

Molecular Characterization of *Clostridium perfringens* Type D and Evaluation of Probiotic and Antibiotic Therapy Against Induced Enterotoxemia in Goat

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ABSTRACT

Enterotoxemia is a major clostridial disease of goats associated with high morbidity and mortality. The present study aimed to characterize *Clostridium perfringens* type D isolated from caprine enterotoxemia cases and to evaluate the therapeutic efficacy of probiotics and antibiotics against experimentally induced infection. Samples collected from clinically affected goats (n = 20) were subjected to bacteriological, biochemical, and molecular analyses. The isolates were identified as Gram-positive, spore-forming rods. Molecular analysis confirmed the presence of the *cpa* and *etx* toxin genes in all isolates, identifying them as *C. perfringens* type D. *In vitro* antimicrobial susceptibility testing revealed the highest sensitivity to enrofloxacin and sulphadiazine/trimethoprim. For *in vivo* evaluation, thirty-five healthy goat kids were randomly allocated into seven experimental groups, including control, probiotic, antibiotic, and combination therapy groups. Clinical observations, survival rate, and hematological parameters were monitored following experimental induction of enterotoxemia. Infected untreated goats developed severe clinical signs and showed the lowest survival rate, whereas probiotic-treated animals exhibited moderate improvement. Combination therapies produced the most favorable outcomes, resulting in rapid clinical recovery, normalization of hematological parameters, and 100% survival. Yeast supplementation demonstrated greater therapeutic benefit than yogurt among probiotic treatments. These findings indicate that probiotics, particularly when combined with antimicrobial therapy, represent a promising therapeutic strategy for the management of caprine enterotoxemia.

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Authors' Contribution

MM performed the experiment and drafted the manuscript. JK Zaman was the supervisor. AH Mirani conceived the title. NH Kalhoro assisted in different tests. B Sahito checked the article.

Key words

Clostridium perfringens, Enterotoxemia, Probiotics, Antibiotics, *In vitro* efficacy and *in vivo* efficacy

INTRODUCTION

Livestock plays a pivotal role in the agricultural economy of many developing countries, providing meat, milk, fiber, and employment to rural populations. Among the livestock species, goats (*Capra hircus*) hold a unique position due to their high adaptability, low maintenance cost, and significant contribution to food security and income generation (Devendra, 2013).

There are over one billion goats in the world, with approximately 80% occurring in Asia and Africa (FAO, 2023). Goats are some of the most economically important animals for their meat and milk in Pakistan (GOP, 2023). Nonetheless, several infections have emerged as significant barriers to increasing the efficiency and productivity of goat rearing (Radostits *et al.*, 2021). Enterotoxemia infection caused by *Clostridium perfringens* has been classified as one of the most serious and economically devastating small ruminant diseases (Silva *et al.*, 2020). It has been shown that enterotoxemia infection commonly affects well-fed animals, especially those fed with high-carbohydrate diets, causing unexpected death without apparent clinical symptoms (Uzal *et al.*, 2022). From a clinical perspective, enterotoxemia infection results in sudden death, nervous system disorders like convulsions and opisthotonus, and diarrhea in subacute infections (Lewis *et al.*, 2010). On postmortem examination, it includes soft pulpy kidneys, hemorrhagic enteritis, and pericardial effusion.

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Enterotoxemia diagnosis is based on clinical signs, pathological findings, and toxin gene molecular detection. Methods of prevention include immunization, but outbreaks have been detected, suggesting genetic diversity among strains (Silva *et al.*, 2021).

C. perfringens is a Gram-positive, anaerobic, spore-forming microorganism found in soil, dust, feces, water, and the intestines of animals and humans. The persistence of this microorganism in the environment is attributed to its capability to form highly resistant endospores that withstand heat and chemical sterilization procedures (Uzal *et al.*, 2014). Such capabilities ensure continuous environmental contamination, allowing contact with *C. perfringens* through the consumption of feed, bedding, and soil in farm environments. Disease-causing capability is linked primarily to the ability of *C. perfringens* to produce vast amounts of toxins and enzymes that trigger necrotic and enteropathic effects. *C. perfringens* is classified into five types, based on their toxins (alpha, beta, epsilon, and iota). These toxinotypes include types A–G, with type A being non-metagenomic (Uzal *et al.*, 2022). Additionally, there are other toxinotypes that are designated as types F and G because they have specific genes coding for the enterotoxin and necrotic toxin B (cpe and netB, respectively) (Uzal *et al.*, 2022). Other accessory toxins produced by *C. perfringens* include β 2-toxin, perfringolysin O, and enterotoxin (cpb2, pfoA, and cpe, respectively) (Allaart *et al.*, 2013).

Despite the significant economic impact of caprine enterotoxemia, information regarding the molecular characteristics of circulating *Clostridium perfringens* type D strains and the comparative therapeutic efficacy of probiotics and antibiotics remains limited in Pakistan. Therefore, the present study was designed to characterize *C. perfringens* type D isolates at the molecular level and evaluate the *in vitro* and *in vivo* effectiveness of selected probiotics and antimicrobial agents against experimentally induced enterotoxemia in goats.

MATERIALS AND METHODS

Isolation and identification of *Clostridium perfringens*

Intestinal contents, of *C. perfringens* and fecal samples were aseptically collected from goats clinically affected with enterotoxemia. Samples were stored at 4°C and processed within 2 h.

For isolation, 1 g of sample was inoculated into pre-reduced reinforced clostridial medium broth (RCMB) and heat-treated at 80°C for 10–15 min to select spore-forming bacteria. Cultures were incubated anaerobically at 37°C for 24–48 h. Enriched cultures were streaked onto selective media including RCMA, Brucella blood agar, and egg yolk agar under anaerobic conditions at 37°C for 24 h. Colonies were selected based on morphology, hemolysis, and lecithinase activity. Purified isolates were identified using Gram staining (Gram-positive, boxcar-shaped bacilli) and biochemical tests including fermentation, catalase, oxidase, gelatin liquefaction, and lecithinase activity. Confirmed isolates were stored at 4°C short-term and in 15–20% glycerol at –80°C long-term.

Molecular identification and toxinotyping

Following phenotypic characterization, PCR analysis was performed to confirm the identity and toxinotype of the isolates. Genomic DNA was extracted from bacterial colonies using the TIANamp Genomic DNA Kit. PCR was performed targeting alpha (*cpa*) and epsilon (*etx*) toxin genes using primers (Table I) described by Van Asten *et al.* (2009). Amplified products were analyzed by 2% agarose gel electrophoresis stained with ethidium bromide.

Experimental animals and study design

This study included thirty-five clinically healthy post-weaned goat kids of either sex, aged 3–6 months and weighing 10–16 kg. Following clinical examination and a 10-day acclimatization period, the animals were randomly assigned into seven groups (n= 5 per group): Group 1 served as the negative control, while Group 2 served as the positive control. Group 3 received

Table I. Toxin genes with respective primer sequences.

Toxin gene	Primer	Sequence 5'→3'	Product size
<i>16S rRNA</i>	0.4 conc. (μM)	F= TAACCTGCCTCATAGAGT R= TTTCACATCCCACTTAATC	481bp
<i>cpa</i> (α -toxin)	CPAlpha	F= GCTAATGTTACTGCCGTTGA R= CCTCTGATACATCGTGTAAG	324bp
<i>etx</i> (ϵ -toxin)	CPEpsilon	F= TGGGAACCTTCGATACAAGCA R= AACTGCACTATAATTCCTTTTCC	376bp

Van Asten *et al.* (2009)

oral yogurt containing *Lactobacillus johnsonii* BS15 (1×10^6 CFU/mL; 50 mL, TID) for five days, and Group 4 received oral yeast containing *Saccharomyces cerevisiae* (>10 billion CFU/mL; 15 mL, TID) for the same duration. Group 5 was treated with Filorphenicol (20 mg/kg, IM, BID), Group 6 received sulphadiazine/trimethoprim (10 mg/kg, IM, BID) in combination with oral yogurt, and Group 7 received enrofloxacin (5 mg/kg, IM, BID) in combination with oral yeast. All treatments were administered for five consecutive days. The clinical signs observed in the experimental groups following treatment are summarized in Table II.

Table II. Clinical signs observed in each group.

Group	Diarrhea Score (0–4)	Abdominal distension	Depression	Anorexia	Dehydration	Neurological Signs
1	0.0 ± 0.0^d	None	None	None	None	None
2	3.8 ± 0.2^a	Moderate	Severe	Present	Present	Present
3	1.0 ± 0.2^b	Mild	Mild	Mild	Mild	Mild
4	1.5 ± 0.2^c	Mild	Mild	Mild	Mild	Mild
5	1.2 ± 0.2^b	None	Mild	None	None	None
6	0.3 ± 0.1^e	None	Mild	Mild	Mild	None
7	0.5 ± 0.1^d	None	None	None	None	None

ANOVA P-value: < 0.001 ; LSD (5%): 0.42; Standard Error (SE): 0.15.

Induction of enterotoxemia, in vitro susceptibility, and in vivo treatment evaluation

Enterotoxemia was induced using a goat-origin *C. perfringens* type D strain cultured anaerobically and adjusted to 1×10^1 CFU/mL. Each animal received intraduodenal inoculation (150 mL) along with 100 mL of 20% sterile starch intra-abomasally. Control animals received sterile medium only. *In vitro* antimicrobial and probiotic susceptibility was assessed using the disc diffusion method on Mueller–Hinton agar under anaerobic conditions, and interpreted according to CLSI (2018) guidelines. *In vivo* treatment efficacy was evaluated based on clinical recovery, survival rate, and biochemical changes. Clinical parameters included rectal temperature, heart rate, respiratory rate, feed intake, fecal consistency, hydration status, and general condition. Supportive IV fluid therapy was provided where necessary.

Blood sampling and hematological study

Blood samples were collected aseptically from all experimental animals for hematological (EDTA vials) and biochemical (plain tubes without anticoagulant) analyses. Hematological parameters analyzed included hemoglobin (Hb), packed cell volume (PCV), total erythrocyte count

(TEC), total leukocyte count (TLC), neutrophil count, and lymphocyte count.

Statistical analysis

Data was statistically analyzed using ANOVA, followed by post-hoc LSD test (Statistix 8.1).

RESULTS

Clostridium perfringens isolated from the field cases of enterotoxemia

The clinical manifestations and postmortem lesions characteristic of enterotoxemia were observed in goats. Eight out of the 20 animals tested died from this disease, implying that enterotoxemia was usually an acute condition. Diarrhea was the most frequently observed clinical sign and was recorded in 12 of 20 goats (60%), followed by abdominal distension in 10 animals (50%) and bloating in 9 animals (45%). Only 7 goats experienced colic (35%), and nervous system related manifestations, including opisthotonos, were noted in 4 out of 20 animals (20%). On the other hand, the postmortem findings revealed that enteritis with hemorrhage occurred in 18 out of 20 (90%) and intestinal distention in 18 animals (90%).

The presumptive isolates showed characteristics of culture and colony of *C. perfringens*. The presence of turbidity along with gas formation in Robertson's cooked meat broth is indicative of active anaerobic growth and was observed in all 20 (100%) samples tested. The double zone of hemolysis was observed in 18 (90%) isolates on blood agar plates and 17 (85%) were found to be positive for lecithinase activity in egg yolk agar. Besides, all the isolates were found to digest meat and form black color colonies in Robertson's cooked meat agar medium. All isolates were Gram-positive, box-like rod bacteria in single or short chain formation. In motility test, all isolates were found to be non-motile with cultures being homogeneous in nature. The biochemical properties of the *C. perfringens* strains exhibited a desirable metabolism for the specific bacteria. In the test for fermentation of carbohydrates, all twenty strains were positive for glucose, lactose, sucrose, and maltose (100%), implying high saccharolytic potential in these strains. Conversely, the strains showed negativity to the tests for catalase and oxidase (0%), which is typical of obligate anaerobes such as *Clostridium* species. Gelatin liquefaction was seen in fifteen of the twenty strains (75%), indicating the ability of these strains to hydrolyze proteins. Lecithin activity, known to be mediated by phospholipase C production, was seen in 17 strains (85%). All the 20 isolates (100%) survived in Robertson's cooked meat broth stored at 4°C and glycerol stocks stored at -80°C, indicating that preservation was efficient both at

refrigerated and ultra-low temperatures.

Molecular identification and toxinotyping of C. perfringens

The PCR-based detection of major toxin genes in *C. perfringens* isolates demonstrated variation in the distribution of toxin-encoding genes among the analyzed samples. The alpha toxin gene (*cpa*), producing an amplicon of 324 bp, was detected in all isolates (100%), confirming that all samples were correctly identified as *C. perfringens*. Similarly, the epsilon toxin gene (*etx*), with an expected amplicon size of 376 bp.

In-vitro antimicrobial sensitivity of C. perfringens

After molecular confirmation, the antimicrobial susceptibility of the isolates evaluated using the disc diffusion method. Inhibition zone of each treatment method for *C. perfringens* was determined via measuring the size of the zone of inhibition. The two most potent antibacterial methods were enrofloxacin and sulphadiazine + trimethoprim combinations; both have shown similar average zones of inhibition (21.0 ± 1.0 mm and 21.0 ± 0.8 mm, respectively), with insignificant difference between their effect ($p > 0.05$). The next in effectiveness was florfenicol (17.4 ± 1.2 mm), followed by probiotic treatments; yogurt (18.0 ± 0.5 mm) and yeast (16.2 ± 0.4 mm) were less effective at controlling bacterial growth. It can be assumed that standard antibiotics had a stronger antibacterial effect compared to probiotics *in vitro*, although the latter also proved their ability to inhibit bacterial growth. Based on these *in vitro* findings, the therapeutic efficacy of the selected probiotics and antimicrobial agents was further evaluated in experimentally induced enterotoxemia in goat kids.

RESULTS

Clinical signs

The *in-vivo* efficacy of treatments was evaluated based on clinical signs, laboratory findings, and survival after experimental enterotoxemia. Group 6 (Sulphadiazine/Trimethoprim + Yogurt) showed the best outcomes with minimal diarrhea (0.3 ± 0.1) and no neurological signs, recovering in 80% of animals. Group 7 (Enrofloxacin + Yeast) also recovered fully by day two (diarrhea 0.5 ± 0.1). Groups 3 (Yogurt) and 5 (Florfenicol) showed moderate improvement (diarrhea 1.0–1.2), while Group 4 (Yeast) exhibited mild-to-moderate signs (diarrhea 1.5 ± 0.2). Negative control (Group 1) remained normal, whereas positive control (Group 2) developed severe diarrhea (3.8 ± 0.2) and systemic and neurological signs.

Rectal temperature, heart rate and respiratory rate

Figure 1 shows rectal temperature, heart rate and

respiratory rate of all groups upto five days. The mean values of the rectal temperatures (1A) heart rate (B) and respiratory rate (C), all the groups varied significantly after the inoculation of *C. perfringens* type D. The negative control (Group 1) maintained constant rectal temperature ($38.5 \pm 0.2^\circ\text{C}$), heart rate (95 ± 2 beats/min) and respiratory rate (22 ± 1 breaths/min). Group 2 showed high fever on day one ($40.5 \pm 0.3^\circ\text{C}$) but recovered slowly on day five ($38.7 \pm 0.2^\circ\text{C}$). They had maximum tachypnea (34 ± 2 breaths/min) lasting up to Day 3. The heart rate increased dramatically to 110 ± 4 beats/min. It remained unchanged up to day three before recovering normally by day five. The groups 3-5 showed intermediate fever (39.0 - 39.8°C) and recovered fast on days three and four. Groups 6 and 7 maintained low fever (38.7 - 38.8°C) and recovered within two days. They experienced moderate increases (102 - 105 beats/min), where recovery took place on days three and four. Respiratory rates were moderately elevated (30 - 32 breaths/min), and these rates became normal by Day 4-5. Groups 5-7 had moderately increased breathing (28 - 30 breaths/min) which became normal by Day 2-3.

Fecal consistency, hydration and appetite scores

Figure 2 shows fecal consistency score, hydration score and appetite scores after inoculation of *C. perfringens* type D. All these scores normal in all groups. The fecal score for group 2 was 4.0 ± 0.0 initially, decreasing gradually to become 2.0 ± 0.2 by Day 5. The score for groups 3-4 was 3.0 ± 0.2 indicating moderate diarrhea which normalized by Day 4-5. Fecal consistency scores for groups 5-7 were 2.0 ± 0.1 , indicating mild diarrhea and fastest recovery.

As well as to hydration subjects in Groups 3-5 demonstrated moderate anorexia (score 1.0-1.5) but had nearly normal appetite on days 4-5 (Figure 2). Groups 6-7 recovered the quickest and by the 2nd day had normal scores. Differences were statistically significant (ANOVA $P < 0.001$, LSD 0.400, SE 0.100).

The dehydration in Group 2 was at its most severe (3.0 ± 0.2) with low recovery rate as they scored 1.2 ± 0.1 on the fifth day. On the fourth day, groups 3 and 4 normalized, group 5 did so on the third day and Groups 6 and 7 did so on the second day.

Behavior scores

Behavioral impairment followed disease severity. Group 1 remained near-normal (0.0 - 0.2), while Group 2 exhibited pronounced lethargy (3.0 ± 0.2) with partial recovery to 1.2 ± 0.1 by Day 5. Groups 3-5 recovered by Days 4-5, and Groups 6-7 normalized earliest, by Day 2, reflecting rapid behavioral restoration (Fig. 3). Differences were statistically significant (ANOVA $P < 0.001$, LSD 0.600, SE 0.200).

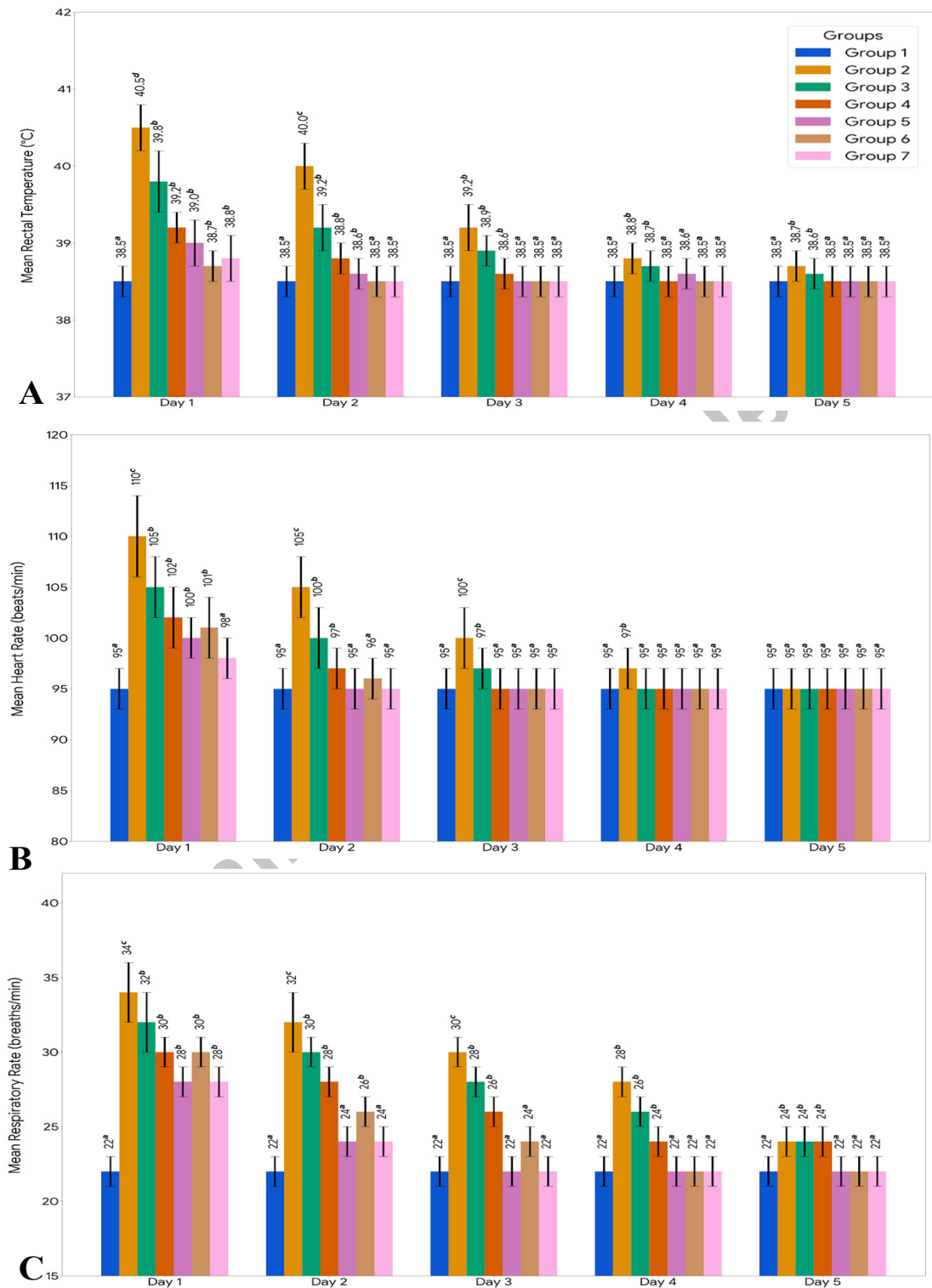


Fig. 1. Mean rectal temperature (A), heart rate (B) and respiratory rate (C) across groups. ANOVA P-values for A <0.005, for B <0.001, for C <0.001. LSD (5%) for A 0.36, for B 3.0, and for C 3.000. Standard Error (SE) for A 0.12, for B 2.0, and for C 1.000.

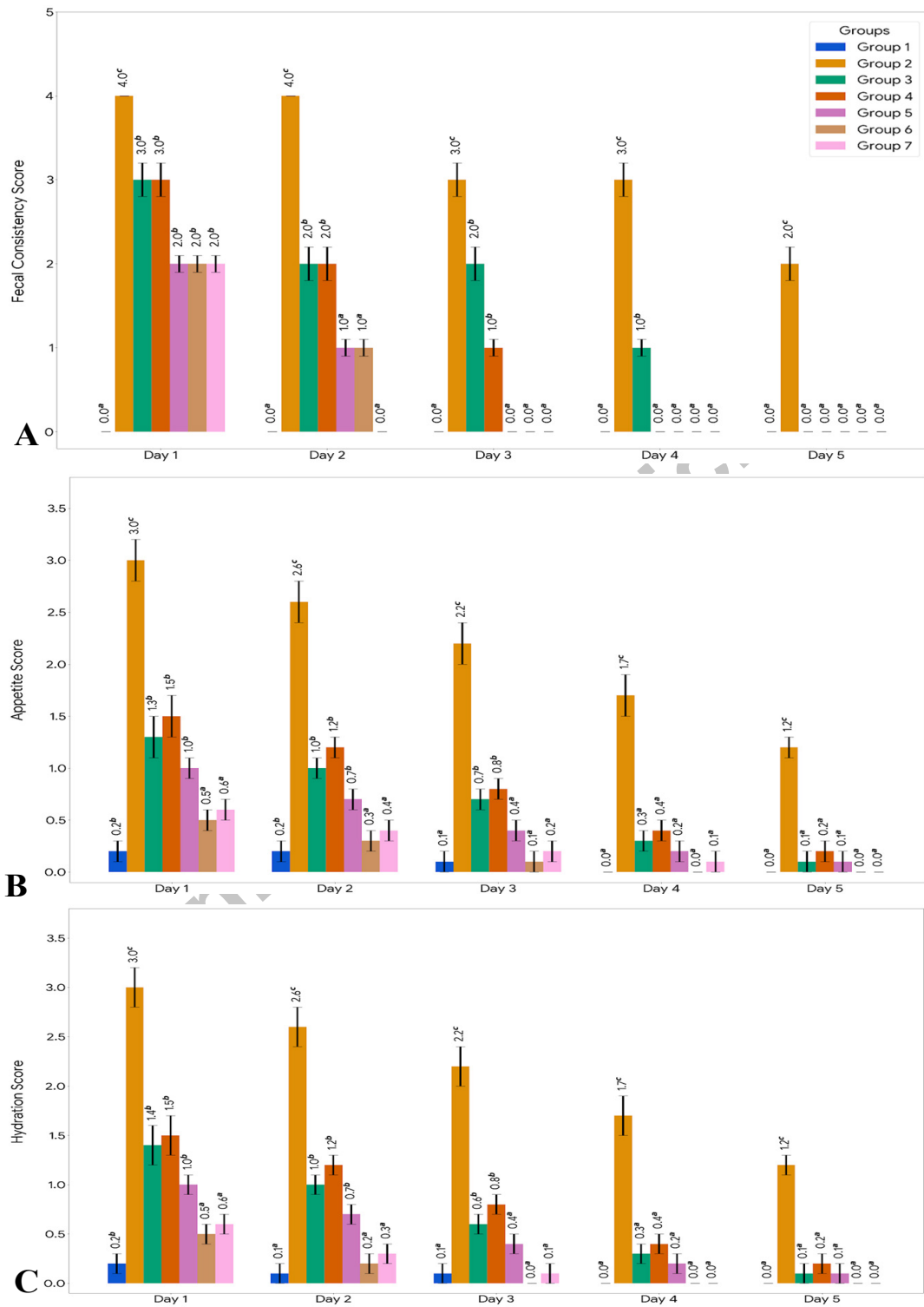


Fig. 2. Fecal consistency scores (A), appetite scores (B) and Hydration scores (C) across experimental groups. Scale for fecal consistency, 0= normal, 4 = severe dehydration; for appetite scores 0 = normal, 3 = severe dehydration and for hydration scores 0 = normal, 3 = severe dehydration. ANOVA P-values <0.001. LSD (5%) for A 0.6; for B 0.4, and for C 0.450. Standard Error (SE) for A 0.20, for B 0.1; and for C 0.300.

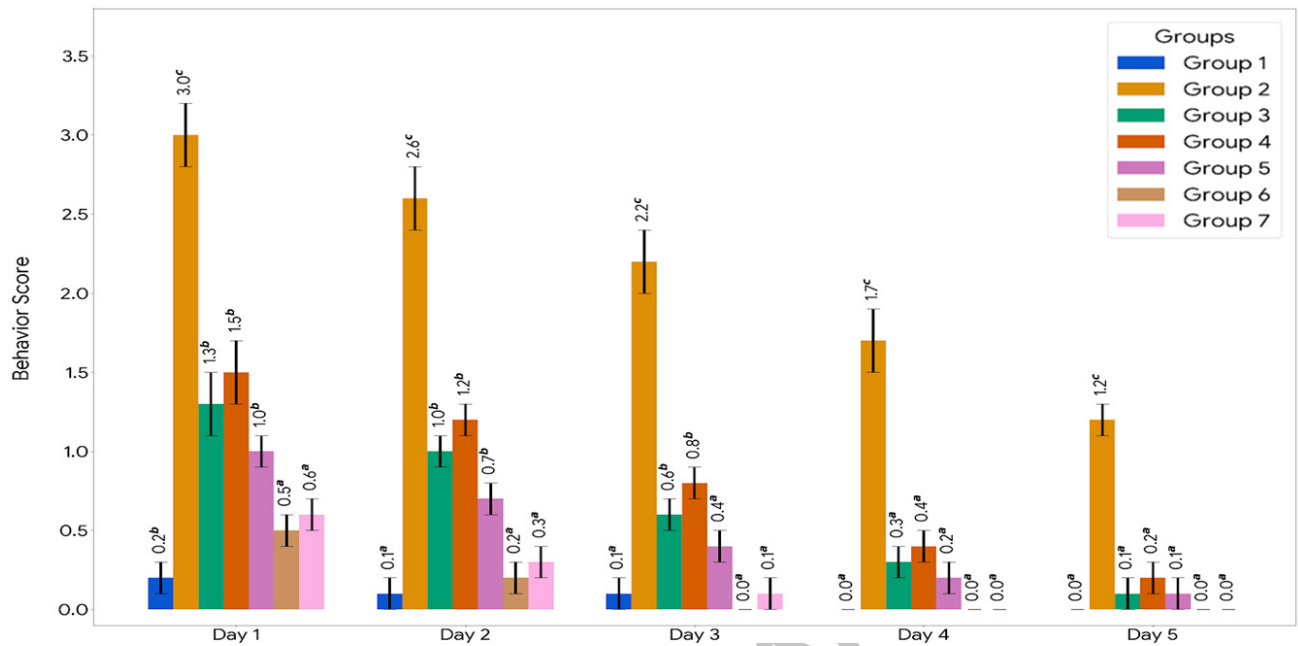


Fig. 3. Behavior scores across experimental groups (0= normal, 3 depressed/ lethargic). ANOVA P-value: <0.001; LSD (5%): 0.570; Standard Error (SE): 0.200.

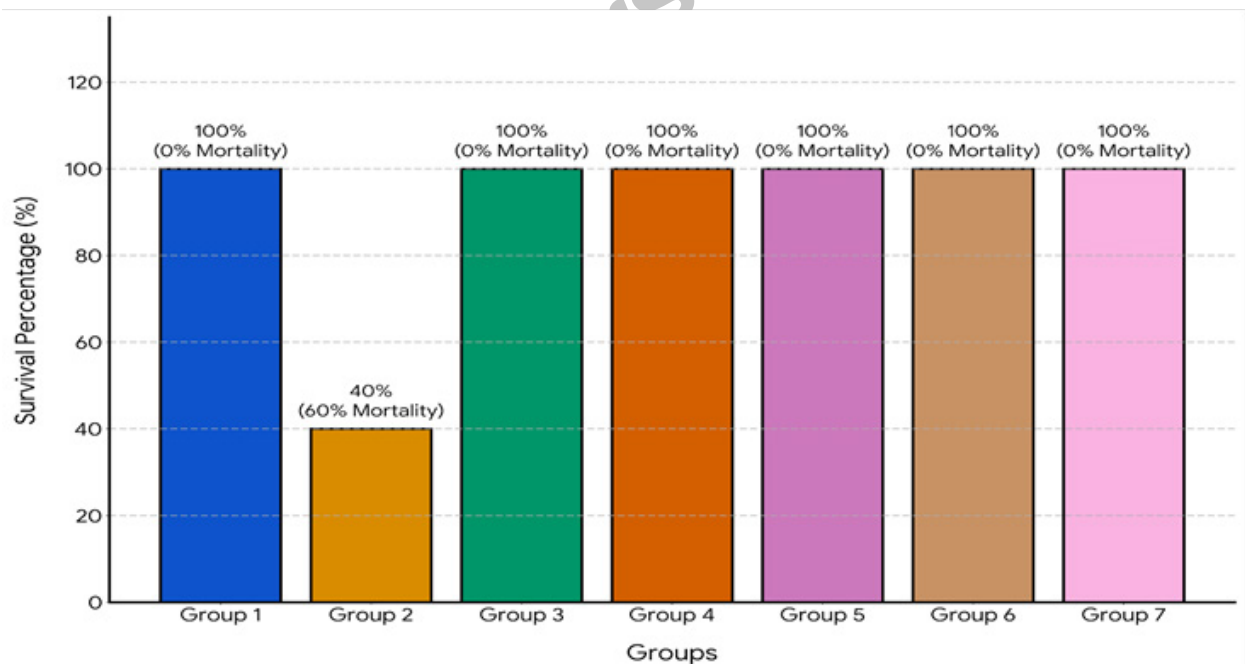


Fig. 4. Survival rate and mortality (%) across groups.

Survival rate and mortality (%)

There was high variance in the survival rate of the various treatment groups after infection with enterotoxemia due to infection by *C. perfringens* type D (Fig. 4). In group

one, the animals had a 100% survival rate, which means that in this case, the animals did not have any infections. For group two, the survival rate was 40% while the death rate was 60%.

Hematological profile

Hematology analysis was done to determine physiological and immunity responses of goat kids upon enterotoxemia induced using *C. perfringens* type D and treated. Hematological parameters that were analyzed

included hemoglobin (Hb), packed cell volume (PCV), total erythrocyte counts (TEC), total leukocyte count (TLC), neutrophils and lymphocytes (Fig. 5). Differences that existed between the groups indicated disease severity and the efficacy of treatments provided.

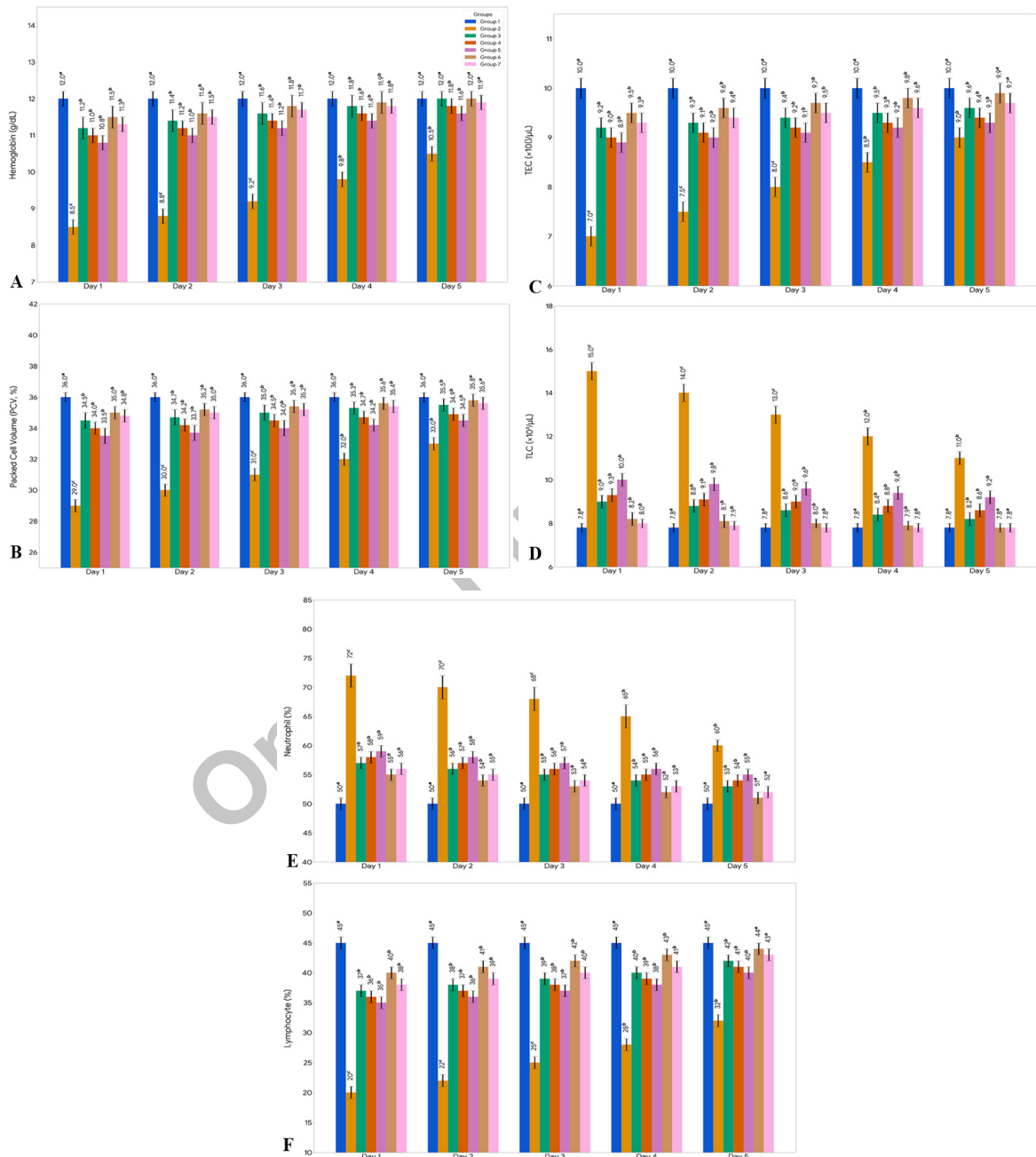


Fig. 5. Haematological profile of experimental groups after inoculation of *C. perfringens* type D isolates (Day 1–Day 5): A, Hemoglobin (Hb, g/dL); B, packed cell volume (PCV, %); C, total erythrocyte count (TEC $\times 10^6/\mu\text{L}$); D, total leukocyte count (TLC $\times 10^3/\mu\text{L}$); E, neutrophil (%) and F, lymphocyte (%). ANOVA P-value < 0.001; LSD (5%) for A, 0.4; B, 0.6; C, 0.3; D, 0.8; E, 2.5; F, 2.0; SE: for A, 0.2; B, 0.2; C, 0.1; D, 0.2; E, 1.0; F, 1.000

The Hb remained stable in the negative control (12.0 g/dL), whereas it decreased in the positive control (8.5 g/dL) (Fig. 5A). Treatment groups showed progressive recovery, with Groups 6 and 7 restoring Hb levels close to normal (10.8–11.9 g/dL by Day 5). The PCV remained constant in the negative control (36%), while it declined in the positive control (29%). Treated groups showed gradual improvement, with Groups 6 and 7 reaching near-normal values by Day 5 (Fig. 5B). The TEC remained stable in the negative control ($10 \times 10^6/\mu\text{L}$), but decreased in the positive control ($7 \times 10^6/\mu\text{L}$). Treatment groups showed recovery, with Groups 6 and 7 approaching baseline values by Day 5 (Fig. 5C). The TLC increased in the positive control ($15 \times 10^3/\mu\text{L}$) compared to negative control ($7.8 \times 10^3/\mu\text{L}$). Treatment groups showed a gradual decline, with the most pronounced reduction observed in Groups 6 and 7 (Fig. 5D). Neutrophil percentage increased in the positive control (72%) compared to negative control (50%). Treatment groups showed gradual normalization, especially in Groups 6 and 7 by Day 5 (Fig. 5E). Lymphocyte percentage decreased in the positive control (20%) compared to negative control (45%). Treated groups showed progressive recovery, reaching near-baseline values in Groups 6 and 7 (Fig. 5F).

DISCUSSION

The clinical and pathological findings of the present investigation strongly indicate that the affected goats were suffering from caprine enterotoxemia caused by *C. perfringens*. The predominance of sudden death (40%) suggests a peracute manifestation of the disease, where rapid toxin production leads to severe systemic toxicity, often resulting in mortality before any therapeutic intervention can be applied (Uzal *et al.*, 2022). Comparable mortality patterns have also been documented in goats infected with toxinotypes B and D under intensive feeding systems (Popoff, 2026). The high occurrence of diarrhea (60%) and hemorrhagic enteritis (90%) aligns with the known pathogenesis of the disease, in which ϵ - and β -toxins disrupt epithelial integrity, increase intestinal permeability, and induce mucosal hemorrhage (Uzal and Navarro, 2018). Neurological manifestations such as opisthotonos (20%) are likely associated with systemic absorption of ϵ -toxin and its neurotoxic effects. Abdominal distension observed in nearly half of the animals can be attributed to excessive gas production resulting from rapid bacterial proliferation and carbohydrate fermentation within the gut (Uzal and Navarro, 2026). Postmortem observations, including intestinal distension and widespread hemorrhages, further corroborate the classical pathological features of the disease (Uzal *et al.*, 2022). Collectively, these findings

provide strong epidemiological and pathological evidence supporting *C. perfringens* as the primary etiological agent.

The enrichment in RCMB followed by successful isolation of 100% anaerobic spore-forming bacteria in all samples highlights the reliability of heat enrichment and anaerobic culture techniques in promoting spore germination while suppressing competing microflora (Mehdizadeh *et al.*, 2021). Observed turbidity, gas production, and tissue blackening due to protein degradation indicate active breakdown of sulfur-containing amino acids, leading to hydrogen sulfide formation (Popoff, 2026). The majority of isolates showing double-zone hemolysis (90%) and lecithinase positivity (85%) further confirm the presence of *C. perfringens*, as lecithinase activity is closely linked with alpha-toxin expression (Flores-Díaz *et al.*, 2016). Variability in lecithinase activity may reflect regulatory differences in enzyme expression rather than absence of the corresponding gene.

Microscopically, all isolates exhibited Gram-positive, box-shaped rods with blunt ends and non-motile characteristics, which are typical diagnostic features of *C. perfringens* and help differentiate it from other clostridial species such as *C. septicum* and *C. sordellii* (Theoret *et al.*, 2026). The development of uniform cultures ensured reliability in biochemical and molecular analyses, as heterogeneity could otherwise compromise toxin typing and sequencing accuracy (Mehdizadeh *et al.*, 2021). Biochemical characterization revealed classical traits demonstrated fermentation of glucose, lactose, sucrose, and maltose, along with negative catalase and oxidase reactions, partial gelatin liquefaction (75%), and lecithinase production (85%) (Uzal *et al.*, 2022). These results collectively reflect the organism's saccharolytic and proteolytic capabilities as well as its pathogenic potential.

The preservation of isolates using both short-term and long-term storage techniques demonstrated excellent phenotypic stability and viability, confirming the efficiency of the preservation methods employed. Refrigerated RCMB storage at 4°C proved suitable for routine handling, whereas glycerol stocks maintained at -80°C ensured long-term genetic preservation (Mehdizadeh *et al.*, 2021). Such stable preservation is essential for future comparative research, vaccine development, and pathogenicity studies.

Molecular analysis confirmed the universal presence of the *cpa* gene across all isolates, identifying it as a defining marker of the species (Ohtani and Shimizu, 2015). The detection of *etx* genes in all isolates indicates predominance of toxinotype D strains associated with caprine enterotoxemia, while the absence of the *iap* gene suggests that toxinotype E was not involved in the studied population (Uzal and Navarro, 2018).

Marked differences in clinical outcomes were

observed among treatment groups. The positive control group (Group 2) exhibited severe diarrhea (mean score 3.8 ± 0.2), abdominal bloating, depression, and a mortality rate of 60%, reflecting the high pathogenicity of *C. perfringens* type D infection (Uzal *et al.*, 2022). Probiotic treatments (yogurt in Group 3 and yeast in Group 4) contributed to symptom reduction, with complete recovery observed by day three in the yeast-treated group. Combination therapies incorporating florfenicol alongside other agents (Groups 5–7) produced more rapid clinical improvement, with Group 7 achieving full recovery within two days.

Physiological parameters supported these observations. Rectal temperatures in the positive control group increased up to 40.8°C due to systemic inflammation, whereas treated groups showed faster normalization, particularly those receiving combination therapy (Liao *et al.*, 2020). Improvements were also observed in cardiovascular and respiratory parameters among treated animals (Qazi *et al.*, 2020).

Gastrointestinal disturbances such as diarrhea, anorexia, and dehydration were severe in untreated animals but resolved more rapidly in treated groups, especially Groups 6 and 7, indicating restoration of gut integrity and appetite. Behavioral recovery further reflected reversal of systemic toxicity and its neurological impact (Uzal *et al.*, 2022).

In vitro antimicrobial testing revealed strong antibacterial efficacy of florfenicol and enrofloxacin, whereas probiotics exhibited minimal direct inhibition of bacterial growth. This suggests that probiotics exert their beneficial effects primarily through modulation of gut microbiota and immune enhancement rather than direct bactericidal activity (Qazi *et al.*, 2020). Survival analysis demonstrated complete mortality reversal in treated groups compared to only 40% survival in the positive control, highlighting the high lethality of untreated *C. perfringens* infection (Uzal *et al.*, 2022).

Hematological evaluation showed anemia, neutrophilia, and lymphopenia in the positive control group, consistent with endotoxin-induced hemolysis, stress-mediated lymphocyte depletion, and systemic infection (Peters and Ball, 2013). In contrast, treated groups exhibited progressive restoration of hemoglobin levels, packed cell volume, total erythrocyte count, and normalization of leukocyte profiles.

Although antibiotics showed superior *in vitro* antibacterial activity, probiotics contributed to clinical recovery and improved hematological responses. The combination of probiotics with antibiotics produced the most favorable outcomes, suggesting a synergistic therapeutic effect in caprine enterotoxemia.

CONCLUSION

This study provides a complete clinical, pathological, microbiological, and molecular demonstration of *C. perfringens* as the cause of caprine enterotoxemia. The experiment has shown the effectiveness of combined treatment involving probiotics and antibiotics to control clinical severity, restore physiological functions, and ensure survival, suggesting their possible application in enterotoxemia field management among small ruminants.

DECLARATIONS

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None.

IRB approval

The research study was approved by the Board of Advanced Studies and Research (BASR), Directorate of Advanced Studies and Research (DAS), Sindh Agriculture University, Tandojam, under reference number DAS/1852.

Ethical approval, biosafety, and study location

Ethical approval was obtained from the Institutional Ethical Review Committee (IERC) for Animal Experimentation, Sindh Agriculture University, Tandojam, in accordance with WOAHS guidelines. All animal procedures were performed under anesthesia with strict adherence to humane endpoints. Due to the pathogenic nature of *Clostridium perfringens*, all biosafety precautions were followed, including PPE use, aseptic handling, and proper sterilization protocols. Infection was induced at the Department of Veterinary Medicine, Sindh Agriculture University Tandojam and Lab facilities to process samples were provided by Sindh Institute of Animal Health Karachi.

Data availability

The data will be shared by the corresponding author on reasonable request.

Generative AI and AI-assisted technology statement

No generative AI or AI-assisted tools were used in preparation of results and data analysis used in this study.

Statement of conflict of interest

The authors have declared no conflict of interest.

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