea with *E. coli* (EPEC 4083). After induction of diarrhoea, the dogs were treated with either probiotic or placebo sachets for 10 days. During the treatment period, time for resolution of diarrhoea, faecal consistency, faecal *E.coli* count and short chain fatty acids (SCFA) in faeces were assessed. Body dehydration and haematology parameters were also assessed during the treatment. Probiotic treatment significantly resolved the duration of diarrhoea (~3 days) as compared to placebo treated dogs (~8 days). There was also a significant improvement in stool consistency in the probiotic treated group as compared to placebo. There was no evidence of dehydration in the probiotic treated dogs. Additionally, probiotic treatment significantly reduced faecal EC count (2.8×10^8 CFU/g faeces at baseline to 2.5×10^7 CFU/g at the end of treatment.) as compared to control (2.5×10^8CFU/g faeces at baseline to 1.0×10^8 CFU/g). Short chain fatty acids-acetate, propionate and butyrate levels in faeces were also elevated in the probiotic treated group as compared to placebo. Haematology parameters remained normal in both the groups. In conclusion, the study demonstrates the efficacy of *B. coagulans* and *B. subtilis* in the treatment of diarrhoea in dogs.

Keywords: Probiotics, diarrhoea, dogs, short chain fatty acids, *B. coagulans* unique IS2, *B. subtilis* UBBS14

Introduction

Gastrointestinal disorders (GI) are common in dogs, etiology of which could be food allergies, bacterial, viral or intestinal parasites, inflammatory and neoplastic conditions including unknown causes [1]. Diarrhoea (acute or chronic) is a major indicator of gastrointestinal disorders apart from vomiting or refusal to eat, which result in electrolyte and fluid disturbance [2]. Acute diarrhoea is common in puppies of chewing and nibbling stage and working dogs due to their high exposure to various environmental conditions. During a diarrhoeal episode, the dog may recover spontaneously or may require medication in the form of antibiotics or supportive therapy which may include dietary modification and oral rehydration for uncomplicated and non-bloody diarrhoea [3]. There have been many concerns over the use of antimicrobial agents and their potential adverse effects by dog owners and veterinarians [4]. Antibiotics cause disruption of the normal intestinal flora which can lead to dysbiosis and growth of some pathogenic organisms which can further lead to antibiotic associated diarrhoea [5-7].

Alternative therapies like probiotics, as opposed to antibiotics are now being sought. "Probiotics are live microorganisms which, when administered in adequate amounts confer a beneficial effect on the host" [8]. Probiotics restore the disturbed microbiota of the gut and reduce the duration of diarrhoea. Even though probiotics are being prescribed for dogs, there have been very few trials on the efficacy of probiotics in the treatment of diarrhoea in dogs. In this study, the effect of two *Bacillus* spp. (*B. coagulans* and *B. subtilis*) on induced diarrhoea in dogs was studied. *Bacillus* spp. in contrast to other vegetative probiotic strains are stable at room temperature and do not require refrigeration and hence have the advantage of stability and long shelf life. They are rod shaped, spore-forming, aerobic or facultative anaerobic, Gram-positive, bacteria. The members of the genus *Bacillus* were recognized as the potential probiotics for human and animals [9, 10].

Commercially, the spore producing *Bacillus* probiotics have a lot of interest due to excellent stability and health claims [11]. Furthermore, the data on several clinical trials in humans indicate safety and efficacy of the strains in host [9]. There are a few studies on the spore forming *Bacillus* probiotics in diarrhoea [12, 13]. Recently, Hatanaka and co-workers [14] demonstrated the efficacy of *B. Subtilis* C-3102 spores in treating loose stools in adult humans.

The effects of probiotics are strain specific [15, 16] and hencethis study was carried out to evaluate the effect of probiotic formulation in the form of sachets containing *Bacillus coagulans* Unique IS2 and *Bacillus subtilis* UBBS-14 on enteropathogenic *Escherichia coli* (EPEC 4083) induced diarrhoea in dogs. EPEC 4083 was chosen to induce diarrhoea in dogs, as it is diarrhoeagenic and not fatal to the dogs with effects being reversible.

Materials and methods

Study design

The study was approved by the Institutional Animal Ethics Committee of PV Narsimha Rao Telangana Veterinary University, Hyderabad, India. A total of 20 Indian, mongrel dogs (12 males and 8 females), aged between 1.7 – 2years (average weight 21.37 ± 3.64 kg), were taken for the study (Table 1). Mongrel dogs were chosen for the study as they have greater resistance and immunity and if efficacy of probiotic formulation in controlling diarrhoea could be established in the model, it would have efficacy in other breeds of dogs as well. The dogs were kept for acclimatization for one week according to the Institutional Animal Ethics Committee (IAEC) guidelines. During the acclimatization period, dogs were observed for any signs of illness. All the dogs were normal and were included in the study. The weights of the dogs were recorded daily. At the same time every day before feeding. The dogs were randomly divided into two groups consisting of 10 dogs each and kept in separate rooms. The dogs were provided with commercial Royal Canin (Medium, Adult) pelleted diet (Table 2) two times daily according to the instructions of the manufacturer. Water was provided *ad libitum*. Diarrhoea was induced in dogs by challenging with the *E.coli* strain EPEC 4083. In 48 hours, all dogs developed diarrhoea. Onset of diarrhoea (after 48 h) was labelled as day 1. The dogs were then administered probiotic or placebo powder which was dissolved in 2 tablespoons of milk. The probiotic sachet (1g) of total strength of 2 billion CFU consisted of *Bacillus coagulans* Unique IS2 (1 billion) and *Bacillus subtilis* UBBS-14 (1 billion). These sachets were a gift from Unique Biotech Limited, Hyderabad, India. Two sachets per day (morning and night) were administered orally to the dogs for a period of 10 days. The placebo was identical to the probiotic except that it lacked the active ingredients, the probiotic strains.

Inoculation of Enteropathogenic-*E. coli* (EPEC 4083) in dogs to induce diarrhoea

Single colony of overnight grown EPEC 4083 was cultivated in 10 ml BHI broth and incubated at 37 °C for 24 h with agitation (120 rpm). The 10 ml growth was further transferred to 100 ml BHI and incubated under same conditions described before. After incubation, bacterial growth was centrifuged at 8000 ×g for 10 min and pellet was suspended in saline (0.85% w/v, NaCl) to McFarland scale 8 (2.4×10^9 bacteria/ml). The animals were experimentally infected with a single oral dose of 1ml bacteria suspension. All the dogs developed liquid-to-pasty diarrhoea within 48 hours.

Examinations

a) **Recording of rectal temperatures:** The rectal temperature of each animal was recorded daily with the aid of thermometer, values were expressed in degree celsius (ºC).

b) **Estimation of frequency of defecation:** Frequency of defecation was noted everyday till the end of treatment (10th day).

c) **Assessment of faecal consistency:** The faecal quality was assessed daily by trained assessors. The faeces were graded on scale 1 to 4,

1. ideal, firm stool,
2. soft amorphous stool,
3. viscous liquid with some particulate matter
4. watery, liquid stool with little or no particulate matter.

d) Estimation of dehydration levels: Skin tenting time was used to assess the percentage of dehydration. In brief, the skin of animal was grasped between two fingers and drag / tented up and held for few seconds then released and the time of skin back to its normal position was noted. Lower arm or abdomen skin was checked for skin tenting time to assess the percentage of dehydration.

e) Faecal sampling and analysis: The faecal samples were collected from all experimental dogs to evaluate *E.coli* count. The faecal samples were collected at day 1, 5 and 10 (morning time) in sterile containers. Immediately after collection, the samples were weighed and diluted in phosphate buffered saline (PBS) to perform (i) CFU count on selective agar plate and (ii) short chain fatty acids (SCFA) like acetate, propionate, and butyrate estimation by gas chromatography.

f) Haematology analysis: Hemoglobin (g/dl), red blood cells (RBC, 10^6/mm^3), white blood cells (WBC, 10^3/mm^3) and pack cell volume (PCV, %) levels were estimated at day 1, 5, and 10 by routine pathological procedures.

Statistical analysis

T test and one-way ANOVA was performed by using GraphPad Prism (GraphPad Software Inc., USA). *p* value less than 0.05 was considered statistically significant.

Results

Baseline ages and body weights were similar between the two groups (Table 1). There was no difference in any of the haematology parameters between the probiotic and placebo treated dogs confirming the safety of the probiotic formulation (data not shown).

Resolution of diarrhoea

Supplementation with probiotic (*Bacillus coagulans* unique IS2 and *Bacillus subtilis*-2 billion CFU) significantly reduced the mean number of days to resolution of diarrhoea compared with placebo (3.0 ± 0.22 versus 8.2 ± 0.13 days respectively; $p<0.001$, Table 3). The daily mean stool scores (consistency of stools) are provided in Table 4 and Figure 1.

Assessment of dehydration and temperature

Skin tenting time used to assess the percentage of dehydration indicated that probiotic treatment prevented dehydration as opposed to the placebo group in which there was evidence of dehydration (Table 5).

Enumeration of E. coli in faeces

Treatment with probiotic reduced *E. coli* count in dog faeces from 2.8×10^8CFU/g (day 1) to 2.5×10^7 CFU/g (day 10), which was significantly lower as compared to the placebo treated (2.5×10^8 to 1.0×10^8 CFU/g) (Fig. 1).

Short chain fatty acid analyses

Probiotic supplementation significantly increased the faecal levels (μmol/g) of acetate (164.32 ±8.15), propionate (119.36 ± 6.51), and butyrate (35.26 ± 3.49) levels at the end of treatment as compared to the placebo (acetate 135.47 ± 7.24; propionate 98.19 ± 7.15; butyrate 28.29 ± 2.71μmol/g of faeces) (Table 6 and Figure 2).

Table 1: Age and Body weight of dogs

Groups	Age (years), Range	Body weight (kg)
Placebo	1.7- 2 years	21.40 ± 3.42
Probiotic	1.7 - 2 years	21.35 ± 3.86

Body weight- Values are Mean ± S.D

Table 2: Nutritive Value of Commercial food (Royal Canin- Medium, Adult)

Composition	Additives (per kg)	Analytical constituents
Dehydrated poultry protein, Maize, Maize flour, Wheat flour, animal fats, Wheat, hydrolysed animal proteins, beet pulp, fish oil, soya oil, yeasts and parts, minerals, hydrolysed yeast (source of manno-oligo- saccharides (0.05%)).	Nutritional additives: Vitamin A: 12500 IU, Vitamin D3: 800 IU, E1 (Iron): 40mg, E2 (Iodine): 4 mg, E4 (Copper): 12mg E5 (Manganese): 52mg E6 (Zinc): 126 mg E8 (Selenium): 0.1 mg Preservatives -Antioxidants	Protein 25.0% Fat content 14.0% - Crude ash: 6.1% -Crude fibers 1.3% - Per kg Omega 3 fatty acids: 6.1g including EPA/DHA: 3.1 g

Dosage: A 240 g per day in two divided doses

Table 3: Days to resolution of diarrhoea in probiotic and placebo treated group

Groups	Days to resolution of Diarrhoea
Placebo	8.2 ± 0.13
Probiotic treatment	3.0 ± 0.22*

Values are Mean ± S.D; **p* value < 0.001

Resolution of diarrhoea was defined as stool scores that improved from 4 to ≤ 2 and remained ≤ 2 for at least 5 consecutive days. Stool score categories: 1=ideal, firm stool; 2=soft amorphous stool;3=viscous liquid with some particulate matter;4=watery liquid stool with little or no particulate matter.

Table 4: Mean values of Stool scores in dogs treated with probiotic group and placebo group

Group	Day 1	Day2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Placebo	4.0 ±0.16	3.5 ±0.19	3.4 ± 0.10	3.4 ± 0.12	3.1 ± 0.23	3.0 ± 0.17	2.7 ± 0.07	2.0 ±0.14	1.8 ± 0.21	1.5 ± 0.24
Probiotic	4.0 ±0.25	3.1 ± 0.15	1.8 ± 0.16	1.1 ± 0.1	1.0 ± 0.16	1.0 ± 0.24	1.0 ± 0.13	1.0 ±0.20	1.0 ± 0.07	1.0 ± 0.06

Values are Mean ± SD

Table 5: Status of dehydration in placebo and probiotic treated group

Groups	Period of study		
	Day 1	Day 5	Day 10
Placebo	Euhydration	Moderate dehydration	Severe dehydration
Probiotic treatment	Euhydration	Euhydration	Euhydration

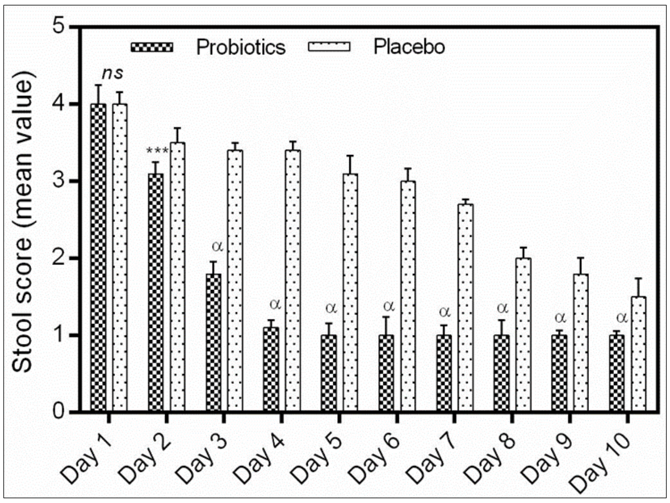
Dehydration scores

Status of Hydration	Score	Percentage	Skin tenting time
Euhydration	0	-	Skin recoils immediately
Mild Dehydration	1	5%	<2 Seconds
Moderate Dehydration	2	5-8%	2-10 seconds
Severe Dehydration	3	> 10%	> 10 seconds

Table 6: Faecal short chain fatty acids (SCFAs).

SCFAs	Probiotic			Placebo			*p* value#
(μmol/g)	Day 1	Day 5	Day 10	Day 1	Day 5	Day 10	
Acetate	135.41 ± 8.41	143.76 ± 7.49	164.32 ± 8.15	137.42 ± 6.15	139.42 ± 6.33	135.47 ± 7.24	< 0.001
Propionate	96.13 ± 5.38	113.43 ± 7.48	119.36 ± 6.51	94.37 ± 6.38	96.08 ± 6.28	98.19 ± 7.15	< 0.001
Butyrate	26.94 ± 2.83	31.84 ± 3.28	35.26 ± 3.49	27.15 ± 3.17	29.45 ± 2.72	28.29 ± 2.71	< 0.001

probiotic and placebo at day 10



ns: non-significant; *** and α: $p< 0.001$.
Stool scores: 1=ideal, firm stool; 2=soft amorphous stool;3=viscous liquid with some particulate matter;4=wateryliquid stool with little or no particulate matter

Fig 1: Effect of probiotic and placebo on stool consistency

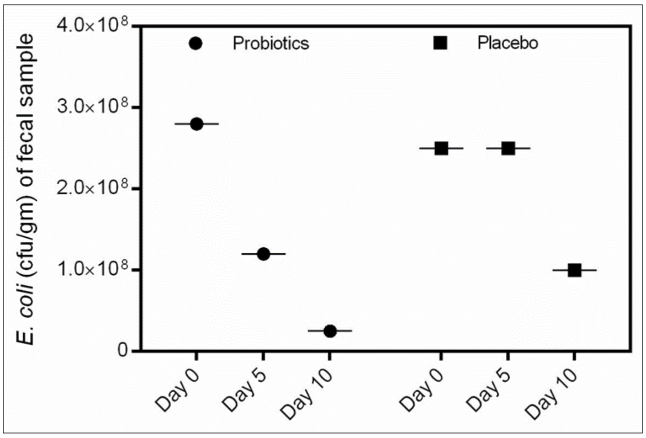


Fig 2: Effect of probiotic and placebo treatment on *E. coli*count in faeces.

Haematology
There were no significant changes in the haematological parameters like haemoglobin, RBC, WBC and PCV between the probiotic and placebo treated groups (data not shown)

Discussion
The probiotic formulation containing *Bacillus* spores (*B. coagulans* Unique IS2 and *B. subtilis* UBBS-14 (2 billion CFU) given twice a day to dogs with diarrhoea (induced by administering EPEC 4083) was effective in resolving diarrhoea as compared to placebo treated group. The number of days to resolve diarrhoea in the probiotic treated group was 3.00 ±0.22 days (mean ± SD) as compared to placebo group which was took more than double the time for diarrhoea to be resolved (8.2 ± 0.13 days). Along with the resolution of diarrhoea, the consistency of stool also improved concurrently (from watery liquid to soft or firm stool by day 3) in the probiotic group as compared to placebo group. Theprobiotic treated group did not show any signs of dehydration at any point of time during the treatment period whereas the placebo treated group showed evidence of moderate to severe dehydration. Our results are concurrent with a few reports on the efficacy of probiotics in the treatment of diarrhoea in dogs. In one study, diarrhoea was induced by the antibiotic Lincomycin and the probiotic used in the treatment was *Saccharomyces boulardii* [17]. A1000 mg/day dose of *S. boulardii* for 10 days in dogs with diarrhoea resulted in significant decrease in duration of diarrhoea as compared to control - 2.9 ± 0.4 days and 6.5 ± 1.2days, respectively. In a separate study [18], placebo or a probiotic cocktail consisting of *Lactobacillus* spp, *Pediococcus acidilactici* and *Bacillus* spp. (2.85 billion live strains per milliliter), was administered according to the animal's weight (1-3ml), three times daily to dogs suffering from acute diarrhoea. The time from initiation of treatment to the last abnormal stools was significantly shorter in the probiotic treated group compared to placebo group. Kelley *et al.* [4] evaluated the supplementation of *Bifidobacterium animalis* at 20 billion CFU/day on the resolution of idiopathic diarrhoea in dogs. There was a significant reduction in the time to resolution as compared to placebo (3.9 ± 2.3 versus 6.6 ± 2.7 days). As probiotic effects are specific to the strains, it was necessary for us to conduct the study with our strains with the required dose to establish efficacy.

The mechanism of action of probiotics in reducing diarrhoea is through inhibition of the growth of pathogens in the gastrointestinal tract. Inhibition of the growth of pathogens in the gastrointestinal tract can be via three distinct mechanisms [19], colonization of free ecological niches, which are no longer available for the growth of other microorganisms; competition for epithelial cell adhesion and production of antibiotics and/or enzymes secreted into the intestinal environment, especially peptide antibiotics. The added advantage of theprobiotic strains used in the present study are that they are spore forming probiotics and hence very stable at room temperature unlike the *Lactobacillus* and Bifidobacterium spp. Being sporeformers, they have the ability to withstand heat and could be added in the pelleted feed of dogs without any loss of viability. The fecal count of *E. coli* in the probiotic and placebo groups indicated that the probiotic formulation was highly effective in combatting the invasion by *E. coli*. By the 10th day, there was a one log reduction in the *E. coli* population as compared to placebo group suggesting the effectiveness of the probiotic strains in targeting the pathogen *E.coli* and in its amelioration. The *E. coli* count in the faeces is indicative of the *E. coli* population in the gastrointestinal tract [20].

There was an increase in the faecal short chain fatty acid concentrations (SCFA's)-acetic propionic and butyric acids in the probiotic treated group as compared to placebo. Increased SCFA production is known to improve colonic health, resulting in improved fecal quality and decreased episodes of diarrhoea [21]. The SCFA -acetate, propionate and butyrate, are end-products of the breakdown of carbohydrates by gut microbial action [22]. In the present study, with the supplementation of probiotics, there was an increase in the levels of SCFA.

The intestinal microbiota plays an important role in the normal physiological intestinal function, preventing intestinal colonization by pathogenic microorganisms and in producing SCFA, particularly acetate, propionate, and butyrate, which are the main energy source of colonocytes [23]. The presence of SCFA stimulates the secretion of glucagon like-peptide 2,which in turn stimulates cell differentiation and proliferation, as well expression of genes related to nutrient transport in the ileum facilitating an improvement in digestive function and hence the resolution of diarrhoea [23-26]. Probiotics come under the Generally Regarded as Safe category (GRAS) and hence can be recommended for use in the treatment of diarrhoea and in maintaining digestive health without the fear of any side effects.

Conclusion

Bacillus coagulans Unique IS2 and *Bacillus subtilis* UBBS14 exhibit tremendous potential in the resolution of diarrhoea and in the prevention of dehydration in dogs. Moreover, there was a notable reduction of *E. coli* load and an increase in the SCFA production which aided in the resolution of diarrhoea and in the restoration of digestive health. The study suggests that the probiotic formulation can be safely used in maintaining digestive health in dogs.

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