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Research Article

Therapeutic efficacy of *Lactobacillus bulgaricus*, zinc glycinate and oyster mushrooms against enterotoxigenic *Escherichia coli* in diarrhoeic pigs

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Abstract

Enterotoxigenic *E. coli*. is a major contributing factor for diarrhoea in pigs. In veterinary medicine, the use of antimicrobials in the management of diarrhoea as a preventative or therapeutic measure in field circumstances is disputed due to resistance development. The current study was designed to assess the therapeutic efficacy of *Lactobacillus bulgaricus*, zinc glycinate, and oyster mushrooms (*Pleurotus ostreatus*) in diarrhoea of pigs. Twenty four diarrhoeic pigs of either sex weighing 2.0 to 25.7 kg possessing enterotoxigenic *E. coli*, confirmed by molecular technique, were divided into four groups viz. Group II, III, IV and V, comprising six pigs in each group alongside the control (Group I) and each group was treated with different combinations of treatment protocol. To assess the therapeutic efficacy, dehydration score, fecal consistency, haemato-biochemical parameters, oxidative stress and zinc level were analyzed. The dehydration score and fecal consistency of diarrhoeic pigs improved significantly ($p<0.01$) in all the four treatment groups at day 7 post-therapy. All the increased and decreased haemato-biochemical parameters were turned to normalcy after the 7th day post-therapy with *Lactobacillus bulgaricus*, zinc glycinate and oyster mushrooms, which were comparable with the healthy control group. The considerable increase in lipid hydroperoxide (LPO), as well as the statistically significant ($p<0.01$) decrease in superoxide dismutase (SOD), glutathione (GSH) and total antioxidant activity (TAOA), and Zn activities indicated oxidative stress in pigs suffering from diarrhoea. These values were successfully turned to normalcy in all the treated groups after the 7th day post-therapy. In conclusion, the results of the present study suggest that combinations of *Lactobacillus bulgaricus*, zinc glycinate and oyster mushrooms can be used in therapeutic measure of diarrhoea in pigs as an alternative to antibiotics.

Keywords: Haemato-biochemical parameters, Oxidative stress, *Pleurotus ostreatus*, Zinc level

Highlights

- One of the major aetiology of diarrhoeic pigs was enterotoxigenic *E. coli*.
- *Lactobacillus bulgaricus*, zinc glycinate and oyster mushrooms produced a better clinicopathological outcome than the antibiotic treated group.
- *Lactobacillus bulgaricus*, zinc glycinate and oyster mushrooms can be used as an alternative to antibiotic therapy to treat diarrhoea in pigs.

INTRODUCTION

Many swine illnesses and disorders influence production, especially in pre-weaned piglets, and diarrhoea alone is thought to account for a decrease of 8-14 g/day average daily weight growth in the first week of life. Every year, high magnitude of health and productivity losses can be linked to piglet diarrhoea worldwide (Kylla *et al*., 2019). Among the various diseases, neonatal diarrhoea is the second highest cause of mortality worldwide. Diarrhoea is caused by a complex interaction of variables, including infectious pathogens, host immunity, and management practices (Wittum *et al*., 1995). Piglet's diarrhoea is one of the major health issues that swine producers encounter. In reality, enteric infections have become one of the major threats to newborn farm pigs, resulting in significant economic losses, particularly in suckling and weaned piglets (Ruiz *et al*., 2016).

Infectious diarrhoea in piglets is a multifactorial disease that is mainly associated with enterotoxigenic *Escherichia coli*, various strains of Coccidia (*Isospora suis*), *Clostridial enteritis*, *Salmonella cholera suis* and *Brachyspira hampsonii*, *B. hyodysenteriae*, rotavirus, coronavirus or some combination of these pathogens among numerous other bacterial infections that thrive in environments with inadequate sanitation, housing, and/or nutrition. Diarrhoea leads to weight loss, stunted growth, and even substantial mortalities in the

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young. Rapid dehydration, electrolyte loss, loose fluid and acidosis were seen in diarrhoeic animals. Though infectious pathogens may only cause initial damage to the gut, dehydration, acidosis, and electrolyte loss are the most common causes of mortality.

Generally, piglet diarrhoea is treated with supportive therapy with antibiotics, i.e. amoxicillin, ceftriaxone, and sulphadimidine (Kylla *et al*., 2019). Uses of antibiotics to treat bacterial infections may become more harmful than invading microbes, especially if taken for a long time (Tandan *et al*., 2017). Antibiotics have been found to dramatically change animal gut flora, necessitating the search for novel treatment approaches for piglet diarrhoea outbreaks (Looft *et al*., 2012). As an alternative therapy, many non-antibiotic methods have been recommended in pig diets, including acidifiers, pre and probiotics, yeast products, and/or plant oil compounds (Liu *et al*., 2018). In field conditions, the use of antimicrobials (AB) as a prophylactic or therapeutic measure is debatable due to their resistance development. As a result, routine use of AB in diarrhoeic cases is not advised. Therefore, nowadays, researchers are advocating alternative therapy for the treatment and control of diarrhoea.

The benefit of using probiotics in acute diarrhoea (mainly *Lactobacilli*, and particularly LGG strain, and *Saccharomyces boulardii*) has recently been established. Probiotics can enhance the intestinal flora and immune system of the host against specific pathogens. Zinc supplementation in diarrhoeic cases proved a significant reduction of faecal excretion, frequency, severity and persistency of diarrhoea (Salvatore *et al*., 2007). The valuable effects of supplementing piglet diets with oyster mushrooms (*Pleurotus ostreatus*) reported by Jilin Agricultural University. The inclusion of mushrooms at 15 g/kg resulted in a 55% lower incidence of diarrhoea. Therefore, this study was performed on the therapeutic efficacy of *Lactobacillus bulgaricus*, zinc glycinate and oyster mushrooms therapy against diarrhoea in pigs.

MATERIALS AND METHODS

The study was conducted in Large White Yorkshire pigs which were showing clinical signs of diarrhoea in the Instructional Livestock Farm Complex, College of Veterinary Sciences and Animal Husbandry, Central Agricultural University, Selesih, Aizawl, Mizoram, India. Twenty four animals including piglets, weaners and growers from day 1 to 6 months old of either sex weighing 2.0 to 25.7 kg, which showed signs of diarrhoea, dehydration, and inappetence, were selected.

Identification of *E. coli* by molecular techniques

Before making the group, samples were streaked on selective MacConkey (Merck, Darmstadt, Germany) agar plates within 12 hr following collection. After 24 hr of incubation at 37°C, at least three lactose-positive colonies of each sample were incubated in Luria-Bertani broth (Merck) at 37°C for one night.

Crude DNA extraction

The bacterial suspension was centrifuged at 13,000 rpm for 30 sec. The pellet was suspended in 50 µL of sterile water, incubated at 100°C for 10 min and centrifuged. The supernatant was used in the polymerase chain reaction (PCR) analysis.

Molecular detection by PCR

All isolates were examined for enterotoxigenic *Escherichia coli*. The primers used are presented in Table 1. For multiplex PCR, 4 µL DNA template was used in the final volume of 25 µL containing 12.3 µL Nuclease free water, 2.5 µL 10X Taq buffer (with $MgCl_2$, 20mM), 2 µL dNTPs (2.5Mm), 10 pM of each primer (1 µL each) for stla (F), stla(R), elt (F), elt (R) and Taq DNA polymerase (0.2 µL) (Cinaclone, Tehran, Iran). The PCR was done with a thermocycler (Eppendorf, Montesson, Franc) with microtubes in the following settings: initial denaturation stage of 94°C for 5 min followed by denaturation in 30 cycles at 94°C for 1 min, annealing at 58°C for 1 min and a final extension at 72°C for 5 min.

Table 1. Primers used in molecular detection of enterotoxic genes of *E. coli*

Primer name	Sequence (5′ -3′)	Expected amplicon size (bp)	Annealing temperature (°C)	Reference
Heat stable toxin ST (*stIa*)	F: TCTTTCCCCTCTTTTAGTCAG R: ACAGGCAGGATTACAACAAAG	166	58	Bolin *et al*. (2006)
Heat labile toxin LT (*elt*)	F: CTCTATGTGCACACGGAGC R: CCATACTGATTGCCGCAAT	322	58	IMCI, 2001

Table 2. Therapeutic protocol in pigs

Group (n=6)	Health Status	Treatment protocol	Duration
Gr. I	Healthy	Placebo (Sterile water), PO, SID	7 days
Gr. II	Diarrhoeic pigs	Antibiotic* + Supportive therapy**	
Gr. III		*Lactobacillus bulgaricus**** @ $1x10^9$ CFU/5 kg body weight orally + Zn Glycinate*** @ 20 mg/kg body weight orally + Supportive therapy	
Gr. IV		*Lactobacillus bulgaricus**** @ $1x10^9$ CFU/5 kg body weight orally + Oyster mushrooms*** @ 15 mg/per kg feed + Supportive therapy	
Gr. V		*Lactobacillus bulgaricus**** @ $1x10^9$ CFU/5 kg body weight orally + Zn Glycinate*** @ 20 mg/kg body weight orally + Oyster mushrooms*** @ 15 mg/per kg feed + Supportive therapy	

*Antibiotic was given as per the antibiotic sensitivity test (ABST) result, and as per the result, sulfadimidine injection was administered @ 14 mg/kg b. wt. intramuscularly twice daily for 7 days, along with supportive therapies; ** Supportive therapy includes electrolyte rehydration solution (ERS); *** *Lactobacillus bulgaricus* (source: Kaypeeyes Biotech Pvt. Ltd., Mysore); *** Zn Glycinate (Source: M/S Chemkari, Bhiwandi); *** Oyster mushrooms (source: Amritatva, Rajasthan)

The amplified PCR products were analyzed by agarose gel electrophoresis. Agarose (1%) was prepared in 0.5 X TBE buffer (pH 8.0). Ethidium bromide was added to get a final concentration of 0.5 µg/mL. 10 µL of PCR product was mixed with 2 µL of 6 X gel loading dye and loaded in the wells. DNA ladder (100bp) was used as a reference to compare the size of amplified products. Electrophoresis was carried out at 80 V/60mA in a horizontal electrophoresis apparatus till the dye reached the last third of the gel. The gel was visualized under a UV trans-illuminator and photographed by a gel documentation system.

Therapeutic grouping

All the ETEC positive pigs of either sex were randomly divided into four groups (II-V) and different treatments were scheduled (Table 2). Six healthy pigs were taken as a healthy control group (Gr. I).

Therapeutic evaluation

Evaluation of comparative and synergistic therapeutic efficacy was done based on the improvement of dehydration score, faecal consistency, haemato-biochemical parameters, oxidant-antioxidant indices and serum and zinc level.

Dehydration score: The dehydration status was assessed [0 - no dehydration, 1 - mild (visible vertebrae), 2 - moderate (prominent vertebrae and eyes retracted in orbit <1 mm), 3 - severe (prominent protruding vertebral and pelvic bones and eyes retracted in orbit >1 mm)] as per the method described by Chethan *et al*. (2017, 2019).

Faecal consistency: Fecal consistency was assessed and classified by the following visual grading scale (Fig. 1)

Chethan *et al*. (2017, 2019):
0 - Dry, hard, well-formed faeces
1 - Soft but formed faeces
2 - Pasty faeces, green or brown
3 - Viscus faeces in light colour, episodic
4 - Fluid faeces in light colour
5 - Watery faeces, continuous

Collection of samples: Blood samples were collected from the anterior vena cava of pigs in a vial of 2 mL with EDTA and another vial of 5 mL without EDTA on day 0^{th}, 3^{rd} and 7^{th} days for haemato-biochemical and oxidative analysis to evaluate the therapeutic efficacy. The serum was collected by micropipette after centrifugation of coagulated blood at 3000 rpm for 8-10 minutes kept in a microtube (Eppendorf) and stored at -20°C for future use.

Haematological parameters: The haematological parameters viz. red blood cell count (RBC), haemoglobin concentration (Hb), packed cell volume (PCV), white blood cell count (WBC), differential leukocyte count (DLC) and platelet count were analyzed with the help of an Automated Blood Cell Counter (Melet Schloesing4e, France).

Serum biochemical parameters: Serum samples were evaluated for the concentrations of total protein (TP), albumin (Alb), total globulin, direct bilirubin, blood urea nitrogen (BUN), creatinine, Na, Cl and K. All these parameters were determined by the Automated

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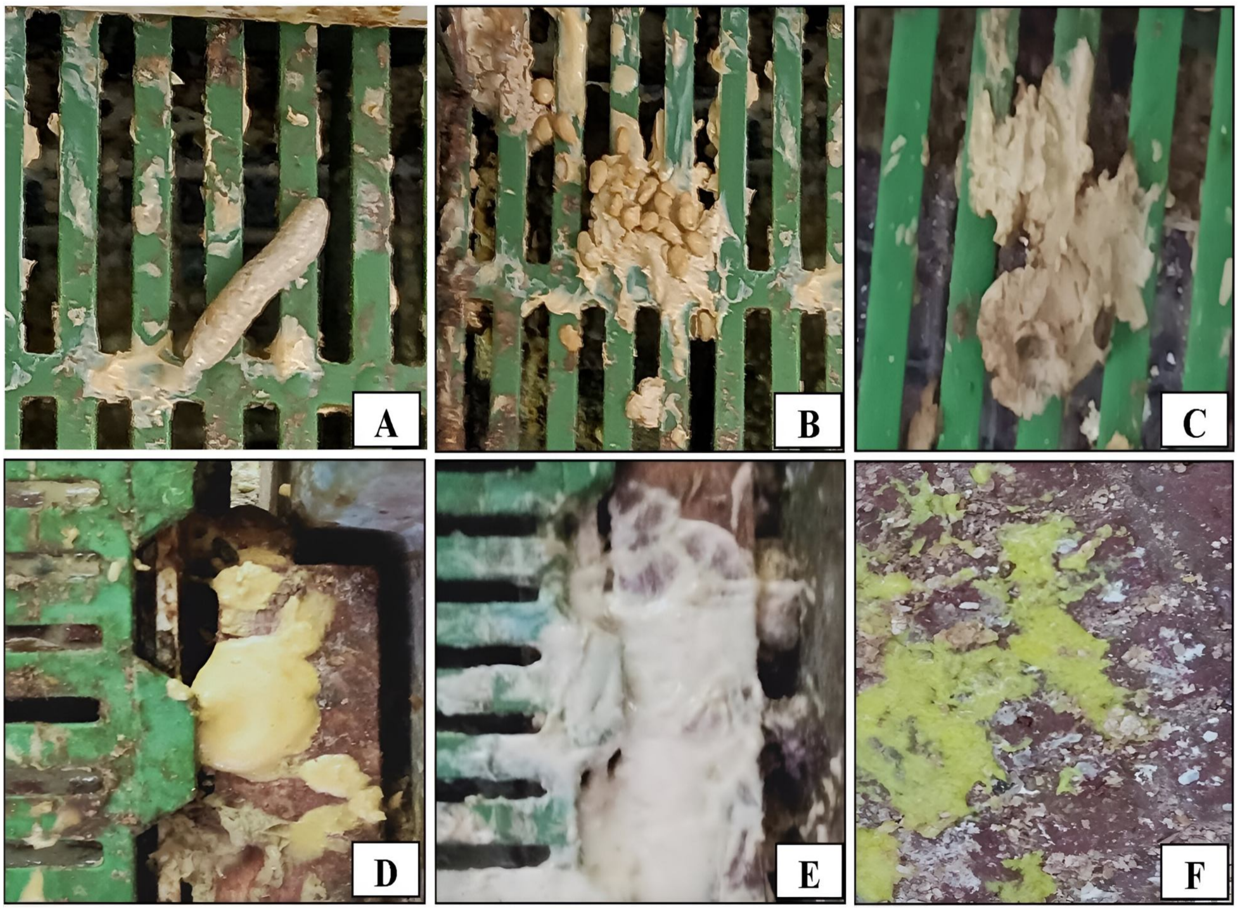


[A. Dry, hard, well-formed faeces; B. Soft but formed faeces; C. Pasty faeces, green or brown; D. Viscus faeces in light colour, episodic; E. Fluid faeces in light colour; F. Watery faeces, continuous]

Fig. 1. Grading of faecal consistency

Biochemical Analyzer (Automated Fuji Drichem 4000 Biochemistry Analyzer, Japan) using commercially available test kits following the manufacturer's instruction.

Evaluation of oxidant-antioxidant indices: Oxidative and antioxidative status was evaluated in serum by measuring lipid peroxidation (LPO) superoxide dismutase (SOD), glutathione (GSH) and total antioxidant assay (TAOA), carried out by commercially available test kits as per manufacturer instruction (Cayman Chemical, USA).

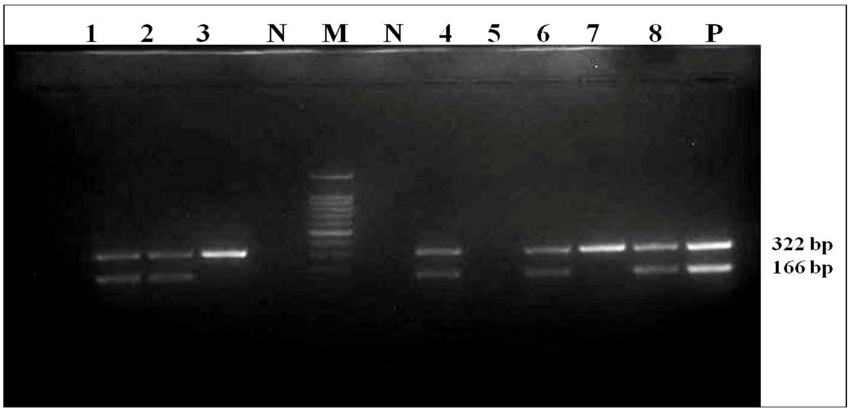
Serum zinc measurement: Zinc concentrations in blood were estimated by atomic absorption spectrophotometer (AA 7000, Shimadzu, USA) at Mizoram University after acid digestion of the samples. Briefly, 5 mL blood sample and 5 mL concentrated nitric acid were mixed properly and kept overnight at room temperature. The next day, the mixture was wet-digested on low heat (below 90°C) using a micro digestion bench after mixing with a double acid mixture (four parts conc. nitric acid and one part 70% perchloric acid) (Kolmer *et al.*, 1951). The concentration of zinc in digested samples was estimated by recording the absorbance of digested samples at specific wave lengths of 324.7 nm and an operating current of 5 mA using acetylene and air mixture as fuel and oxidant. The analytical quality was maintained by running a reagent blank and standard with each batch.

Statistical analysis

All the data were analyzed statistically as per the method outlined by Snedecor and Cochran (1995) using two-way ANOVA by Statistical Package SPSS 20.

RESULTS

Out of 51 positive samples, 72.55% (37/51) cases were diagnosed as ETEC by faecal cultural, biochemical test and molecular techniques. Out of 37 ETEC-positive cases, 35.14% (13/37) cases were diagnosed as ETEC heat-stable toxin (ST), 24.32% (9/37) were ETEC heat-labile toxin (LT) and 40.54% (15/37) cases were confirmed as ETEC (LT+ST) by multiplex PCR (Fig. 2).



[Lane M: 100-bp ladder; Lane P: *E. coli* LT/ST positive; Lane N: Negative; Lane 1 2, 4, 6, 8: positive for LT and ST; Lane 3, 7: positive for LT; Lane 5: negative for both]

Fig. 2. Simultaneous identification of *E. coli*-specific LT and ST enterotoxin-specific genes in bacterial strains using a multiplex PCR system

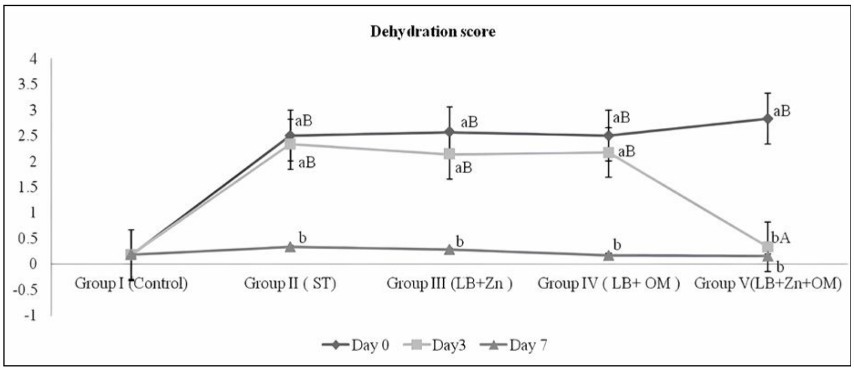


Fig. 3. Therapeutic efficacy of *Lactobacillus bulgaricus*, zinc glycinate, and oyster mushrooms on dehydration score of diarrhoeic pigs in comparison with healthy control group

Therapeutic efficacy of *Lactobacillus bulgaricus*, zinc glycinate, and oyster mushrooms on dehydration score of diarrhoeic pigs: During the study period, it was observed that the dehydration score of all the treated groups was significantly ($p<0.01$) increased as compared to the control group at 0 day, but there was no significant difference among the treatment groups (Fig. 3). The dehydration score at days 7 post-therapy of diarrhoeic pigs improved significantly ($p<0.01$) in all the treatment groups, and there was no significant difference with the healthy control group at day 7 post-therapy.

The critical analysis revealed that the Gr. V animals performed better than all other treatment groups. The value of the dehydration score of Gr. V was almost similar to the healthy control group.

Therapeutic efficacy of *Lactobacillus bulgaricus*, zinc glycinate, and oyster mushrooms on faecal consistency of diarrhoea in pigs: Therapeutic efficacies of *Lactobacillus bulgaricus*, zinc glycinate, and oyster mushrooms on faecal consistency are depicted in Fig. 4. The clinical trial revealed that the faecal consistency of Gr. II, Gr. III, Gr. IV and Gr. V were significantly (p<0.01) raised as compared to Gr. I, but there was no significant difference between the treatment groups in 0 days. On day 7 post-therapy, it was observed that the faecal consistency in all the treatment groups returned to normalcy, which was comparable to that of the healthy control group.

The critical analysis revealed that the faecal consistency in Gr. V showed normal consistency on 3rd day post-therapy onwards. But among the four groups, Gr. V showed better results (Fig. 4.).

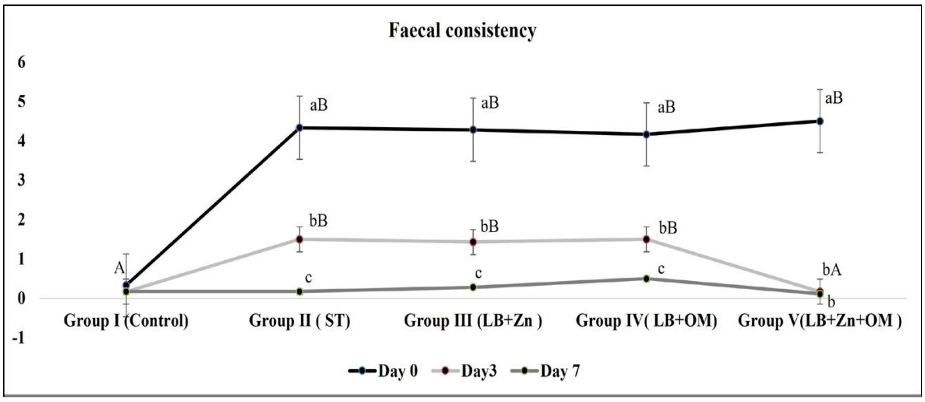


Fig. 4. Alteration of faecal consistency on 0 days, 3rd and 7th days in diarrhoeic pigs treated with *Lactobacillus bulgaricus*, zinc glycinate, and oyster mushrooms as an alternate therapy with antibiotic (Mean ± SE)

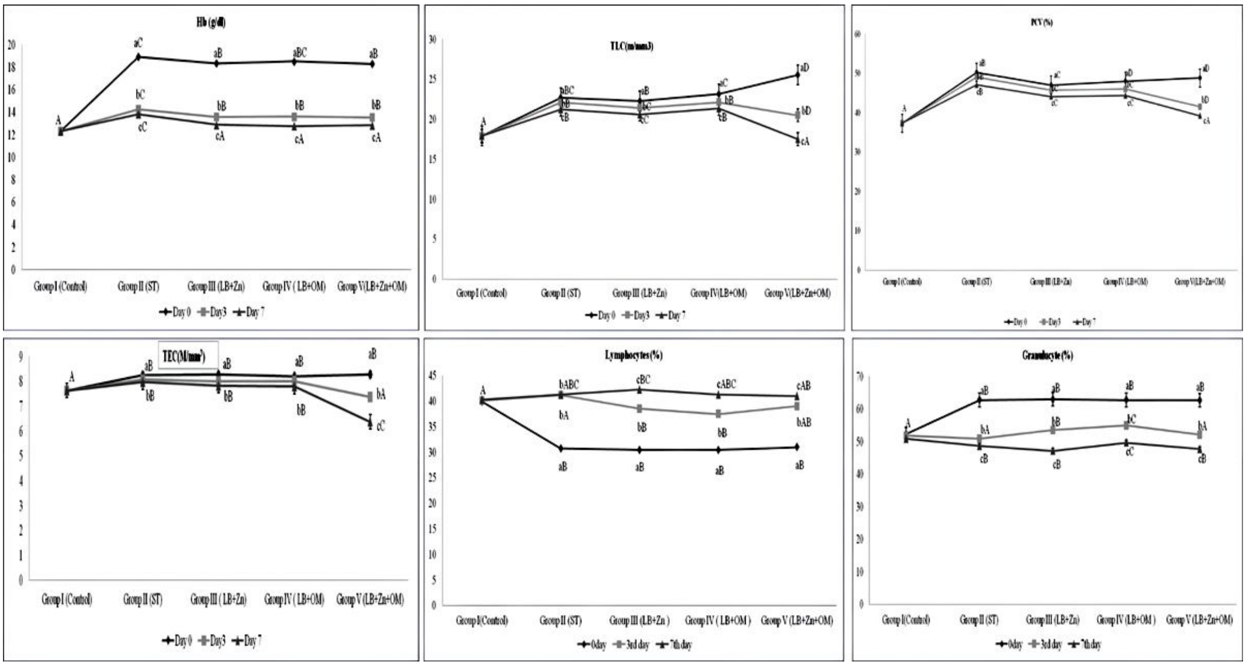


Fig. 5. Therapeutic efficacies *Lactobacillus bulgaricus*, zinc glycinate, and oyster mushrooms as an alternate therapy to antibiotic on haematological parameters of diarrhoeic pigs in comparison with healthy control group

Therapeutic efficacies *Lactobacillus bulgaricus*, zinc glycinate, and oyster mushrooms on haematological parameters of diarrhoeic pigs: The haemoglobin (Hb), TEC, PCV and TLC values of all the treatment groups increased significantly (p<0.01) as compared to the healthy control group before treatment (Fig. 5). But the level of all treatment groups became normal range on 7th post-treatment day.

The granulocyte values of all the treatment groups were significantly (p<0.01) increased as compared to the healthy control group on day 0 (Fig. 5). After

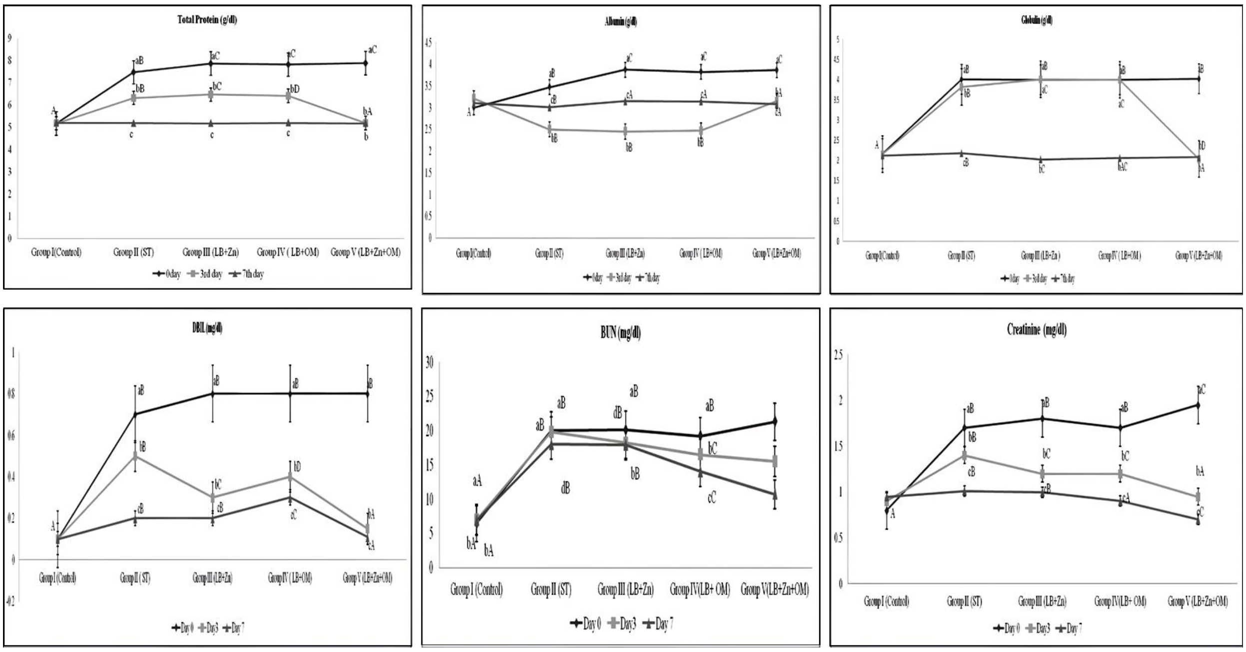


Fig. 6. Therapeutic efficacies *Lactobacillus bulgaricus*, zinc glycinate, and oyster mushrooms as an alternate therapy to antibiotic on biochemical parameters of diarrhoeic pigs in comparison with healthy control group

therapy, the values were decreased in the entire treated group. On the other hand, the lymphocyte values of all the treatment groups were significantly ($p<0.01$) decreased as compared to the healthy control group on day 0 (Fig. 5). After therapy, the values were significantly ($p<0.01$) increased in the entire treated group on 3rd-day post-therapy onwards and the values were no significant difference with the healthy group.

Therapeutic efficacies *Lactobacillus bulgaricus*, zinc glycinate, and oyster mushrooms on biochemical parameters of diarrhoeic pigs: The total protein, albumin, globulin, direct bilirubin, BUN and creatinine were significantly ($p<0.01$) increased on 0 days as compared to the healthy control group (Fig. 6). After treatment of all diarrhoeic pig groups, the value was turned to normal as compared to the healthy control group on 7th-day post therapy.

Therapeutic efficacies *Lactobacillus bulgaricus*, zinc glycinate, and oyster mushrooms on electrolyte concentration of diarrhoeic pigs: The Na, and Cl concentration was significantly ($p<0.01$) decreased in all treatment groups on 0 days as compared to the healthy control Gr. I and the concentration were significantly ($p<0.01$) increased on the 7th day of treatment and turned to normalcy. But the value of Gr.V showed better results than the other treatment groups on the 3rd day onwards.

On the other hand, the potassium values of all the treated groups were significantly ($p<0.01$) increased before treatment as compared to the healthy control group. The values were significantly ($p<0.01$) decreased in all the treated groups after day 7 post-therapy and Gr. V showed better results as compared to other treated groups and the value was turned to normalcy on day 3rd post-therapy onwards (Fig. 7).

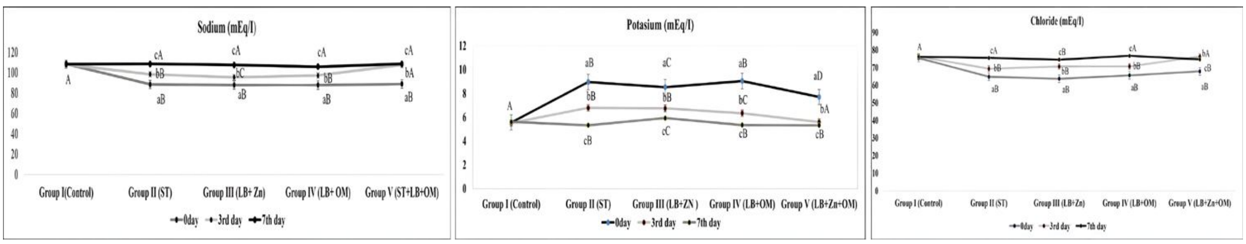


Fig. 7. Therapeutic efficacies *Lactobacillus bulgaricus*, zinc glycinate, and oyster mushrooms as an alternate therapy with antibiotic on electrolyte concentration of diarrhoeic pigs on 0 days, 3rd and 7th day in comparison with healthy control group

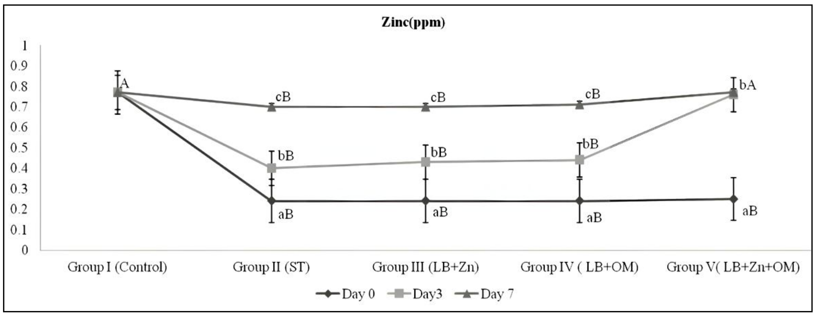


Fig. 8. Therapeutic efficacies *Lactobacillus bulgaricus*, zinc glycinate, and oyster mushrooms as an alternate therapy to antibiotic on zinc concentration of diarrhoeic pigs on 0 days, 3rd and 7th day in comparison with healthy control group

Therapeutic efficacy *Lactobacillus bulgaricus*, zinc glycinate, and oyster mushrooms on zinc concentration of diarrhoeic pigs: The zinc concentration of all 4 treatment groups significantly ($p<0.01$) decreased on day 0 as compared to the healthy control group (Fig. 8). After continuous treatment, the values were significantly ($p<0.01$) improved from day 3rd post-therapy onwards and turned to normal concentration on day 7th post-therapy which was non-significant with healthy control Gr. I. The critical analysis revealed that the value of zinc in Gr. V significantly ($p<0.01$) improved on day 3rd onwards as compared to other treated groups, and the level was similar to the healthy control group.

Therapeutic efficacies *Lactobacillus bulgaricus*, zinc glycinate, and oyster mushrooms on oxidant and antioxidant concentration of diarrhoeic pigs: The LPO level in Gr. II, Gr. III, Gr. IV and Gr. V was significantly ($p<0.01$) increased on day 0 as compared to Gr. I. After treatment, the values were significantly ($p<0.01$) decreased on day 7 in all the treated groups and there was no significant difference between all treatment groups with healthy control Gr. I. The critical analysis revealed that the LPO value of Gr. V was significantly ($p<0.01$) decreased on day 3rd post-therapy and turned to normal, which was not significant with healthy control group I (Fig. 9).

The GSH, SOD and TAOA levels in all the treated groups were significantly ($p<0.01$) decreased on day 0 as compared to Gr. I (Fig. 9). After treatment, the values were significantly ($p<0.01$) increased on day 7 in all the treated groups and there was no significant difference between all treatment groups with healthy control Gr. I. The critical analysis revealed that the values of Gr. V significantly ($p<0.01$) increased on day 3rd post-therapy and turned to normal, which was not significant with healthy control Gr. I (Fig. 9).

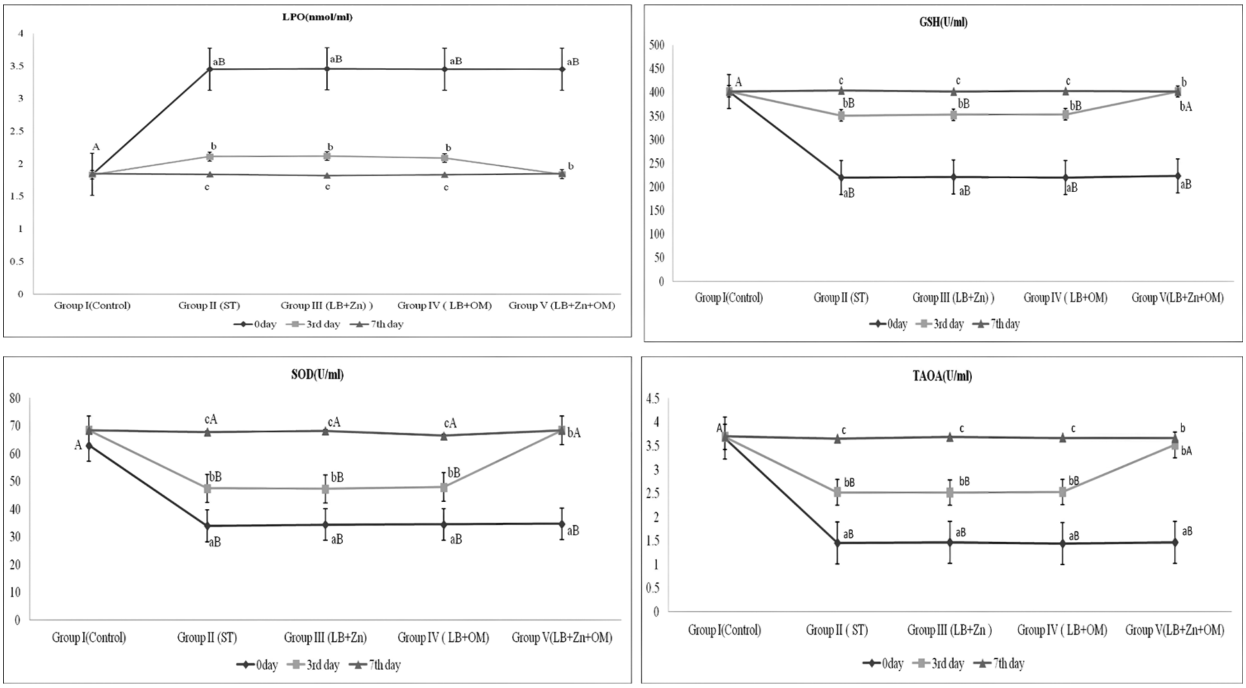


Fig. 9. Therapeutic efficacies *Lactobacillus bulgaricus*, zinc glycinate, and oyster mushrooms as an alternate therapy to antibiotic on oxidant and antioxidant concentration of diarrhoeic pigs

DISCUSSION

The use of antibiotics to treat bacterial and parasitic infections may not be beneficial, especially over long-term medication (Rhouma *et al*., 2017). Another problem is the multi-drug resistance capacity that is often developed by specific microbial strains and hampers treatment (Mathew *et al*., 1999). Finally, the animal gut flora has been severely affected by using antibiotics, which urged us to develop new remedies to treat piglet infections following epidemic diarrhoea outbreaks. Keeping this point of view, an alternative remedy was tried to find against the piglet diarrhoea. To achieve this, a combined formulation of *Lactobacillus bulgaricus*, zinc glycinate and oyster mushroom was used against diarrhoeic pigs. As per the available literature, this is the first study to determine the therapeutic efficacy of *Lactobacillus bulgaricus*, zinc glycinate and oyster mushroom against diarrhoea in pigs caused by enterotoxigenic *E. coli*. Before treatment, it was observed that dehydration score, faecal consistency, and haemato-biochemical parameters particularly total protein, albumin, globulin, direct bilirubin, BUN and creatinine were significantly ($p<0.01$) increased in the entire diarrhoic pigs as compared to the healthy group. The Na and Cl concentration was significantly ($p<0.01$) decreased, whereas potassium (K) was significantly increased in diarrhoic pigs before treatment. Duncan (1994) also reported that decreased ECF water in diarrhoea results in loss of Na+-rich fluids and leads to hypo-natraemia. The present finding was also in agreement with earlier reports. The values of serum chloride (Cl) increased from day 0 to day 7, which might be due to prolonged or increased loss of chloride ions in the intestinal tract during diarrhoea, failure of gastric H+ and Cl- ions to be reabsorbed by villous of the small intestine. The hyperkalaemia observed in all the diarrhoeic pigs agreed with the findings of Sridhar *et al*. (1988). The clinical symptoms in all treated groups improved following treatment on day 7. All of the sick animals were more attentive and had a better appetite. After treatment, all the haemato-biochemicals were restored to normal.

Many studies have initiated the assessment of the potential role of reactive oxygen species (ROS) in the pathogenesis of various infections in recent years. They often indicate that infections caused by various pathogens are associated with a significant increase in lipid peroxidation (Crnogaj *et al*., 2010). Malondialdehyde is one of the end products of lipid peroxidation and is excreted in the urine, blood, and other body fluids, so it serves as a marker of lipid peroxidation and the presence of oxidative stress (Marks *et al*., 1996). The manifestation of oxidative stress occurs due to either inadequate availability of antioxidant enzymes or uncontrolled production of free radicals in the body. LPO is a significant phenomenon in free radical-induced tissue damage and has been implicated in many pathological conditions. In the present study, the level of LPO was significantly ($P<0.01$) increased in diarrhoeic pigs as compared to the healthy group. But superoxide dismutase (SOD) value and total antioxidant assay (TAOA) were significantly ($P<0.01$) decreased in diarrhoeic pigs as compared to healthy. The production of arachidonic acid metabolites in the lipid peroxidation process can contribute to intestinal dysfunction including diarrhoea. Ranjan *et al*. (2006) stated that the enhanced production of free radicals was evident from the increased level of lipid peroxide in the erythrocytes of diarrhoeic calves. Analysis of oxidative stress parameters showed that there were significant decreases in the activity of SOD in diarrhoeic calves when compared with apparently healthy calves (Sharma *et al*., 2011). It is also stated that there was a significant ($P<0.01$) decrease in the activity of Zn in diarrhoea-infected piglets in the healthy group. Decreased SOD level is also responsible for decreased Zn concentration. The lower blood zinc level in diarrhoeic calves could be attributed to poor absorption and loss of zinc in the gastrointestinal tract during diarrhoea, to increased demand for zinc by the immune system and utilization and restoration of zinc at the tissue level for the synthesis of antioxidant enzymes as a compensatory mechanism to counter excessive free-radical production (Ranjan *et al*., 2006). After treatment, all the haemato-biochemical and oxidant-antioxidant alterations were restored to normal.

The critical analysis revealed that combined therapy of *Lactobacillus bulgaricus*, zinc glycinate and oyster mushrooms produced a better clinicopathological outcome in the treatment of pigs with diarrhoea than the other treated groups. The use of antibiotics in piglets with diarrhoea is debatable. Over the last 15 years, the usage of probiotics in farm animals has skyrocketed (Chaucheyras-Durand and Durand, 2010). The greatest significant impacts of probiotics have been found when they were included in the diets of animals during times when the gut microbiota was severely stressed (Chaucheyras-Durand and Durand, 2010). Probiotics help to establish a microenvironment in the gut that favours beneficial microorganisms and reduces the colonization of pathogenic bacteria by (1) creating a hostile environment for harmful bacteria species (through the production of lactic acid, SCFA, and reduction in pH); (2) competing for nutrients with undesired bacteria; (3) production and secretion of antibacterial substances (e.g. bacteriocins by *Lactobacillus*, *Bacillus* spp.); and (4) inhibition of bacterial adherence and translocation (Brown, 2011). Probiotics may help animals by facilitating the bacterial population of the stomach through competitive exclusion or direct effects of harmful pathogenic species, as well as reducing the length of diarrhoea. According to Tanabe *et al*. (2014), probiotic supplements reduced the incidence of diarrhoea. They found that when *Lactobacilli*-based probiotics were given, the incidence of diarrhoea was reduced from 75.9% (control) to 30.7% (probiotic). Silva *et al*. (1987) reported that *Lactobacillus* was able to produce an unknown antimicrobial substance against *E. coli.* Thus, the adhesion of *E. coli* and other aerobic bacteria to gastrointestinal mucosa may have been inhibited by the complex *Lactobacilli* preparation.

Zinc supplementation promotes water and electrolyte absorption, improves intestinal epithelial regeneration, increases brush border enzyme levels, and boosts the immunological response, allowing infections to be cleared more quickly (Bajait and Thawan, 2011). Zinc is shown to have important roles in maintaining the gut epithelial barrier integrity and function (Vallee and Falchuk, 1993). Recent research has demonstrated that zinc is an essential factor in minimizing the morbidity and mortality associated with acute and chronic diarrhoeal diseases (Walker and Black, 2010). Jang *et al*. (2023) reported that zinc glycinate (ZnGly) could be an alternative to the pharmaceutical use of antibiotics without negatively affecting the growth of nursery pigs by enhancing intestinal Zn absorption, reducing intestinal inflammation and oxidative stress, and improving the jejunal mucosa-associated microbiota activity. They found that supplementation of ZnGly at a range of 400 to 675 mg/kg reduced the negative impacts of F18+ *E. coli* by decreasing intestinal inflammation and oxidative stress with improving jejunal microbiota activity, leading to improved growth performance and reduced diarrhoea.

Oyster mushrooms (OM) are commonly utilized as a critical component in traditional Chinese medicine and as a diet by the majority of Asians (Kawagishi *et al*., 2000). These mushrooms are high in carbohydrates, minerals, proteins, and vitamins while being low in fat (Manzi *et al*., 1999). Alkaloids, lactones, polyphenols, sesquiterpenes, sterols, terpenoids, glucans, and glycoproteins are all secondary metabolites found in mushrooms. OM's bioactive compounds, such as fibre, glucans, phenols, and other active substances, may effectively reduce the population and activity of enteric bacteria (Coliform *E. coli*, etc.) in piglets' intestines, reducing diarrhoea incidence (Adams *et al*., 2019). The addition of oyster mushrooms to piglet diets was also discovered to have beneficial effects by researchers at Jilin Agricultural University in Changchun, China. They discovered that adding mushrooms to the feed at 5 g/kg led to a 51% reduction in diarrhoea compared to the control group, and 15 g/kg resulted in a 55% reduction in diarrhoea.

In this study, administration of *Lactobacillus bulgaricus*, zinc glycinate and oyster mushroom showed promise in becoming a complementary treatment option for enterotoxigenic *E. coli* diarrhoea in the swine population. It was also shown that *Lactobacillus bulgaricus,* zinc glycinate and oyster mushroom combination also helped to improve the immunity of piglets in terms of oxidative stress. Randomized controlled trials that incorporate *Lactobacillus bulgaricus*, zinc glycinate and oyster mushrooms in the treatment of diarrhoeal diseases may potentially lead to improved utilization of the traditional and virtually universally applied antimicrobial chemotherapy.

Conflict of interest: Authors have no conflict of interest in this study.

Author's contribution: DN, KS, PR, GEC, GK, JBR, SKB, HP, CD: Conception and design; TCT: Performed statistical analysis of data.

Data availability statement: This research paper is based on M.V.Sc. Programme. All data are available with the corresponding author. Whenever require, it will be ready to submit.

Ethical statement: The study protocol was approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (No. 1476/GO/Re/SL/11/CPCSEA).

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