Bacillus Coagulans SKB LAB-19: A Potential Probiotic in Humans and Animal Healthcare

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Research Article

Keywords: Bacillus coagulans SKB LAB-19, animal feed, enzyme activity, castor oil, E. coli, anti-diarrhoeal

Posted Date: June 28th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-640678/v1

Abstract

Probiotics are live microorganism which when administered in adequate amounts confer health benefits to the host. Different probiotic strains are being used in animal feeds. The main goal of current study was to screen the isolate for potential probiotic characteristics *viz.* different enzyme production, antimicrobial properties, pH/bile salt tolerance, temperature stability, antidiarrheal activity against *E. coli* and castor oil induced diarrhoea in non-ruminant's Swiss albino mice and Wistar rats; and acute oral toxicity in mice using OECD guidelines. The results showed that the isolate SKB LAB-19 produces 8 potential enzymes, effective against *E. coli* and *C. perfringensis*, tolerant to bile salt and gastric pH, stable at 40-90^{o}C and shows no cytotoxicity. In *in vivo* studies, SKB LAB19 was found to be safe and displayed promising results to reverse *E. coli* and castor oil induced diarrhoea in Albino mice and Wistar rats, respectively. An attempt was made to explain mechanism of action and immunomodulatory effects of *Bacillus coagulans* SKB LAB-19 on reversal of diarrhoeal symptoms, haematological parameters, concentration of immunoglobulins in blood and increases the weight of spleen and thymus. Histopathological pictures showed repair to damaged mucosal epithelium cells and also improves integrity of the goblet cells of colon. Thus, *Bacillus coagulans* SKB LAB-19 could be an effective probiotic alternative to standard anti-diarrhoeal drugs in humans and animals.

Highlights

- Lactic acid producing soil isolate was identified as *Bacillus coagulans* SKB LAB-19
- *B. coagulans* was found to produce nine different potential enzymes
- *B. coagulans* showed promising antimicrobial/antidiarrheal/antioxidant activities
- *B. coagulans* showed tolerance to bile salts, gastric pH and temperature (40-90°C)
- *B. coagulans* can be used as potential probiotic strain in animal and human being

Introduction

As per FAO (Food and Agriculture Organisation of the United Nation) and WHO (World Health Organisation), probiotics are defined as healthy living microorganisms, which when administered in adequate amounts confer a health benefit on the host [1]. There has been growing interest in probiotics over last two decades results in more than 6000 publications in biomedical literatures, with more than 60% research papers published in last 5 years [2, 3]. Lactic acid bacteria and *Bifidobacteria* are most commonly used as probiotics, although yeast strains and other bacteria are also used [4]. The beneficial effects of the probiotics are specific to strain and success/failure of one probiotic strain cannot be correlated with another strain. Therefore, identification and characterization of the probiotic strain using novel techniques are necessary [5].

As per guidelines suggested by ICMR DBT for evaluation of probiotic in food [6]; A probiotic strain to qualify as safe; needs to pass following criteria's *viz*. A) Genus, species and strain identification, B) *in*

vitro tests such as tolerance to gastric acid and bile acid, antimicrobial activity against pathogenic bacteria, capability to inhibit the adhesion of pathogen to surfaces, and possesses bile salt hydrolase activity [7]. C) *in vivo* safety studies in animal models: evaluation of the acute/sub-acute/chronic toxicity of probiotic strains. D) *In vivo* efficiency studies in animal models: appropriate validated animal models must be used prior to human trials.

Present study attempted to evaluate an indigenous strain of *Bacillus coagulans* SKB LAB-19 as a potential probiotic for human and animal feed as a direct fed microbial. SKB LAB-19 is a gram positive, spore forming soil isolate and was identified as *Bacillus coagulans* using 16s-rDNA technique. It has unique ability to produce high concentration of L-lactic acid [8]. *Bacillus coagulans* in combination with other microbial strains has been reported for treatment antibiotic-associated diarrhoea [9], bacterial vaginosis (*B. coagulans* ATCC PTA-11748) [10] and immunological support (*B. coagulans* GandenBC30) [11]. The current study demonstrates *in vivo* and *in vitro* properties of *Bacillus coagulans* SKB LAB-19 for it to qualify as an essential probiotic.

Materials And Methods

Materials

Bacillus coagulans SKB LAB-19 strain was obtained from S K Biobiz Pvt. Ltd. Nasik, Maharashtra, India, Loperamide (standard reference anti-diarrhoeal drug) was purchased from Johnson and Johnson Pvt. Ltd, Mumbai. Castor oil (laxative agent) was purchased from Vishal Chemicals, Mumbai. *E. coli* (ATCC No.8739) was procured from NCIM, Pune, Maharashtra. *Clostridium perfringens* (MTCC 450) was procured from MTCC, Chandigarh.

Chemicals *viz.* potassium dihydrogen orthophosphate, dipotassium hydrogen ortho phosphate, hydrogen peroxide, soluble starch, dextrose, ammonium sulphate, magnesium sulphate, calcium chloride, manganese sulphate, ferrous sulphate, potassium chloride, sodium taurocholate and oxy-taurocholate, PBS were procured from Himedia Laboratories Mumbai, India. Ascorbic acid and DPPH (2, 2-Diphenyl-1-picrylhydrazyl) were purchased from Sigma Aldrich, India.

Media components like GYEA, GYEB, Nutrient agar, Nutrient broth, Tryptone, Yeast extract, Pikovskaya agar medium, Sheep Blood agar, Mueller-Hinton Agar, Tryptic soya agar, Antibiotic assay discs were procured from Himedia Laboratories Mumbai, India. Substrates for enzyme analysis like Tributyrin, Sodium phytate, Uric acid, Sodium Carboxymethyl Cellulose were procured from Sigma, USA.

Experimental animals**:** 36 Swiss albino mice (weight 20-25 g) and 30 Wistar rats (weight 180-200 g) were procured from LACSMI Biofarm Pvt. Ltd. Pune, India. The mice and rats were kept at temperature of 25 $\pm 2^{o}$C and RH of 50-60% under 12 hrs light and dark cycle. The mice and rats were allowed to adapt for 15 days before study. The protocol (MGVPC/CPCSEA/XXXVI/01/2019/01) was approved by Institutional Animal Ethics (IAE) committee.

Methods

Isolation and identification of the lactic acid producing bacteria

Soil samples were collected from of the industrial area of the S K Biobiz Pvt Ltd. Nashik, India. Soil samples were collected from the top layer of the soil and transferred aseptically into sterile bag. Soil sample (1 g) was inoculated aseptically into glucose yeast extract (GYE) broth and incubated in an incubator shaker (160 rpm, 37°C for 48 h). The 48-h grown culture was streaked on glucose yeast extract agar (GYEA) and incubated in an incubator (37°C for 24 h). Based on morphology and growth characteristics, five different types of colonies were isolated. These colonies were picked up individually and inoculated into GYE broth under aseptic condition and further incubated in an incubator shaker (160 rpm, 37 °C and 24 h). The isolated colonies were further purified on GYE agar plates.

Morphological, physiological and biochemical characteristics of the soil isolate

SKB LAB-19 isolate was assessed for its shape, motility, and purity using a light microscope (Olympus CX31). The morphology and growth characteristic of the isolated colonies were assessed on GYE agar plate. The biochemical tests and Gram staining characteristics were carried out by using KB020 HiLacto Identification Kit (HiMedia, India).

Partial gene sequencing by 16S rRNA and phylogenetic analysis

SKB LAB-19 isolate was identified by 16S rRNA partial gene sequencing. The extraction and isolation of DNA from the SKB LAB-19 isolate was carried out as per the reported protocol [12]. The extracted DNA was analysed by agarose gel (1.2%) electrophoresis, results showed the presence of single band of high molecular weight DNA. The resultant 1525 nucleotide bases were compared with GenBank database using the BLAST server at NCBI. Neighbour-joining method was adopted for construction of phylogenetic tree, while Molecular Evolutionary Genetic Analysis (MEGA) phylogenetic software was used for genetic analysis. The nucleotide sequences were submitted to GenBank databases under Accession No. MW750514.

In vitro determination of enzyme activity

SKB LAB-19 isolate was screened for production of different enzymes *viz.* catalase, *α*-amylase, lipase, protease, phytase, uricase, phosphatase, cellulase and nitrate reductase. SKB LAB-19 was grown in GYE broth (HiMedia M963) at 37°C, 160 RPM for 24 h in a shaking incubator (Remi CIS-24 PLUS, India). The 24 h old culture broth was subjected for centrifugation (2000 × *g* for 15 min), the cells so obtained were washed thrice with a sterile saline solution (0.9%w/v) to remove the residual media components. Then, different dilutions (10^{-1} to 10^{-10}) of the inoculums was placed on GYEA (HiMedia 963) and incubated in an incubator (37°C for 48 h) to determine the concentration of viable cells (cfu/mL). For evaluation of enzyme activity, 20 µl (10^{8}cfu/mL) suspension of SKB LAB-19 was placed at the centre of well dig in

each selective media plate. After incubation, zones of clearance were measured in millimetres (mm). Relative enzyme activity (REA) was evaluated by using the following formula,

$$REA = Diameter\ of\ zone\ of\ clearance\ (mm) / Diameter\ of\ well\ (mm)$$

All the *in-vitro* enzyme activity determination experiments were evaluated in triplicate and values of relative enzyme activities were represented in Table 2.

Production of catalase

SKB LAB-19 culture was grown on GYEA plates for 48 h at 37^{O}C and then flooded with 3 mL of 10% v/v hydrogen peroxide solution. Production of gas bubbles on the colonies indicates production catalase enzyme by the culture [13].

Production of amylase

Amylase activity was determined using starch agar media composed of (g/L) of tryptone 10, starch soluble 3, KH_2PO_4 5, yeast extract 10, and agar 15. For evaluation of amylase activity, 20 µL of 48 h old culture of SKB LB-19 (grown in GYE broth) was inoculated in 8 mm well in starch agar plates and were incubated in an incubator (37°C for 48 h). For observation of zone of clearance, the plates were flooded with Gram's iodine solution (5 mL) [14].

Production of lipase

The production of lipase was evaluated on tributyrin agar composed (g/L) of tributyrin- 10 and nutrient agar-13 with pH-7. Twenty µL of 48 h old culture of SKB LB-19 (grown in GYE broth) was inoculated in 8 mm well on tributyrin agar plates. These plates were subjected for incubation in an incubator (37°C for 24-48 h). The zone of clearance around the halo indicates the lipase production [14, 15, 16].

Production of protease

Protease activity was evaluated using skim milk agar (SMA) containing (g/L) of skim milk-25, bacteriological agar-20. Twenty µL of 48 h old culture of SKB LAB-19 (grown in GYE broth) was inoculated in 8 mm well in SMA agar plates. These plates were subjected for incubation in an incubator (37°C for 24 h). The zone of clearance was observed around the halo [17].

Production of phytase

For determination of phytase activity *SKB LAB-19* was inoculated in phytate agar medium containing (g/L) of dextrose-10, ammonium sulphate-0.3, magnesium sulfate-0.5, calcium chloride-0.1, manganese sulfate-0.01, ferrous sulphate-0.01, sodium phytate- 5, and bacteriological agar-15. Twenty µL of 48 h old culture of SKB LAB-19 (grown in GYE broth) was inoculated in 8 mm well in phytate agar plates. These

plates were subjected for incubation in an incubator (37°C for 120 h) and zone of clearance was observed after incubation [18].

Production of uricase

Uricase activity was determined using uric acid agar composed of (g/L) of nutrient agar 10, uric acid 10, bacteriological agar 10 with pH 6.8. Twenty µL of 48 h old culture of SKB LAB-19 in GYE broth was inoculated in 8mm diameter wells cut using a well cutter and the plates were subjected for incubation in an incubator (37°C for 24-48 h), and the diameter of the zone of clearance was determined [19].

Phosphate solubilization assay

Phosphate solubilization assay was performed using Pikovskaya agar. Twenty µL of SKB LAB-19 (grown in GYE broth) was inoculated in 8 mm well in agar plates and these plates were subjected for incubation in an incubator (37°C for 7 days). The zone of clearance around the colonies was observed after 5 days of incubation [20].

Screening for cellulase producers

Cellulase activity was evaluated using carboxymethyl cellulose agar containing (g/L) of yeast extract 0.5, potassium dihydrogen orthophosphate 1, sodium carboxymethyl cellulose 1.2, magnesium sulphate 0.5, sodium nitrate 0.3, potassium chloride 0.5, bacteriological agar 15, and supplemented with marine salts 15 [21]. Twenty µL of 48 h old culture (grown in GYE broth) was inoculated in 8 mm well in cellulose agar plates and then plates were incubated for 5-10 days (37°C) in an incubator. For determination of cellulase activity, the plates were flooded with 0.01% w/v Congo red dye for 15 minutes, and then plates were washed with 1M NaCl solution to observe cellulase activity. The cellulase activity was indicated by presence of an orange zone surrounding the colony against a red media background [22].

Acid and bile salt tolerance

For acid tolerance assay, 1 mL of SKB LAB-19 culture (10 Billion cfu/mL) was transferred into 9 mL of phosphate buffer saline (PBS) with pH 2.5 (adjusted with 5 M HCl) and then subjected for incubation in an incubator (30°C). The number of viable cells in the PBS was determined after 0, 1, 2 and 3 hrs of incubation periods on a GYE agar plates in triplicates.

For the bile salt tolerance assay, 1 mL of SKB LAB-19 culture (10 Billion cfu/mL) was transferred into 9 mL of GYE broth containing bile salt (0.05 -0.3% w/v), and then subjected for incubation in an incubator (37°C). The number of viable cells was enumerated after 0 and 4 hrs of incubation periods [23].

Hemolysis

The safety assessment of SKB LAB-19 culture towards its use as potential probiotics was confirmed by its non-hemolytic activity on sheep blood agar plate. SKB LAB-19 culture was streaked on sheep blood

agar plates, and then subjected for incubation in an incubator (30°C for 48 h) [24]. The hemolysis assay was performed in duplicate.

Evaluation of antimicrobial activity of probiotic bacteria

The antimicrobial activity of the SKB LAB-19 isolate was performed using Mueller-Hinton Agar (MHA). The inhibitory and antagonistic effects of probiotic bacteria on the *E. coli* and *Clostridium perfringens* were evaluated using well diffusion agar methods [25].

Well diffusion agar method for antimicrobial efficacy against E. coli

Suspension of *E. coli* cultured in tryptic soya broth (TSB) were spread on Mueller-Hinton Agar, then 10 µL of 48 h old SKB LAB-19 culture (grown in GYE broth) was inoculated in 8 mm well in the MHA plates and incubated in an incubator (37°C for 24 h). The growth inhibition zone was observed after incubation [25].

Well diffusion agar method for antimicrobial efficacy against Clostridium perfringens

Clostridium perfringens ATCC 13124 were inoculated into fluid thioglycolate broth and subjected for incubation under anaerobic conditions (37°C). A 12-hr old culture of *C. perfringens* was streaked on the surface of tryptone soya yeast extract agar using a sterile cotton swap. SKB LAB19 culture was grown in GYE broth and subjected for incubation in an incubator (37°C under 5% CO_2). Twenty µL of 48 h old SKB LAB-19 culture was inoculated into the 8 mm wells of tryptone soya yeast extract agar plates previously streaked with *C. perfringens* and incubated in an incubator (37°C under anaerobic conditions) for 18 h. The diameter of growth inhibition zone was determined after incubation.

Effect of temperature on viability SKB LAB-19 spore count

A 100 mL spore suspension of SKB LAB-19 (10 Billion cfu/mL) was prepared in previously sterilized saline solution (0.9% w/v). This spore solution was divided into 10 sterile test tubes each containing 10 mL of suspension and then subjected to heat treatment at 45^{o}C for 60 minutes, 60^{o}C for 45 minutes, 70^{o} C for 30 minutes, 80^{o} C for 20 minutes and 90^{o}C for 10 minutes. The viability of the spores in the treated sample was assessed by serial dilution method. The serial dilution method was performed until the appropriate dilution was attained (approximately 30-100 cfu/mL). The enumeration of spore was carried out by pour plate technique. The plates were subjected for incubation in an incubator (37°C for 48 h). At least six plates were counted and the average count per plate was calculated. The number of cfu per unit (mL or gram) of sample was calculated by employing the following equation [11].

$$Colony forming\ units\ (cfu/mL) = Average\ number\ of\ colonies/Dilution\ factor$$

In-vitro antioxidant activity

DPPH radical scavenging activity

Diphenylpicryl hydrazine (DPPH) radical scavenging activity is an indicator of hydrogen-donating capability. A 0.8 mL of 0.1mM DPPH solution (prepared in 95% methanol) was added to 0.2 mL of different concentrations (5, 10, 20, 50 mg/mL) of SKB LAB-19 suspension and subjected for incubation (30 ± 2 °C for 30 min). After incubation the samples were centrifuged (8385 × *g* for 5 min) and supernatant so obtained was used for measuring the absorbance at 517 nm. The antioxidant activities of the different samples of the SKB LAB-19 were compared with ascorbic acid (50 mg/mL) [26, 27, 28]. The antioxidant activity was determined by following equation,

DPPH scavenging activity (%) = [Absorbance sample / Absorbance control] -1) × 100

Reducing power assay

Different concentrations (5, 10, 20, 50 mg/mL) of SKB LAB-19 (0.2 mL) were added to 0.2 mL of phosphate buffer (0.2 M, pH 6.6) and 1% w/v potassium ferricyanide. The reaction mixture was allowed to incubate at 50 °C for 20 min, after incubation 0.5 mL of trichloroacetic acid (10% w/v) was added. This reaction mixture was subjected for centrifugation (755 × *g*, 10 min). The supernatant was separated and added to deionized water and mixed with ferric chloride (0.1% w/v) at ratio of 1:1:1 (v/v/v) and the absorbance of solution was measured at 700 nm. Reducing power is directly proportional to absorbance of the solution [26, 27, 28]. Reducing power of the different concentrations of SKB LAB-19 was compared with ascorbic acid (50 mg/mL).

Ascorbate auto oxidation inhibition

Different concentrations (5, 10, 20, 50 mg/mL) of SKB LAB-19 (0.2 mL) were added to 0.1 mL of ascorbic acid solution (5 mM) and 9.7 mL of phosphate buffer (0.2M, pH 7) and subjected for incubation (37 °C for 10 min). Absorbance of the solution was measured at 265 nm [26, 27, 28]. Ascorbate auto-oxidation inhibition (%) was calculated as,

Ascorbate auto-oxidation inhibition (%) = [(Absorbance sample /Absorbance control)-1] × 100

In vivo studies

Acute oral toxicity assay

Nulliparous and non-pregnant female albino mice (8-10 weeks age and 25 ± 2 g weight) were randomly selected according to OECD test guidelines 425. The mice were kept under standard laboratory conditions for seven days. The limit test was performed using as single dose (2000 mg/kg p.o.) and mice were fasted for 3-4 h before dosing but allowed to take water *ad libitum*. The mice were monitored for first 30 min, then for 4 h and then food was given 1-2 h of postdosing. The treated mouse was checked for its survival and four additional mice were administered with same dose under same conditions. Vehicle control group (5 mice) were administered with same volume of saline solution as like treated group. Both the groups were observed for any toxic effects initially for 4 h and then for 14 days at regular interval. Body weight and behavioural pattern was observed [29].

Castor oil induced diarrhoea

Swiss albino mice were screened for induction of diarrhoea by administering castor oil (0.5 mL). The mice suffered from diarrhoea were subjected for further studies. Mice were randomly assigned as 5 animals in each group (n = 5). Mice were administered with three different doses of SKB LAB-19 (5, 20 and 50 Billion cfu/kg) for 8 days. On eighth day, Group I were administered with 10 mL/kg, p.o. of sterile water for injection, Group II were administered with 0.5 mL castor oil p.o. for induction of diarrhoea, Group III were administered with 3 mg/kg, p.o. of loperamide, Group IV were administered with SKB LAB-19 (5 Billion cfu/kg, p.o.), Group V were administered with SKB LAB-19 (20 Billion cfu/kg, p.o.), and Group VI were administered with SKB LAB-19 (50 Billion cfu/kg p.o.). After 1h, all mice were administered with castor oil p.o (0.5 mL) and all mice were evalauted for diarrhoeal index. The parameters of evaluation included, onset of diarrhoea (latency), frequency of defecation, total faecal weight and percentage inhibition of defecation [30. 31, 32].

E. coli induced diarrhoea

Wistar rats were screened and standardized for induction of diarrhoea by administering *E. coli* suspension. Rats were administered with three different doses of SKB LAB-19 for 15 days. *E. coli* suspension was administered for 3 subsequent days for induction of diarrhoea on eleventh day. Group I (vehicle control) were administered with sterile water for injection (10 mL/kg p.o.), Group II (diarrhoeal control) were administered with *E. coli* suspension (1 mL, 1 Billion cfu/mL), Group III were administered with SKB LAB-19 (5 Billion cfu/kg, p.o.), Group IV were administered with SKB LAB-19 (20 Billion cfu/kg, p.o.) and Group V were administered with SKB LAB-19 (50 Billion cfu/kg, p.o.) by oral gavage. The parameters monitored were total faecal output, faecal consistency, faecal water content on 12^{th}, 13^{th} and 14^{th} day and all rats were sacrificed on 15^{th} day of its treatment. Blood was collected and analysed for immunoglobulin level and WBC count. Colon tissues were isolated for histopathological investigation whereas spleen and thymus were isolated for evaluation of immune organ index [12, 33, 34].

$$Faecal\ water\ content = Weight\ of\ wet\ stool - Weight\ of\ dry\ stool\ \times 100/Weight\ of\ stool$$

$$Relative\ immune\ organ\ index = \ Weight\ of\ orgam\ (mg)/Body\ weight\ (gm)$$

Haematoxylin and Eosin staining

Colons isolated from the rats were deep in neutral buffered formalin solution (10% v/v) for fixing the tissues. The colon tissues were fixed in paraffin and divided into 3 µm slices on glass slides. The slides were deparaffinized and stained with haematoxylin followed by eosin. The slides were dehydrated in alcohol, cleared in xylene, and covered for microscopic examination. The slides were observed blindly by a pathologist and the colonic damage was evaluated [34].

Statistical analysis

The experimental data were analysed by PRIMER statistical software and expressed as Mean ±SEM. Statistical analysis was performed using one-way ANOVA, followed by Dunnett's test. $^{\#}$P value < 0.05 and $^{\#\#}$P value < 0.01was considered statistically significant.

Results And Discussions

Isolation and identification of the lactic acid producing bacteria

The lactic acid producing bacteria was isolated from the soil. Based on the single colony characteristics, five different cultures were isolated.

Morphological, physiological and biochemical characteristics of lactic acid producing bacteria

The colonies of the isolate SKB LAB-19 were white, smooth, shiny, circular and convex. The isolate was found to be Gram positive, motile, facultative anaerobe, rod shaped, spore forming, catalase positive, methyl red positive and nitrate reductase negative.

16S rRNA gene sequencing and phylogenetic analysis

Based on 16S rRNA gene sequencing, the isolate SKB LAB-19 showed 98.41% similarity with *Bacillus coagulans* (partial sequence, accession number: NR_115727.1, query coverage: 98%, E value: 0.0), 98.72 % similarity to *Bacillus coagulans* (partial sequence, accession number: NR_041523.1, query coverage: 96%, E value: 0.0), 97.11% similarity to *Bacillus coagulans* (partial sequence, accession number: NR_115580.1, query coverage: 92%, E value: 0.0), and 96.55% similarity to *Bacillus coagulans* (partial sequence, accession number: NR_118954.1, query coverage: 92%, E value: 0.0). Based on phylogenetic tree, phenotypic and phylogenetic characteristics of SKB LAB-19 isolate showed closest homology with *Bacillus coagulans* (Supplementary data Fig S1). Therefore, it was identified as *Bacillus coagulans* SKB LAB-19 and considered as a new strain of *Bacillus coagulans.*

In vitro determination of enzyme activity

Animal feed are complex and contain material rich in nutrient though hard to digest. The main constituents of the commercial animal feed are grains such as soybeans, corn, sorghum, oats and barley etc. Supplementation of animal diet with a probiotic enhanced digestibility of dry matter (DM), weight gain, feed intake, average daily gain, and feed conversion ratio (FCR). Moreover, probiotics enhanced apparent ileal digestibility (AID) of the essential amino acids, and increased bioavailability of essential ions like calcium [33].

Spore forming bacteria such as *B. subtilis and B. amyloliquefaciens* secrets extracellular enzymes such as α-amylase, protease, metalloproteases and cellulase etc. [35, 36] enhances nutrient digestion. Supplementation of probiotics improved enzyme activity in the gastrointestinal tract of animals. It is

evident from Fig. 1 and Table 1 that *Bacillus coagulans* SKB LAB-19 is capable of producing eight extracellular enzymes at good concentrations when a suitable substrate is available. The enzyme produced by SKB LAB-19 can increase the digestibility of complex carbohydrates, non-starch polysaccharides, proteins, fats and also provide protections against pathogens, thereby increasing overall health of the animal.

Table 1
Relative enzyme activity (REA) values produced by *Bacillus coagulans* SKB LAB-19.

Sr No.	Enzyme	Ability to produce enzyme	REA
1.	Catalase	Positive	+ve
2.	Amylase	Positive	2.10 ± 0.3
3.	Lipase	Positive	3.50 ± 0.2
4.	Protease	Positive	3.70 ± 0.4
5.	Phytase	Positive	2.50 ± 0.1
6.	Uricase	Positive	2.10 ± 0.3
7.	Phosphatase	Positive	2.30 ± 0.4
8.	Cellulase	Positive	2.20 ± 0.2
9.	Nitrate reductase	Negative	-ve

Acid and bile salt tolerance of SKB LAB-19 isolate

Probiotic survival at lower pH is very crucial for resisting initial stress in the stomach [37]. Table 2 illustrates the survivability of *Bacillus coagulans* SKB LAB-19 strain under simulated conditions of low pH. The results indicate that the studied strain resist a simulated phosphate buffer saline solution (pH 2.5) showed an over 87% survival rate after 3h. Additionally SKB LAB-19 was also found to be stable in presence of 0.05–0.3% bile salt with 95% and 70% retention of activity in presence of 0.05 and 0.3% bile salt, respectively (Table 3). It is evident from the results that spores of SKB LAB-19 qualifies as potential probiotic

Table 2 Viability of *Bacillus coagulans* SKB LAB-19 under simulated acid conditions

Sr No	Incubation time under acidic condition, H	Total viable count (cfu/mL)	Retention of activity (%)
1.	Control (0 h)	10.00×10^9	100
2.	1 h	9.52×10^9	95.27
3.	2 h	9.00×10^9	90.00
4.	3 h	8.76×10^9	87.62

Table 3
Viability of *Bacillus coagulans* SKB LAB-19 in presence of varying concentration of bile salts

Sr. No.	Bile salt concentration (% w/v)	Total viable count (cfu/mL)	Retention of activity (%)
1.	Control	10.00×10^9	100
2.	0.05	9.44×10^9	94.47
3.	0.10	8.62×10^9	86.25
4.	0.15	8.26×10^9	82.62
5.	0.20	7.88×10^9	78.88
6.	0.25	7.35×10^9	73.53
7.	0.30	7.04×10^9	70.41

Haemolysis

Effect of temperature on viability Bacillus coagulans SKB LAB-19 spore count

Spore forming bacteria such as *Bacillus* are used as probiotics in animal feed. Spores of the *Bacillus* are tolerant to heat, UV radiation and desiccation [38, 39] hence preserving their viability during feed pelleting, storage and handling. As illustrated in Table 4, SKB LAB-19 when exposed to variable time and temperature incubation conditions was found to be stable and retained more than 80 % activity at 90^o C

and 10 minutes and more than 90% activity when exposed to 70^{o} C for 30 minutes. These properties of SKB LAB-19 make it a probiotic of choice to be incorporated in animal feed during pelletization.

Table 4
Viability of *Bacillus coagulans* SKB LAB-19 on exposure to conditions of incubation at variable temperature and time

Sr. No	Incubation temperature and time	Total viable count (cfu/mL)	Retention of activity (%)
1.	45^{o}C for 60 minutes	10.00×10^{9}	100
2.	60^{o}C for 45 minutes	9.50×10^{9}	95
3.	70^{o}C for 30 minutes	9.30×10^{9}	93
4.	80^{o}C for 20 minutes	8.90×10^{9}	89
5.	90^{o}C for 10 minutes	8.30×10^{9}	83

Evaluation of antimicrobial activity of SKB LAB-19

Inhibition of growth of food borne pathogen is one of the desirable characteristics of probiotic bacteria. Recently there is lot of development on *Lactobacillus* probiotics in food and feed industry, however there is still an urgent need for commercial Direct Feed Microbials (DFM) that are cost-effective and shelf-stable. In this context, different *Bacillus* spp. has been isolated as an active member from poultry and pigs [40]. Furthermore, endospores of some *Bacillus* spp. have been extensively studied as DFM; results showed that these spores are safe and reliable prophylactic agents to reduce GI diseases in livestock and humans [41, 42].

In current study, SKB LAB-19 showed antimicrobial activity against *E. coli* and *C. perfringens* (Fig. 3). This might be due to the capability of SKB LAB-19 to synthesize antimicrobial metabolites. *Clostridium* is an anaerobic spore forming bacteria and is a major etiological agent of nosocomial diarrhoea in patients under antibiotic therapy. Therefore, *Bacillus coagulans* SKB LAB-19 could be an appropriate alternative as DFM to diminish the occurrence of bacterial GI diseases in humans and animals, including cases of *E. coli* and *Clostridium* infections.

In-vitro antioxidant activity of SKB LAB-19

The radical scavenging capacity of DPPH is commonly used to investigate the antioxidant capacity of probiotics. *Bacillus coagulans* SKB LAB-19 showed significant DPPH scavenging, reducing capacity and ascorbate auto-oxidation inhibition effect. DPPH scavenging and reducing ability increased with an increase in *Bacillus coagulans* SKB LAB-19 concentration (Fig. 4a and 4b). *Bacillus coagulans* SKB LAB-19 (50 mg/mL) showed maximum radical scavenging activity as 82.93% at 517 nm (Fig. 4a). *Bacillus coagulans* SKB LAB-19 exhibited highest reducing ability as demonstrated by an absorbance of 0.166 ±

0.00 for 20 mg/mL and 0.174 ± 0.00 for 50 mg/mL at 700 nm (Fig. 4b). *B Bacillus coagulans* SKB LAB-19 exhibited highest ascorbate auto-oxidation inhibition activity as 86.67% for 20 mg/mL and 94.62 % for 50 mg/mL at 265 nm (Fig. 4c).

Bacillus coagulans SKB LAB-19 showed scavenging/reducing/ascorbate auto-oxidation inhibition activity. The results suggest that, the reducing potential of *Bacillus coagulans* SKB LAB-19 contributes to its significant antioxidant property [43, 44].

In-vivo studies

Safety assessment

Body weight and behavioural pattern

The body weights of both control and SKB LAB-19 treated groups were increased gradually throughout the study period. Jørgensen et al., 2016 reported that, probiotic supplementation increased growth rate by 7.9% in wean to finish pig [45]. The test animals did not show any abnormal behaviour after SKB LAB-19 dosing indicating non-toxic nature of the *Bacillus coagulans* SKB LAB-19. However, the primary results recommended that *Bacillus coagulans* SKB LAB-19 is a safe for animal consumption.

Effect of 8 days treatment of SKB LAB-19 on castor oil induced diarrhoea in mice

Castor oil induced diarrhoea is a well-known and effective model for screening of new anti-inflammatory, antimotility and anti-secretory compounds. Castor oil is degraded to ricinoleic acid (hydroxylated fatty acid) by the action of intestinal lipases in the gut which stimulates peristaltic activity in the small intestine, causes changes in permeability of electrolyte in the intestinal mucosa and thus, increases the volume of intestinal content by preventing the reabsorption of electrolyte and water. Its action induces release of inflammatory mediators such as prostaglandin E and histamine, results in stomach cramp and diarrhoea due to the effect on the smooth muscle and hypersecretory response. Gut microbiota plays very important role in curing gastrointestinal diseases like diarrhoea [46].

New practice for management of diarrhoea by using probiotics is very common. Probiotics containing lactic acid bacteria reported to have various health benefits such as counteract with harmful microorganisms in gut to restore the gut epithelial and immune homeostasis [27, 47, 48]. SKB LAB-19 administered for 8 days with three different doses, significantly ($P < 0.01$) controls the onset of diarrhoea compared with diarrhoeal control group (Fig. 5a). There was significant reduction in frequency of defecation ($P < 0.05$) (Fig. 5b) and weight of stool ($P < 0.01$) (Fig. 5c) in medium and high dose (SKB LAB-19) treated group compared to diarrhoeal control group. The percent inhibition of defecation with three different doses of SKB LAB-19 were found to be 54.55%, 71.29%, 72.25% and was more or less similar to Loperamide treatment group (68.42%) (Fig. 5d). SKB LAB-19 significantly reduces the diarrhoeal index and strongly inhibits diarrhoea in low, medium and high doses.

In case of castor oil induced diarrhoea, castor oil induces the release of prostaglandins which stimulates vasodilation, contraction of smooth muscle and secretion of mucus which results in diarrhoea. SKB LAB-19 prevents release of prostaglandin which in turn delayed onset of diarrhoea, reduced frequency of defecation and faecal output. It is also assumed that antidiarrheal activity of SKB LAB-19 probably involves the nitric oxide (NO) pathway. *Bacillus coagulans* LAB-19 may inhibit the production of NO which leads to inhibition of diarrhoea. From the observed results, it is clearly evident that *Bacillus coagulans* LAB-19 proves to be strong anti-diarrhoeal through the anti-secretory properties.

E. coli (5×10^9cfu/mL) induced diarrhoea: 15 days treatment of SKB LAB-19

E. coli induced diarrhoea is a well-known model for assessing anti-diarrhoeal potential of probiotic strains against the infectious diarrhoea. Researchers have discovered that pathogenesis of *E coli* occurs due to different virulence factors which leads to different pathological processes. *E. coli* adheres to intestinal mucosa results in destruction of microvilli and formation of 'pedestals' of epithelial cell membrane at site of EPEC adhesion [49]. EPEC confers firstly to enterocytes microvilli, causing their localized effacement. Colonized *E. coli* attaches to intestinal cell surface results in swelling of plasma membrane around attached bacteria. Changes in cell organelles indicates intracellular damage as a result of heavily colonized enterocytes, finally leads to death and loss of cells from villus surface. These observations also evident in histopathology studies of colon in diarrhoeal control rats (Fig. 6a-6e).

E. coli induced diarrhoea, results in disruption of absorptive and secretary mechanism of intestinal fluid and electrolytes which further destructs mucosa and increases intestinal permeability. SKBLAB-19 acts by secreting various antimicrobial peptides like as bacteriocins, reuterin, and hydrogen peroxide. Lactic acid produced by SKB LAB-19 prevents the growth of pathogens by producing the acidic environment and probably by its bactericidal effect. It was also evident in our *in vitro* study that SKB LAB-19 showed good antimicrobial activity against *E. coli* (Fig. 3a). SKB LAB-19 reduced faecal output (Fig. 7a), improved faecal consistency (Fig. 7b) and faecal water content (Fig. 7c) in a dose dependent manner. It is also evident from histopathology study of SKB LAB-19 treated group, which exhibited no damage to the mucosal epithelium with retention of complete crypt cell architecture and integrity of goblet cells and no erosion nor cell infiltration observed (Fig. 6, Table 6 and Fig. 7a-7d).

Table 6
Effect of SKB LAB-19 on *E. coli* induced colonic changes

Colonic condition	Vehicle control	Diarrheal control	SKB LAB-19 5×10^9 cfu/kg	SKB LAB-19 20×10^9 cfu/kg	SKB LAB-19 50×10^9 cfu/kg
Epithelial cell damage	-	+++	++	+	+
Disruption crypt cell architecture	-	+++	++	-	-
Inflammatory cell infiltration	-	+++	+	-	-
Mucosal erosion	-	+++	+	-	-
- No damage, +++ high, ++ Moderate, + Low					

Effect of SKB LAB-19 on relative weight assay and WBC count in E. coli induced diarrhoeal rats

Spleen and thymus are the key immune organs of body. Fifteen days treatment of *Bacillus coagulans* SKB LAB-19 significantly ($P < 0.05$) increased relative organ weight of spleen and thymus in all three doses of *Bacillus coagulans* SKB LAB-19 treatment. The increase in weight of thymus and spleen results in propagation of lymphocytes which in turn imitates the state of immune response [27, 50, 51]. Thymus is related with differentiation, development and maturation of T lymphocytes.

Spleen produces lymphocytes, purifies the blood and store white blood cells [52]. SKB LAB-19 treated group showed significant increase in relative organ weight index of thymus and spleen compared with diarrhoeal and vehicle control group. Different probiotic strains reported to increase the spleen and thymus index which further improves the immune system of the animals. Present results showed that *Bacillus coagulans* SKB LAB 19 promotes anti-infective capability by enhancing growth of spleen and thymus and also promotes spleen recovery in rats infected with *E. coli*. (Fig. 7d).

Leukocyte count is measure of infectious process in the body. A reduced WBC count results in leukopenia which indicates altered immunity or existence of infection that limits the supply of certain WBC's. A reduced WBC count in diarrhoeal control compared to vehicle control explains resolution of infection. In the present study, there was no significant difference in WBC count in treated group as compared to vehicle control. However, WBC count was significantly higher in all SKB LAB-19 treated (5 Billion cfu/kg, 20 Billion cfu/kg and 50 Billion cfu/kg) groups as compared to diarrhoea control group (Fig. 8a).

The results can be better explained considering the differential WBC count. Neutrophils are primary defence system able to respond in different ways through chemotaxis, phagocytosis, exocytosis, intracellular and extracellular killing of microorganisms [53]. In present study, neutrophils percentage was significantly higher ($P = 0.001$), suggesting that supplementation of probiotic results in modulation of non-specific immune response. The present results corroborate with the earlier study by Babar et al who reported significant increase in neutrophils in Wistar rats after administration of probiotic [54].

The results also showed there is a decrease in lymphocytes in infected group (Fig. 8a) which may be due to stress of infection, which produces a moderate to marked absolute decrease in lymphocytes. Lymphocytes play an important role in immune system function and helps body to fight against infection. SKB LAB-19 stimulated significantly increased levels of lymphocytes (P = 0.001) in all treated rats as compared to the diarrhoeal control. This is also in correlation to our observation with immune organ development which shows increased thymus weight/development which is major site for production of lymphocytes. Thus, *Bacillus coagulans* SKB LAB-19 shows a probiotic effect by relieving the stress form infection by increasing lymphocyte count.

The production of antibodies in the serum via soluble antigens could be promoted by probiotics and that plays crucial role to enhance the innate immunity [55]. It can be hypothesised from the results that, SKB LAB-19 improved host defence in the gut by production in specific antibodies (Ab) against pathogens, and the overall increase in total IgA and IgM in a dose dependent manner. There was no significant effect observed in secretion of IgG antibodies in the blood (Fig. 8b). Similarly, enhanced IgA concentration was reported after administration of *Lactobacillus acidophilus* and *Lactobacillus casei* probiotics [56]. Increased levels of IgG and IgA was reported after administration of probiotics containing *Bacillus subtilis and Lactobacillus acidophilus* and Chinese herbal combinations to milking cows [57]. Naqid et al., 2015, demonstrated significant enhancement of IgG, IgM and IgA responses of the host to the bacterial infection after probiotic treatment [58]. These results prove that *B. coagulans* SKB LAB-19 improved cell-mediated immunity and systemic humoral immunity.

Conclusions

A new strain of *B. coagulans* SKB LAB-19 was isolated and characterized. Owing to its properties *viz.* enzyme production, gastric pH and bile salt stability, temperature stability, non-cytotoxic nature, production of antimicrobial metabolites, its proven *in vivo* efficacy towards treatment and reversal of castor oil and *E. coli* induced diarrhoea in mice and rats and its safe nature based on acute toxicity studies and its immunomodulatory effects. *Bacillus coagulans* SKB LAB-19 can serve as an ideal candidate to qualify as probiotic in human and animal feed industry. Further studies to show efficacy in humans and animals is warranted.

Declarations

Ethical Approval

The protocols for experimentation and handling of animals were approved by Institutional Animal Ethics (IAE) committee (MGVPC/CPCSEA/XXXVI/01/2019/01).

Consent to Participate: Not applicable

Consent to Publish: Not applicable

Authors' contributions: Conceptualization-Khushal Chuadhari, Mahalaxmi Mohan, Parag Saudagar, Chetna Sable and Dattatray Bedade, Writing-original draft preparation-Khushal Chuadhari and Dattatray Bedade; Writing-review and editing- Dattatray Bedade, Parag Saudagar, Mahalaxmi Mohan; Supervision-Mahalaxmi Mohan and Parag Saudagar; Project administration-Parag Saudagar; All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Competing Interests: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Availability of data and material: Data supporting reported results can be found in publicly archived datasets such as SciFinder and Scopus.

Conflict of Interest: Author declares no conflict of interest

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Figures

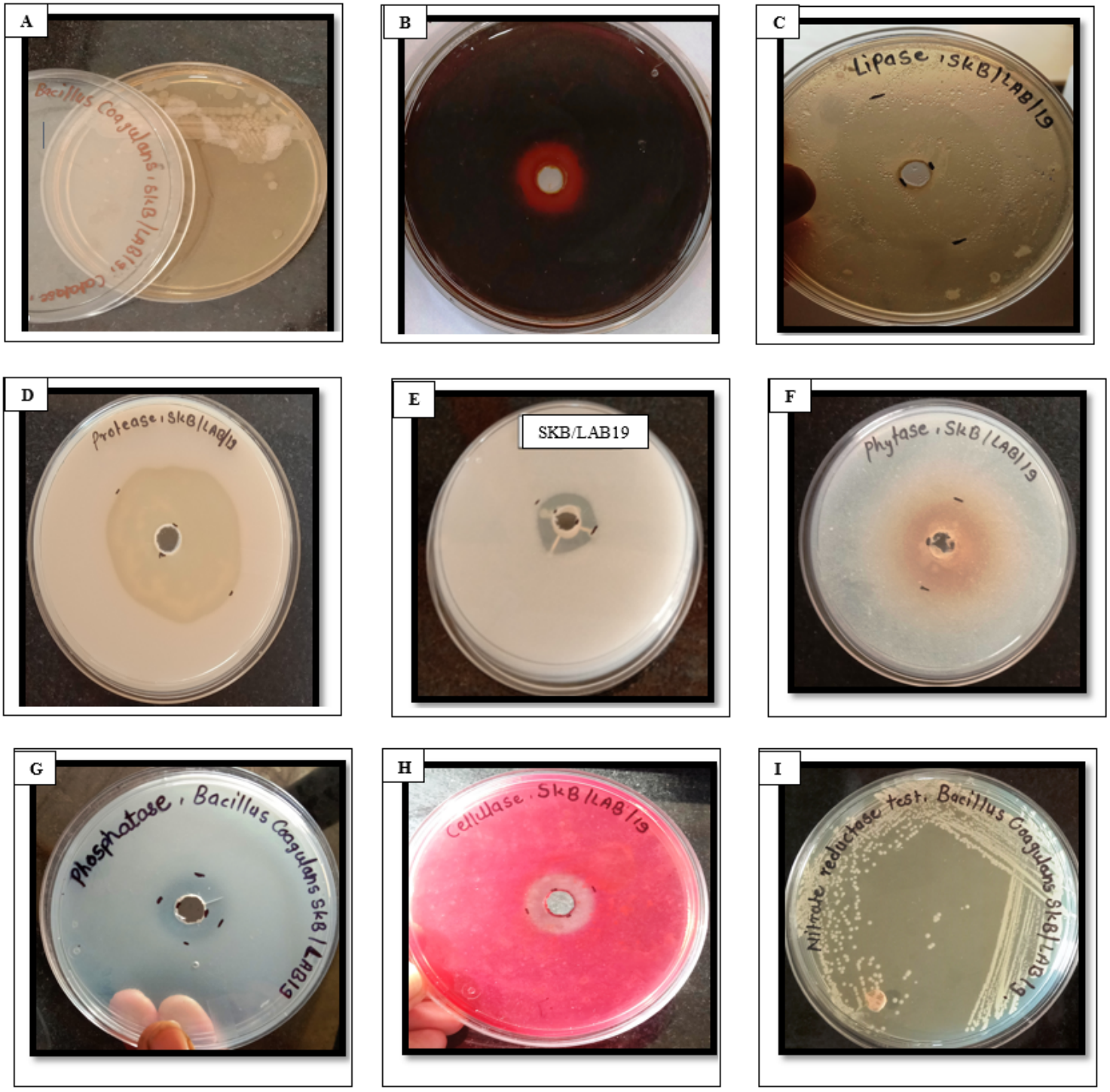


Figure 1

Representative examples of microbial enzyme activity using a different selective media for each enzyme under evaluation. An area of clearance around a bacterial colony can be observed, representing enzyme production of (A) Catalase, (B) Amylase, (C) Lipase, (D) Protease, (E) Phytase, (F) Uricase, (G) Phosphatase, (H) Cellulase, (I) Nitrate reductase. All the screening experiments were conducted in triplicate.

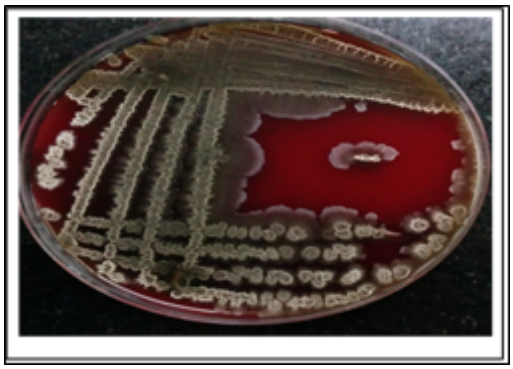


Figure 2

Growth of Bacillus coagulans SKB LAB-19 on sheep blood agar

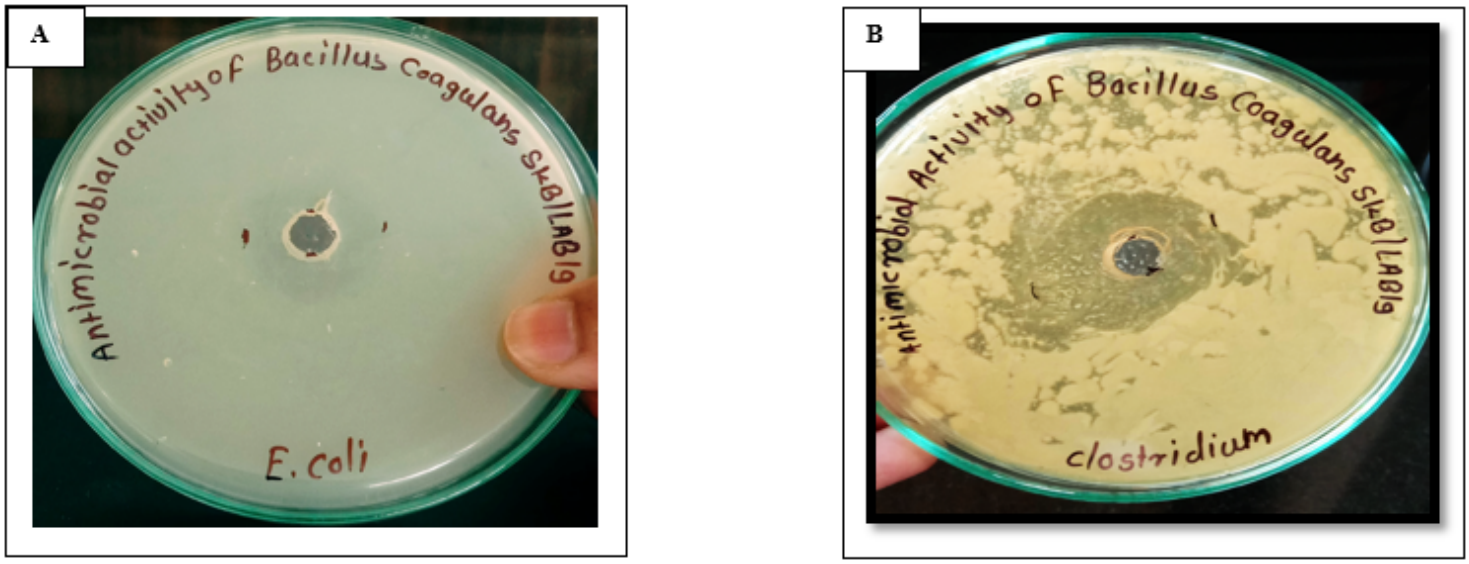


Figure 3

Evaluation of antimicrobial activity from SKB LAB-19 using an overlay method. A zone of inhibition is shown surrounding a tested bacterial colony located in the middle of the plate against (A) E. coli, (B) C. perfringens. All the experiments were conducted in triplicate.

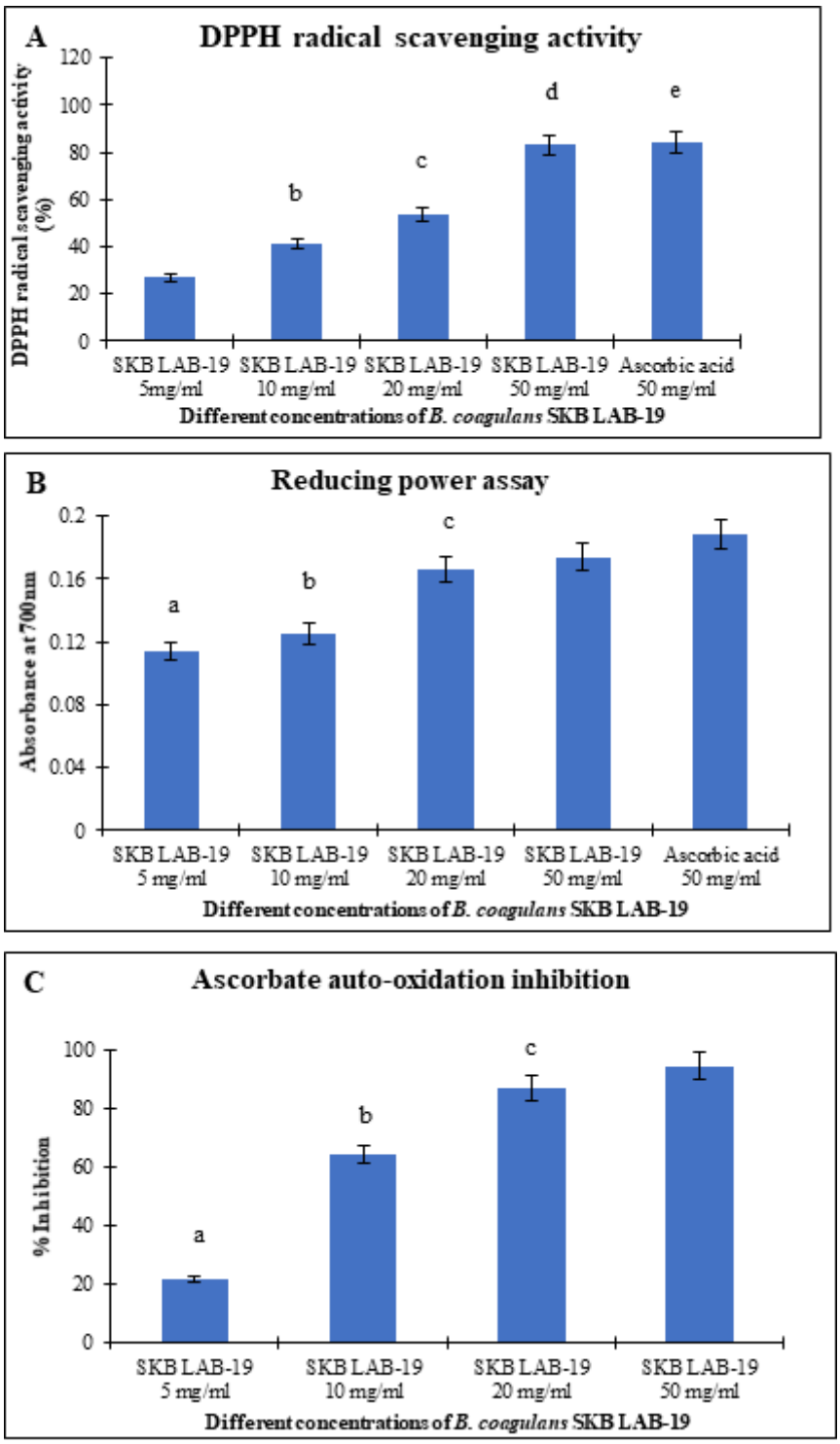


Figure 4

Antioxidant activity of the Bacillus coagulans SKB LAB-19 (A) DPPH radical scavenging activity (B) Reducing power activity (C) Ascorbate auto-oxidation inhibition assay (The dissimilar alphabets (a to e) above the bar indicate that the corresponding mean value belongs to different subsets at 95% confidence interval of the mean).

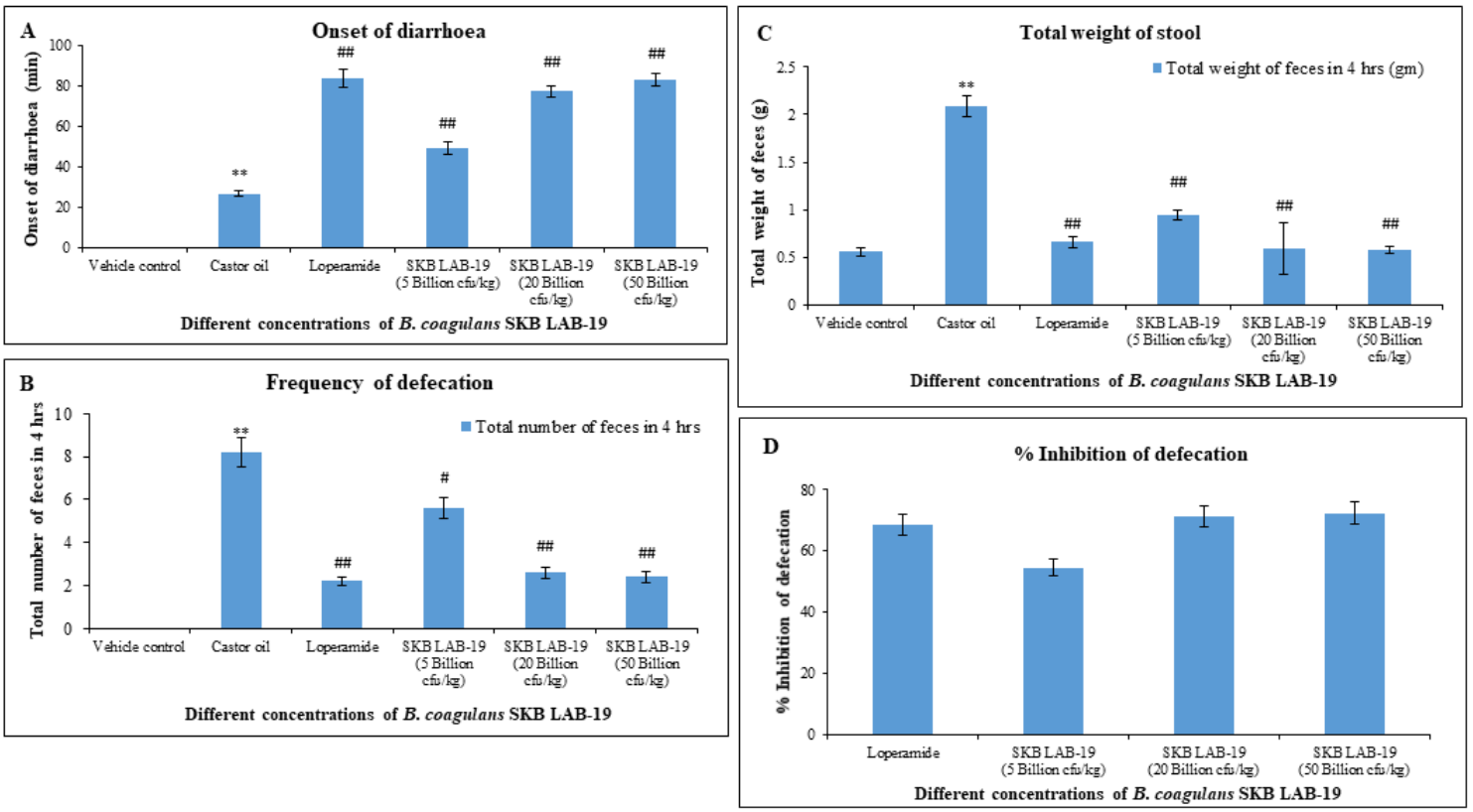


Figure 5

Effect of B. coagulans SKB LAB-19 on (A) onset of diarrhoea in castor oil induced diarrhoea in mice. (B) frequency of defecation in 4 hrs in castor oil induced diarrhoea in mice (C) weight of stool of castor oil induced diarrhoea in mice (D) percentage inhibition of defecation in castor oil induced diarrhoea in mice (All values are expressed as mean ±SEM (n=5), #p□0.05 and ##p□0.01 as compared with the diarrhoeal control group and * p□0.05 and **p□0.01 as compared with the vehicle control group. All data are analysed by one-way ANOVA followed by Dunnett's test).

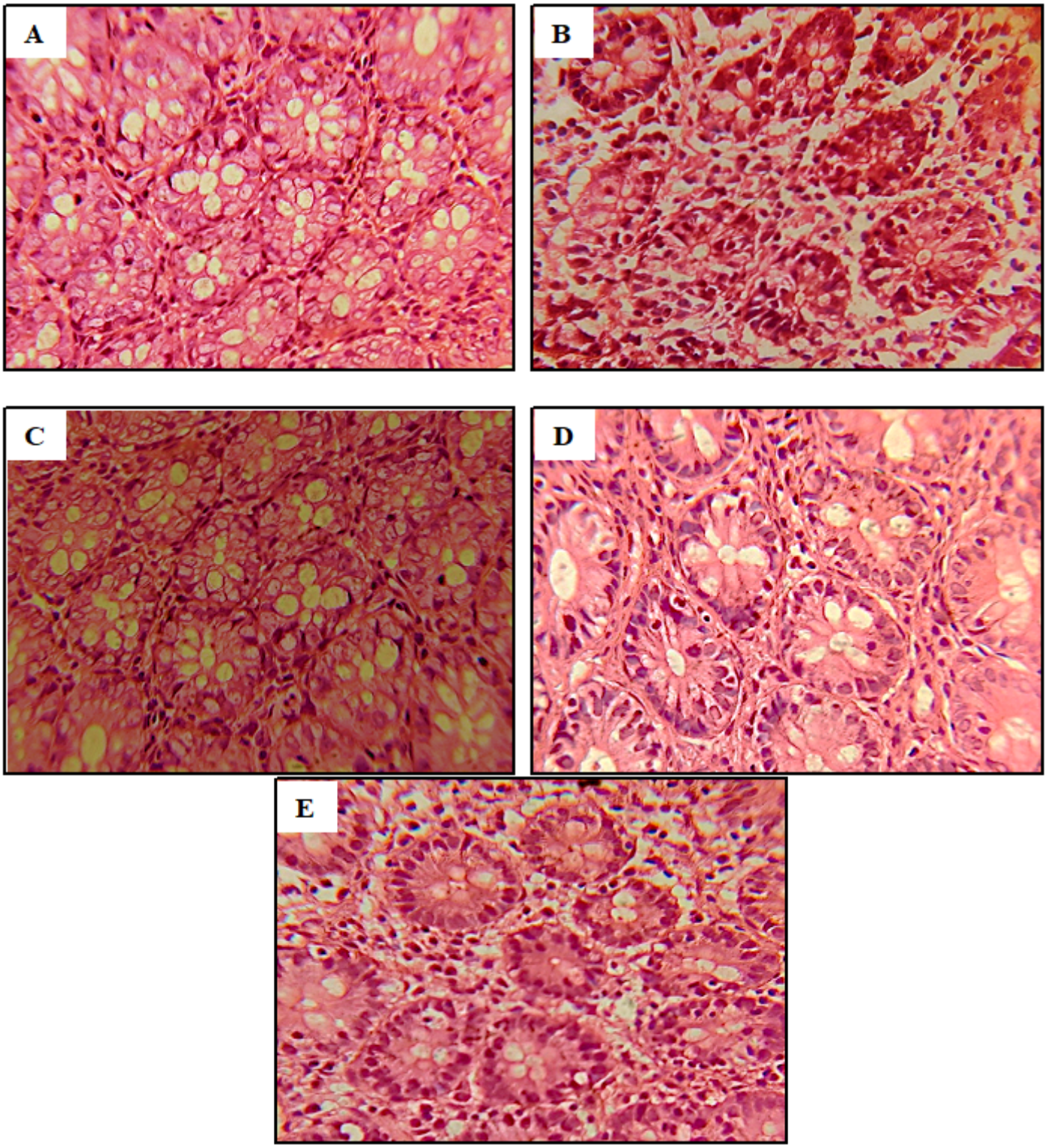
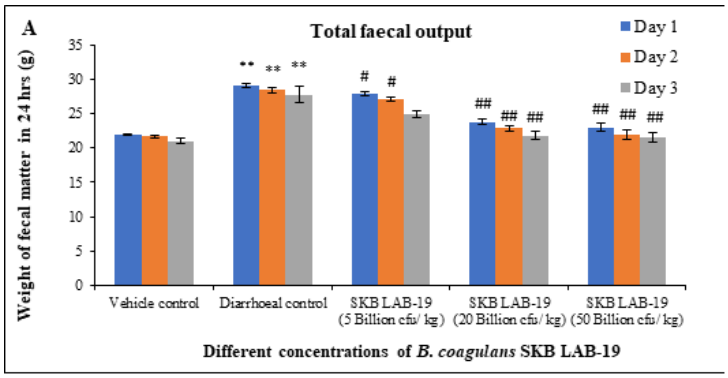
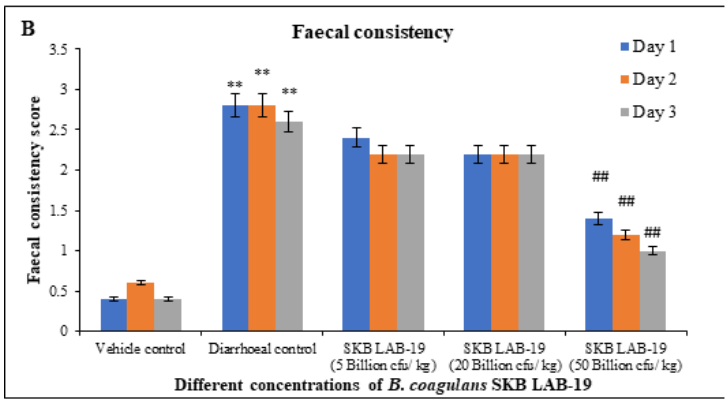


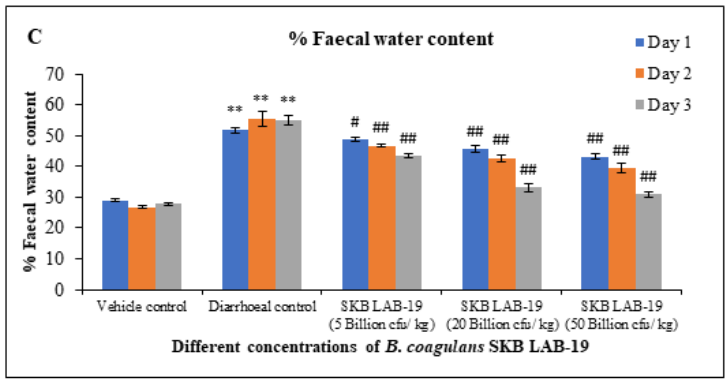
Figure 6

Histopathological study rat colon tissue in various treatment groups in E. coli induced diarrhoeal model (A) Vehicle control: Representation photomicrographs of Hematoxylin and Eosin stained [40X images] vehicle control rats group showed normal mucosa covering surface intact epithelium. There is no crypt cell architecture damage, complete goblet cell with integrity of crypt with mucus filled vacuoles observed and no infiltration of leucocytes, (B) Diarrhoeal control: Representation photomicrographs of Hematoxylin

and Eosin stained [40X images] E. coli (5×109 cfu/mL) group showed damage of mucosal epithelium, goblet cell integrity and crypt cell architecture compared to vehicle control group. (C) Representation photomicrographs of Hematoxylin and Eosin stained [40X images] Bacillus coagulans SKB LAB-19 (5×109 cfu/kg, p.o.) group showed recovery of mucosal epithelium and crypt cell architecture compared to diarrhoeal control group, (D) Representation photomicrographs of H and E stained [40X images] Bacillus coagulans SKB LAB-19 (20 ×109 cfu/kg, p.o.) group showed recovered or no damage to the mucosal epithelium with complete crypt cell architecture and integrity of goblet cells and no erosion nor cell infiltration observed, (E) Representation photomicrographs of H and E stained [40X images] High dose Bacillus coagulans SKB LAB-19 (50×109 cfu/kg, p.o.) group showed recovered or no damage to the mucosal epithelium with complete crypt cell architecture and integrity of goblet cells and no erosion nor cell infiltration observed.







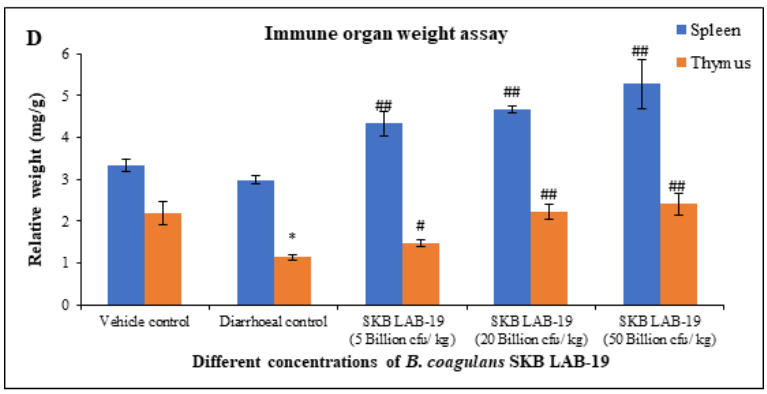


Figure 7

Effect of B. coagulans SKB LAB-19 on (A) faecal output in 24 hrs in E. coli induced diarrhoea in rat (B) faecal consistency in E. coli induced diarrhoea in rats (C) faecal water content (%) in E. coli induced diarrhoea in rats (D) relative weight index of spleen and thymus in E. coli induced diarrhoea in rats. (All values are expressed as mean ±SEM (n=5), #p□0.05 and ##p□0.01 as compared with the diarrhoeal

control group and * p≤0.05 and **p≤0.01 as compared with the vehicle control group. All data are analysed by one-way ANOVA followed by Dunnett's test).

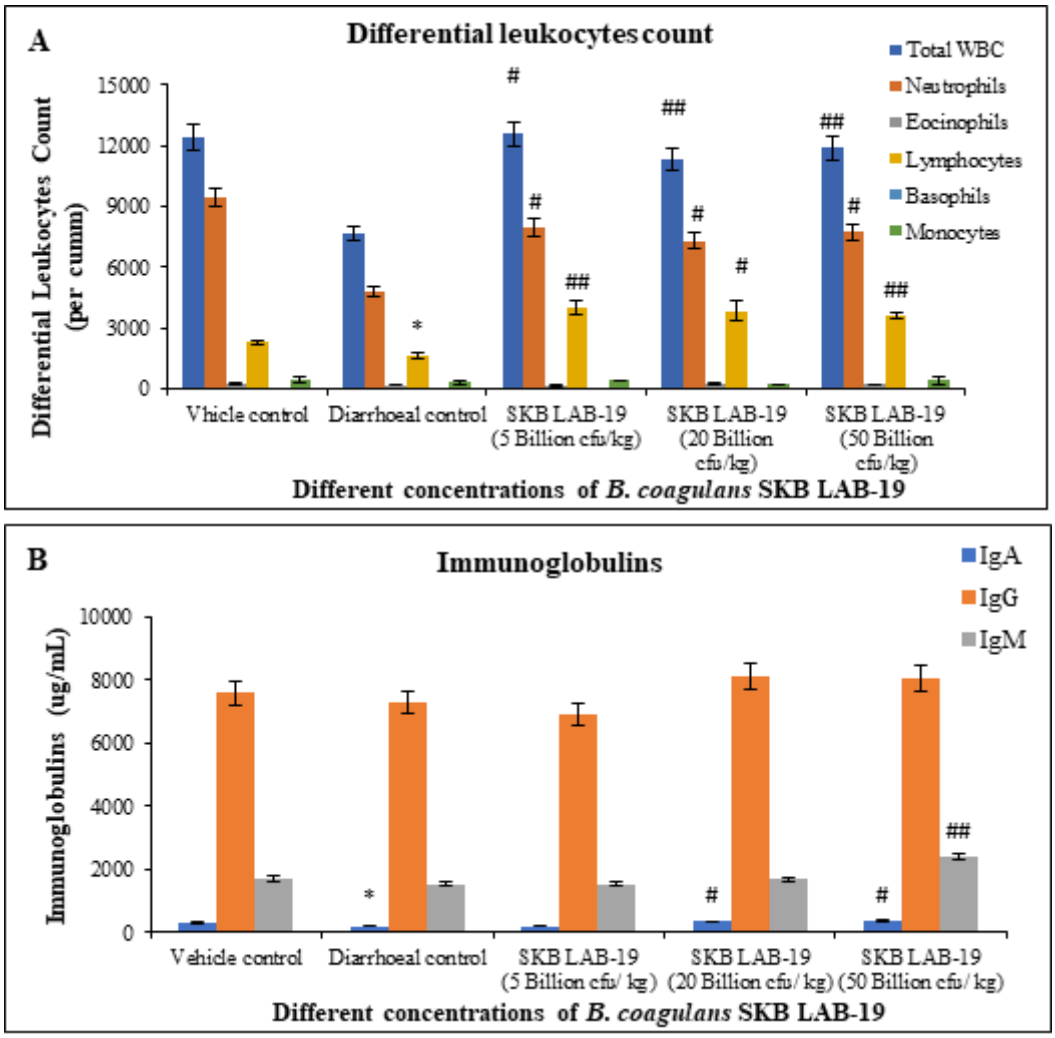


Figure 8

Effect of Bacillus coagulans SKB LAB-19 on (A) differential leukocytes count in E. coli induced diarrhoea in rats (B) immunoglobulin level on E. coli induced diarrhoea in rats (All values are expressed as mean ±SEM (n=6), *p≤0.05 and **p≤0.01 as compared with the diarrhoeal control group. All data are analysed by one-way ANOVA followed by Dunnett's test).

Supplementary Files

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- 05Supplementarydata.docx