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Molecular and Immunological Evaluation of Some Bacteria Causing Calf Diarrhea

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Abstract

One issue that intensive farming has to deal with is neonatal calf diarrhea, and probiotics are seen to be a viable solution to improve the health of calves. The aim of this study was to assess the effects of short-term administration of an antibiotic or probiotic to diarrheal calves on total leukocytic count, serum bactericidal activity, and phagocytosis, as well as to identify potential bacteria that may cause calf diarrhea in early life stages. 55 diarrheal calves, ranging in age from 1 to 20 days, were sampled for feces at a private dairy farm in the Sharkia governorate. *E. coli*, *Salmonella* spp., and *Clostridium* species were among the isolated bacteria, with prevalence rates of 58.2%, 8%, and 12%, respectively. The O26, O111, O119, O128 and O125 serogroups of *E. coli* isolates were the identified serotypes. While *S.* Typhimurium and *S.* Enteritidis were the recovered *Salmonella* serotypes. The results of the polymerase chain reaction (PCR) showed that the *invA* gene was amplified in the four *Salmonella* isolates but the *phoA* gene was amplified in all *E. coli* isolates. Since only the alpha toxin gene was present in each isolate of *C. perfringens*, they were all type A. Antibiogram test results showed that most isolated species were multi-drug resistant to the ten commonly used antibiotics. On the other hand, isolates of *E. coli*, *Salmonella*, and *Clostridium* that demonstrated sensitivity for amoxicillin with percentages of 93.3%, 100%, and 66.7%, respectively, represented the medicine that was most successful. Regardless of infection, probiotic or antibiotic treatment, the total leukocytic count values in all diarrheal calves were considerably greater than those in the control group. Serum bactericidal activity in probiotic-treated calves was significantly higher than in the control and antibiotic-treated groups, while it was significantly lower in the antibiotic-treated group. Calf polymorphonuclear leukocytes treated with antibiotics or probiotics had considerably increased phagocytic activity than the control group. In conclusion, probiotics supplementation is an effective strategy for the prevention and control of calf diarrhea.

KEYWORDS
Calf diarrhea, *Salmonella*, *E. coli*, *Clostridium*, Probiotics

INTRODUCTION

Calf diarrhea has been recognized as a serious disease due to its detrimental impact on the cattle industry (Cho and Kyoung-Jin, 2014). One of the most typical disorders recorded in calves up to three months old is calf diarrhea; a multifactorial disease entity that can have major financial and animal welfare repercussions in both dairy and beef sucker herds. *Salmonella* spp., and *Escherichia coli* are the most prevalent and significant economically among the bacterial causes of diarrhea in newborn food animals. Additionally identified bacteria involve species of *Campylobacter* and *Clostridium perfringens* (Muktar *et al*., 2015). Although there are many potential causes of calf diarrhea, Kumar *et al*. (2012) believe that bacterial infections account for more than 50% of occurrences in newborn calves. A Gram-negative, mobile, facultative anaerobic bacteria called *E. coli*. Enterotoxigenic *Escherichia coli* (ETEC) and Enterohemorrhagic *Escherichia coli* (EHEC) are the pathogenic *E. coli* strains that can cause diarrhea (Mohammed *et al*., 2019). According to Jenkins *et al*. (2003) and Lin *et al*. (2011), the most significant serogroups of *E. coli* that cause disease in humans and animals are O157, O26, O111, O145, O91, O119, O121, and O128, which are primarily associated with STECs (STECs that produce the shiga toxin). *Salmonella* is also a Gram-negative facultative anaerobic bacteria, which cause infections associated with clinical symptoms such as diarrhea, septicemia and extraintestinal infections affecting different organs (Berge *et al*., 2008; Huang *et al*., 2018) *Salmonella* harbored The *invA* gene which represents a standard gene in its diagnosis of *Salmonella* because it is only found in *Salmonella* species (O'Regan *et al*., 2008). While *C. perfringens* generally lives in a healthy gut microbiota, stressors like fasting and prolonged use of anthelmintics and antibiotics increase the pathogenicity of the organism, which can lead to a variety of illnesses such enteric infection, food poisoning, and enterocolitis. According to their capacity to assemble the following main toxins: alpha (α), beta (β), epsilon (ε) and iota (ι) (Freedman *et al*., 2015; Heida *et al*., 2016; Navarro, 2018), *C. perfringens* strains were divided into five groups from (A to E). The failure of transfer or partial failure of transfer of passive immunity is more common in calves with diarrhea, and bacteremia is more common in these calves. According to Thomas *et al*. (2004), 20% to 30% of calves with diarrhoea or systemic sickness have bacteremia, primarily with *E. coli*.

Although calf diarrhoea is frequently treated with antibiotics, their effectiveness is debatable (Kim *et al*., 2021). Additionally, the

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use of antibiotics has been shown to have a deleterious effect on the microbial makeup of the calf gut (Ianiro *et al*., 2016; Ramirez *et al*., 2020). Lactic acid bacteria (LAB) may be used as an alternative treatment as the use of antibiotics for the prevention of infections in animals should be avoided (Bujnakova *et al*., 2014). It is promising to employ probiotics to cure neonatal calf diarrhoea. The balance of intestinal flora is supported by probiotics, which also prevent pathogenic bacteria from adhering to and invading the epithelium and create antimicrobial compounds that are toxic to both Gram-positive and Gram-negative bacteria (Rolfe, 2000).

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MATERIALS AND METHODS

All animal testing was done in accordance with the regulations set forth by the Agriculture Research Centre of Egypt's animal health research institute. From October through December of 2021, feces were collected from a private dairy farm in the Sharkia governorate. From calves 1 to 20 days old, 55 fecal samples were taken. In a nutshell, sterile cotton swabs were used to collect each fecal sample from the rectal-anal junction (RAJ). Swabs with fecal samples were put in a 15 ml conical tube on ice and transferred within an hour to the laboratory of the animal health research institution for additional processing. As previously mentioned (Fan *et al*., 2020), swab samples were suspended in a mixture of 2 ml of Luria-Bertani (LB) broth and 2 ml of 30% glycerol.

A commercial version of Lactobacillus acidophilus is called Lacteol Fort. The lactobacilli (*Lactobacillus delbruekii* and *Lactobacillus fermentum*; Rameda, Egypt) are 10 billion each sachet.

Synulox is a ready-to-use suspension for injection from Haupt Pharma that contains 140 mg/ml of amoxicillin and 35 mg/ml of potassium clavulanate for Zoetis Italy. MOH Registration No. 1532A/2010.

Experimental Design

Twenty calves were kept in individual hutches for housing as part of clinical research that was randomized. The control group (C, n.= 5) consisted of five calves which appeared healthy and had no evidence of diarrhoea, whereas the other fifteen calves had moderate diarrhoea (based on the findings of clinical indicators). Five calves were left untreated and served as the infected group (I, n.=5). Five other calves were treated with Synulox (7.0 mg amoxicillin, 1.75 mg clavulanic acid) as the recommended dosage rate is 8.75 mg/kg bodyweight, equivalent to 1 ml of suspension per 20 kg bodyweight treatment by intramuscular route once/24 hours for at least three days (A, n.=5), and the remaining five calves served as the probiotic group (P, n.= 5) and were given Lacteol Fort 3 sachets daily for three consecutive days (Abd-El-rahman, 2011).

Sampling for bacteriological examination

Fecal samples were transported to the lab in an ice chest and either cultured that day or refrigerated at 4°C for culture. Samples were streaked on MacConkey agar and separated into lactose fermenters and non-lactose fermenters for bacteriological analysis in order to isolate *E. coli* and *Salmonella*. Each lactose fermenter sample was divided into one colony, which was then streaked on eosin methylene blue agar and incubated at 37°C in sterile distilled water. On the surface of the XLD (xylose-lysine-deoxycholate agar), non-lactose fermenters were streaked. *C. perfringens* was isolated anaerobically at 37°C for 24 hours using GasPakTM (Oxoid Limited, Thermo Fisher Scientific Inc., UK) and a cooked beef medium (Becton, Dickinson and Company, USA). These were then splattered on a surface of 5–10% sheep blood agar that contained 200 g/mL neomycin (Knoeman *et al*., 1992).

Using protocols published by Quinn *et al*. (2002), the isolation and identification of *E. coli*, *Salmonella*, and *Clostridium* species from the collected samples were confirmed based on their morphological, culture, and biochemical properties. At the Serology Unit, Animal Health Research Institute, Dokki, Giza, Egypt, we used commercial antisera (Difco, Detroit, MI, USA) in accordance with the manufacturer's instructions to perform serological identification of *E. coli* and *Salmonella* isolates.

Antimicrobial susceptibility testing

The antimicrobial susceptibility of the isolates was assessed using Mueller Hinton agar plates and commercial antibiotic discs (Oxoid, Basingstoke, Hampshire, England, UK) using the agar disc diffusion method, as described by Markey *et al*. (2013). The parameters outlined in the CLSI recommendations were used to interpret the sizes of the inhibition zones (CLSI, 2019).

Polymerase chain reaction procedure

The QIAamp DNA Mini kit (Qiagen, Germany, GmbH) was used to extract DNA from samples, with some modifications made to the manufacturer's instructions.

Table 1 lists the primers that were provided by Metabion (Germany). Primers were used in a 25-µl reaction that contained 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer at a concentration of 20 pmol, 5.5 µl of water, and 5 µl of DNA template for the PCR amplification necessary to confirm the isolates. Thermal cycler 2720 from Applied Biosystems was used to carry out the process.

A 50- µl reaction including 25 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer at a concentration of 20 pmol, 12 µl of water, and 5 µl of DNA template was used to perform a multiplex PCR for the detection of *C. perfringens* toxins. Thermal cycler 2720 from Applied Biosystems was used to carry out the process. PCR amplification products were examined using an agarose gel electrophoresis.

Blood Sampling and immunological parameters

Jugular venipuncture was used to collect three blood samples following therapy (Radostits *et al*., 2000). 5 ml of blood in plastic tubes containing EDTA were used to calculate the total leukocyte count. Until processing, samples were stored at 4°C. For

Table 1. Oligonucleotide primers and cycling protocols for specific genes of the isolated bacteria

Target gene	Primers sequences	PCR Product (bp)	Amplification (35 cycles)			Final extension	References
			denaturation	Annealing	Extension		
Alpha toxin	GTTGATAGCGCAGGACATGTTAAG CATGTAGTCATCTGTTCCAGCATC	402 bp	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Yoo *et al*. (1997)
Beta toxin	ACTATACAGACAGATCATTCAACC TTAGGAGCAGTTAGAACTACAGAC	236 bp					
Epsilon toxin	ACTGCAACTACTACTCATACTGTG CTGGTGCCTTAATAGAAAGACTCC	541 bp					
Iota toxin	GCGATGAAAAGCCTACACCACTAC GGTATATCCTCCACGCATATAGTC	317 bp					
Salmonella invA	TCATCGCACCGTCAAAGGAACC TCATCGCACCGTCAAAGGAACC GCGGGACGCATCATCTCATA	284 bp	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Oliveira *et al*. (2003)
E. coli phoA	CGATTCTGGAAATGGCAAAAG CGTGATCAGCGGTGACTATGAC	720 bp	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Hu *et al.* (2011)

the purpose of measuring phagocytic activity, 5 ml of blood was received in heparinized tubes. An additional 5 ml of blood was collected without the use of an anticoagulant, clotted at room temperature for 20 minutes, spun at 3000 rpm for 10 minutes, and then the clear, non-hemolyzed serum samples were separated and stored at 20°C until needed. Using a Neubauer counting apparatus, a total leukocyte count was performed six hours after manual extraction (Ióvine and Selva, 1985). Wilkinson (1977) and Goddeeris *et al*. (1986) both provide descriptions of how to determine the phagocytic activity of bovine polymorphonuclear leukocytes (PMNL). By counting 100 phagocytes on each slide while using a microscope, the phagocytic index (PI) was calculated. A suspension of *E. coli* was adjusted for serum bactericidal activity by comparing it to 0.5 McFarland turbidity standards ($1.5x10^8$ CFU). Five cycles of serial PBS dilution (1:10) were applied to this bacterial suspension. In sterile microtubes, 20 µl of *E. coli* suspension (fifth dilution) and 20 µl of calf serum were incubated for 1 h at 37°C in order to measure the serum's bactericidal activity. Following this procedure, 20 µl of the resulting suspension was cultured in Müller Hinton Agar, and plaques were incubated overnight at 37°C, including a positive group made up of Müller Hinton Agar plates containing bacteria and PBS suspension rather than calf serum (Kajita *et al*., 1990; Rao *et al*., 2006). After a 24-hour incubation period, the colonies from the resulting mixture were counted on Muller Hinton Agar plates in duplicate (two plates per sample) to determine the quantity of viable bacteria. According to Maqsood *et al*. (2010), the bactericidal activity of the test serum was expressed as a ratio of colony forming units in the test group to those in the control group.

Statical analysis

Utilizing SPSS software version 23, statistical analysis was performed. The significant differences between the analyzed groups (C, I, P, and A) were examined using a one-way ANOVA test. Multiple comparisons between the averages of the various study groups were made following the ANOVA test using the Duncan test, where the averages denoted by the same letter are not statistically different from one another at the 0.05 probability level (P).

RESULTS AND DISCUSSION

Incidence of E. coli, Salmonella and Clostridium isolates among the examined samples

Out of 55 fecal samples collected from diarrheic calves, *E. coli*, *Salmonella* and *Clostridium* species were recovered at 32 (58.2%), 4 (8%) and 6 (12%), respectively (Table 2). Among the examined samples, 3 had mixed *E. coli* and *Salmonella* infection, 4 samples had mixed *E. coli* and *Clostridium* infection. While none of the samples had mixed *Salmonella* and *Clostridium* infection. Twenty *E. coli* isolates were sero-grouped into 5 pathotypes, namely, O26, O111, O125, O119, and O128. The predominant serogroups were O26 and O125 (6 isolates each, 30%) followed by O111 (4 isolates, 20%) and O119, O128 (2 isolates each, 10%). Serotyping of 4 *Salmonella* isolates revealed that 3 of the isolates belonged to *S.* Typhimurium and one isolate belonged to *S.* Enteritidis serotype (Table 2). In agreement with the obtained results of the current investigation, *E. coli* and *Salmonella* spp., were isolated from fecal samples collected from diarrheic calves in Bangladesh (Ansari *et al*., 2014). Besides, Muktar *et al*. (2015) reported that *Salmonella* and *Escherichia coli* species are the most prevalent and commercially significant bacterial causes of diarrhoea in newborn food animals. Other causes for enteric illnesses in calf diarrhoea include *Clostridium perfringens* and *Campylobacter* species. Neonatal calf diarrhoea may be brought on by non-infectious reasons such inadequate colostrum intake, poor sanitation, stress, crowding in the calf quarters, and cold weather.

Table 2. Recovery rates and serotypes of isolated bacteria.

Bacterial isolate	Recovery rate	Serotypes	No. of isolates
E. coli	32 (58.2%)	O26	6
		O125	6
		O111	4
		O128	2
		O119	2
Salmonella	4(8%)	*S.* Typhimurium	3
		S. Infantis	1
Clostridium	6(12%)	*Clostridium perfringens*	6

Antibiogram test of E. coli, Salmonella and Clostridium species isolates

Results of antibiogram test demonstrated the occurrence of multi-drug resistance in most of the isolated bacterial species recovered from diarrheic calves against the ten tested antibiotics. *E. coli* isolates were resistant to the tested antimicrobials including Sulfamethoxazole/trimethoprim, erythromycin, ampicillin, streptomycin, ciprofloxacin, chloramphenicol, colistin and gentamycin at 100%, 100%, 86.7%, 86.7%, 80%, 73.3%, 66.7%, and 60%, respectively. While all recovered *Salmonella* spp. and *C. perfringens*

isolates revealed absolute resistance (100%) to the same antimicrobials except for chloramphenicol (75%, and 83.3%) and gentamycin (50%, and 83.3%) in *Salmonella* species and *C. perfringens*, respectively. On the other hand, amoxicillin represented the most effective drug for the isolated *E. coli*, *Salmonella* spp., and *Clostridium* spp., as the recovered isolates showed marked sensitivity for amoxicillin at 93.3%, 100%, and 66.7%, respectively (Table 3). Similarly, Constable (2004) demonstrated that on the basis of published evidence for the oral administration of antimicrobial agents, only amoxicillin can be recommended for the treatment of diarrhea in calves. Likely, Ansari *et al.* (2014) reported that the recovered *E. coli* and *Salmonella* spp., recovered from fecal samples collected from diarrheic calves in Bangladesh had a high sensitivity towards ciprofloxacin, levofloxacin, azithromycin and cefotaxime, and marked resistance against tetracycline.

Molecular detection of virulence and toxin producing genes in the recovered bacteria

PCR was applied on 4 randomly selected *E. coli* isolates for the detection of *phoA* gene and 4 *Salmonella* isolates for the presence of *invA* gene and the results showed that the examined genes were detected in all examined isolates. A multiplex PCR protocol for the detection of *C. perfringens* toxins was performed on the recovered 4 isolates and only the gene of alpha toxin was amplified in all tested isolates as shown in Figs. 1-3. In accordance with the obtained results of the present study, Liu *et al.* (2022) reported that *invA*, *phoA*, and cpA are major virulence determinants associated with *Salmonella*, *E. coli*, and *C. perfringens* causing calf diarrhea.

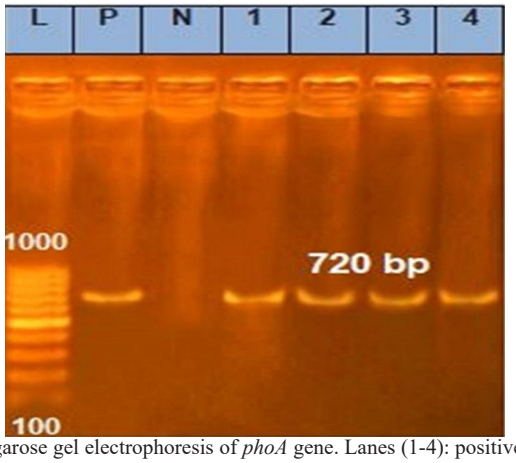


Fig. 1. Agarose gel electrophoresis of *phoA* gene. Lanes (1-4): positive amplification of target genes at 720bp. M: 100 bp DNA marker. P: positive control. N: negative control.

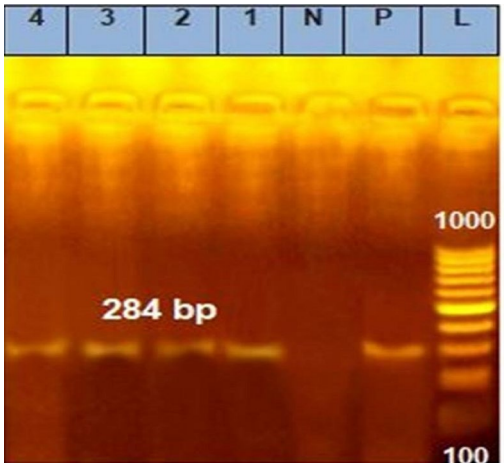
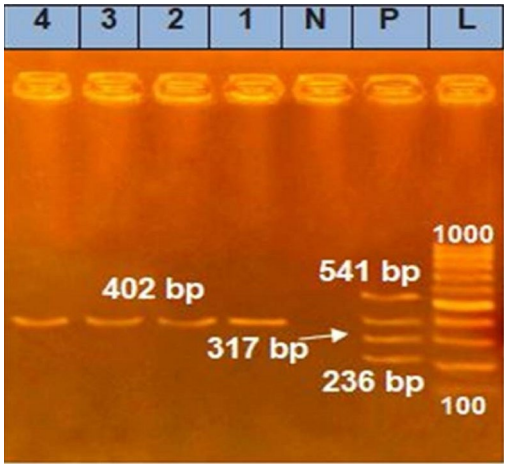


Fig. 2. Agarose gel electrophoresis of *invA* gene. Lanes (1-4): positive amplification of target genes at 284bp. M: 100 bp DNA marker. P: positive control. N: negative control.



Fig, 3. Agarose gel electrophoresis of *Alpha, Beta, Epsilon* and *Iota* genes. Lanes (1-4): positive amplification of target genes at 402bp. L: 100 bp DNA marker. P: positive control. N: negative control.

Immunological parameters

This study was extended in order to evaluate the protective effects of either probiotics (*Bacillus subtilis*), or antibiotics (amoxicillin) against cases of calf diarrhea reported in the present study. Therefore, early immunological markers were examined to assess the short course of treatment by the selected antibiotic or probiotic. Total leukocytic count has the potential for use as biomarkers of health and performance (Mohri *et al.*, 2007; Roland *et al.*, 2014; Marcato *et al.*, 2018). The total leukocytic count values in all

Table 3. Antibiotic sensitivity profile of isolated bacteria.

AMA / symbol	Potency (µg)	*E. coli* (n=30)		*Salmonella* (n=4)		*C. perfringens* (n=6)	
		S	R	S	R	S	R
Amoxicillin (AX)	10	28 (93.3%)	28 (93.3%)	4(100%)	0	4(66.7%)	2(33.3%)
Ampicillin (AMP)	10	4 (13.3%)	4 (13.3%)	0	4(100%)	3(50%)	3(50%)
Colistin (CT)	10	10(33.3%)	10(33.3%)	0	4(100%)	0	6(100%)
Gentamycin (CN)	10	12(40%)	12(40%)	2(50%)	2(50%)	1(16.7%)	5(83.3%)
Streptomycin (S)	10	4(13.3%)	4(13.3%)	0	4(100%)	0	6(100%)
Amikacin (AK)	30	18(60%)	18(60%)	2(50%)	2(50%)	0	6(100%)
Chloramphenicol (C))	30	8(26.7%)	8(26.7%)	1(25%)	3(75%)	1(16.7%)	5(83.3%)
Sulfamethoxazole + Trimethoprim (SXT)	25	0	30(100%)	0	4(100%)	0	6(100%)
Erythromycin (E)	15	0	30(100%)	0	4(100%)	0	6(100%)
Ciprofloxacin (CIP)	5	6(20%)	6(20%)	0	4(100%)	0	6(100%)

Table 4. Immunological parameters of diarrheic calves treated with antibiotic and Probiotic.

Groups	WBCs ($x10^3$/ml	Serum bactericidal activity (percentage of CFU/control	Phagocytic percentage	Phagocytic percentage index
Control	6.50±0.28c	48.00±3.74 b	55.67±3.48b	2.19±0.18c
Infected group	8.47±0.19b	62.00±3.74 a	44.60±2.25c	1.83±0.08c
Probiotic treated group	9.58±0.17a	26.00±2.45 c	78.67±2.33a	3.350±0.21b
Antibiotic treated group	9.53±0.16a	72.00 a±5.83	88.00±2.31a	4.05±0.05a
Pr > F	0	0	0	0
Significant	Yes	Yes	Yes	Yes

Data are expressed as mean±S.E.

diarrheic calves whether treated or not were significantly higher than that of the control group ($p < 0.05$). That may be a response to infection as stated before (Malik *et al*., 2013) as diarrheic calves due to *E. coli* showed a significant increase in total leukocytic count than control. Treated groups were significantly higher in total leukocytic count than infected groups. This agreed with previous studies that reported an increased leukocytic count in amoxicillin-treated sheep (Elmajdoub, 2014). This can be related to amoxicillin-induced tissue damage (Hussain *et al*., 2022). Besides, Mousa *et al*. (2019) mentioned the potential immune-enhancing effects of probiotic supplementation in Barki lambs, but Moslemipur *et al*. (2014) stated that there were no variations in the hematological and biochemical parameters between probiotic treated calves and the control group. Serum bactericidal activity in calves treated with probiotics was significantly higher than that of other groups. Probiotics support the balance of intestinal flora, inhibit epithelial adhesion and invasion by pathogenic bacteria, and produce antimicrobial substances that are inhibitory to both gram positive and gram-negative bacteria (Rolfe, 2000). Likely, Shehta *et al*. (2022) reported a significant decrease in total protein, serum albumin in diarrheic calves compared to healthy calves. Phagocytic activity of calf polymorphonuclear leukocytes of groups treated with antibiotic or probiotic was significantly higher than that of the control group. While phagocytic activity of the infected group was significantly the lowest (Table 4). This agreed with Zoltan (2018) who proposed that antibiotics' administration can improve the phagocytic function of macrophages, but Labro (2000) suggested that the presence of certain antimicrobial drugs may cause modifications in phagocyte and lymphocyte functions and reduce the production of pro-inflammatory cytokines and oxidation processes. Species of lactic acid bacteria have been suggested to promote host innate immune function by influencing activity of phagocytic cells and modulating pathogen-induced inflammatory responses (Kotzamandis *et al*., 2010; Marranzino *et al*., 2012).

CONCLUSION

The results of the present study showed that diarrheic calves due to bacterial infection when treated with a short course of probiotic (*Bacillus subtilis*) could significantly improve calves' innate immunity with minimum adverse effects compared with antibiotic (amoxicillin) administration.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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