Scientific Research Publishing

Advances in Microbiology, 2026, 16(3), 55-73
https://www.scirp.org/journal/aim
ISSN Online: 2165-3410
ISSN Print: 2165-3402

Immunomodulatory Properties of Probiotic Lactic Acid Bacteria from Kossam, a Cameroonian Traditionally Fermented Milk

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How to cite this paper: Toukam, T.L.L., Vanessa, A.B.T., Felicite, D.-D., Gertrude, B.T., Eyong, E.M., Bernard, T. and Bertrand, T.F. (2026) Immunomodulatory Properties of Probiotic Lactic Acid Bacteria from Kossam, a Cameroonian Traditionally Fermented Milk. *Advances in Microbiology*, **16**, 55-73.
https://doi.org/10.4236/aim.2026.163005

Received: February 22, 2026
Accepted: March 23, 2026
Published: March 26, 2026

Abstract

Background: "Kossam", a traditionally processed milk in Cameroon and sub-Saharan Africa, has been described as potential source of probiotic lactic acid bacteria (LAB). However, to our knowledge, no research work has been done on their immunomodulatory properties. **Aim:** This study was aimed at isolating and characterising lactic acid bacteria from "Kossam", a traditionally fermented cow milk and investigating their immunomodulatory properties in rat model. **Materials and Methods:** Lactic acid bacteria were isolated from "Kossam" using pour plating technique. The strains exhibiting probiotic potential were selected and their identities were confirmed using the sequencing of 16S ribosomal RNA gene. Probiotic isolates were administered to rats for 2 consecutive days and a post ingestion period of 10 days was observed. Full blood count, differential blood count, total spleen protein and pro-inflammatory cytokines (TNF-α, IFN-g, IL-1-β and IL-6) concentrations were detected in the sera after 10 days. **Results:** A significant increase ($p < 0.05$) in the level of RBCs, lymphocytes, monocytes and total spleen proteins was observed in rats treated with the different isolates. However, a significant decrease ($p < 0.05$) in circulating WBCs and neutrophils count was observed in animals treated with the isolates A20, A27, A46, and A56 that had the highest effect ($p < 0.05$) on pro-inflammatory cytokines level in serum. These strains were identified using 16S rRNA gene sequence as strains of *Lacticaseibacillus paracasei* (A20), *Limosilactobacillus fermentum* (A27), *Enterococcus faecium* (A46), *Lactiplantibacillus plantarum* (A56). **Conclusion:** These isolates showed immunomodulatory properties in rats and were able to prevent the incidence of diarrhoea associated with purgative components.

Keywords

Probiotics, Immunomodulatory Properties, Lactic Acid Bacteria, "Kossam"

1. Introduction

Probiotics are live microorganisms that upon ingestion in adequate amount exert health effects beyond inherent basic nutrition [1]. Several probiotic strains have drawn special attention in recent years for their encouraging abilities to increase animal and human health, especially through the prevention of chronic diseases and the improvement of natural immune protection). Recently, there has been much interest in Lactic acid bacteria (LAB) due to their "Generally Recognized as Safe status (GRAS) and their beneficial effects" in health [2]. In particular, lactobacilli form a source of potential modulators of the immune system. The immunological benefits of probiotics are said to be due to activation of local macrophages and modulation of IgA production locally and systemically, to changes in pro/anti-inflammatory cytokine profiles, or to the modulation of response towards food antigens [3]. In the event of their action as immune modulators, probiotics may either have up- or down-regulatory effects on various indices of immune response.

Immunomodulation is a key issue in tissue homeostasis for the physiological stability of organisms. A number of chemically synthesized compounds have been used as immunodulators for the past few years; however, there are limitations to the general use of these agents due to their related adverse effects on health. Hence, natural immunodulators are potential agents to replace them in therapeutic regimens [4]. Previous studies have reported that probiotics are one of the most important of these natural agents, which are friendly; microbiota can boost immunity and reduce inflammation and infection in the host [5].

"Kossam" is a Cameroonian traditionally fermented food and a rich source of LAB whose probiotic properties have not yet been fully explored [6]. To our knowledge, there are surprisingly no studies done on the immunomodulatory properties of the lactic acid bacteria from this food, though it has been speculated that the Fulani owe their strong health to the daily consumption of this food. This study therefore aimed at providing knowledge base as well as bridging the existing gap in knowledge with regard to immunomodulatory properties of probiotics from "Kossam". The results of this investigation would be crucial in order to promote indigenous strains with interesting new functional properties that may help to design probiotics for specific prophylactic or therapeutic purposes.

2. Materials and Methods

2.1. Sample Collection

This study was carried out in the Life Science Laboratory of the University of Buea.

Samples were collected from four different sites in Bamenda, North West region of Cameroon: Mendakwe, Bambui, Bandja and Old town. A total of 50 samples of traditionally fermented cow milk were purchased from Mbororos and cattle rearing communities around Bamenda. The purchased samples were collected directly and aseptically introduced inside sterile plastic bottles and kept in a flask containing ice until delivery to the laboratory.

2.2. Isolation of Lactic Acid Bacteria

The bacteria were isolated following aseptic laboratory procedures. One milliliter of each milk sample was serially diluted up to the ten logarithmic fold in sterile test tube containing 9 ml of 0.85% saline solution (Nacl). Demann Rogosa and sharpe (MRS, Sigma-Aldrich, Germany) agar, a selective medium for lactic acid bacteria isolation was measured and prepared according to the manufacturer's instructions. 1 ml aliquot of three different dilution factors (10^{-4}, 10^{-6} and 10^{-8}) were poured into plates and about 20 ml of the selective medium was added and allow to solidified at room temperature. The plates were sealed with parafilm and incubated at 37˚C for 2 - 3 days under anaerobic conditions. Upon growth, isolated colonies were picked up and tested for biochemical (catalase activity, gram stain and API) and physiological properties (acid and bile tolerance). Gram positive rods and catalase negative isolates were selected for preliminary screening for acid and bile tolerance. Bacterial plates were more purified by sub-culturing continually on MRS agar media, and the colony morphologies (colour, size and shape) were recorded. Well isolated bacterial colonies were picked up and stored in MRS broth for further investigations.

2.3. Study of the Functional Properties of Isolated LAB

2.3.1. Resistance of Isolate to Acid

Cells were harvested by centrifugation for 10 min at 5000 rpm and 4˚C. Pellets were washed once in phosphate-saline buffer (PBS at pH 7.2). The cell pellets were resuspended in 5 ml of MRS broth whose pH had been adjusted (pH 2.5) using 1N HCL or NaOH, then incubated at 37˚C. 1 ml of each of the suspensions was serially diluted up to the ten logarithmic fold and the viable microorganisms (10^{-6} and 10^{-8} diluted factor) were enumerated on MRS agar after incubation at 37˚C for 0 and 3 h reflecting the minimum and maximum time which food spends in the stomach. Isolates that exhibited final counts $\geq 10^3$ cfu/ml or $\geq 10^6$ cfu/ml at low pH for 3 hours, were considered to have moderate or good resistance, respectively.

2.3.2. Resistance to Bile Salt

LAB isolates were cultured in MRS broth, for 24 h at 37˚C, the cells were harvested by centrifugation for 10 min at 5000 rpm and 4˚C. The pellets were washed once in phosphate-saline buffer (PBS at pH 7.2) then suspend into 1 ml MRS broth containing 0.2% (w/v) and 0.4% (w/v) oxgall-bile (Sigma-Aldrich) respectively. Broth without oxgall-bile served as control. The cultures were incubated at 37˚C and the optical density (O.D.) at zero hour and after 4 h was read at 650 nm using

a spectrophotometer (Thermo Fisher). The O.D of the 24 h cultures was also recorded. Growth leading to increase in the O.D. was considered as the tolerance ability of the LAB isolates to bile and hence, survival. The survival percentage of LAB was calculated according to the formula by Fossi *et al.* [7] as shown below:

$$\text{Survival rate} = \frac{OD_{xH,y} - OD_{0H,y}}{OD_{xH,0\%} - OD_{0H,0\%}} * 100$$

$x = 4$; $y = 0.2\%$ or 0.4% for a same wavelength and the same isolate.

2.4. Molecular Characterisation of the Selected Bacterial Isolates

The bacterial isolates exhibiting probiotic potential were identified at genomic level using 16S rRNA gene sequencing. The total genomic DNA was isolated following the protocol of Sambrook *et al.* [8] with some modifications. The DNA was finally dissolved in 50 µl of 1% sterile tris-EDTA (TE) and stored at −20˚C for further analysis. Concentration of the DNA extracted was determined by gel electrophoresis. PCR amplification of the 16S rDNA gene from each sample was performed to confirm the identity of the bacterial species and the small sub unit 16S rDNA genes was amplified from the genomic DNA using universal primers:

- F (5'-AGAGTTTGATCCTGGCTCAG-3);
- R (5'-ACGGCTACCTTGTTAACGACTT-3).

The primers were used to amplify a 1.5 kilobase (kb) fragment of part of the 16S rRNA gene of the selected bacteria. The PCR products were separated by agarose gel electrophoresis. After completion of electrophoresis, the gel was taken out of the chamber and examined using UV transilluminator and subsequently photographed by Bio RadGel Doc system (Bio-Rad, Hercules, CA, USA). The PCR amplicons obtained by amplifying PCR products were sent together with the primers to Inquaba in South Africa for nucleotide sequencing (Sanger nucleotide sequencing technique). Translated nucleotide sequences were then analysed for similarities by using NCBI blast tool (http://www.ncbi.nlm.nih.gov/BLAST/).

2.5. Animal and Ethical Considerations

Wister albinos rats (*Rattus norvegicus*) aging between 16 to 18 weeks and weighing 200 ± 10 g, were obtained from the animal house of the Department of Animal biology, University of Dschang, Cameroon. The rats were housed under standard conditions with a 12 h light and 12 h dark cycle. Temperature was maintained at 23˚C ± 2˚C and relative humidity at approximately 50%. The rats were acclimatized for 7 days prior to use. Investigations were conducted in accordance with the institutional guidelines defined by the University of Buea Institutional Animal Care and use Committee (UB-IACUC) guiding principles in the care and use of animals. The investigations were performed in accordance with the institutional protocols established by the University of Buea Institutional Animal Care and Use Committee (UB-IACUC), with ethical clearance permit number: UB-IACUC no. 015/2019. Experiments were performed in the Life Science Laboratory of the Uni-

versity of Buea and animal protocol was approved by the local ethic committee for experiments.

2.5.1. Preparation of Probiotic Solutions

Bacteria cells for *in vivo* immunomodulatory studies were grown on MRS broth overnight at 37˚C, then separated from the culture supernatant by centrifugation (15 min at 3000 rpm) at 4˚C, washed three times with ice cold phosphate saline buffer (PBS) (pH = 7.2) and resuspended in PBS. The final concentration of the mixture was adjusted to McFaland standard 0.5 (1.5×10^8 cfu/ml).

2.5.2. Experimental Design

This study was a complete randomized control trial design. Animal grouping and feeding was performed according to the protocol of Tiepma *et al.* [9] with slight modifications. 30 albinos Wister rats were randomly assigned to 6 groups designated A, B, C, D and two control E and F based on their body weight and age so that the mean body weight and mean age of each group should not differ significantly at the beginning of the experiment. Each group comprised 3 males and 2 females. Group F (negative control), Group E (positive control) was placed on basal diet and challenged with 1 ml oral gavage of castor oil, while group A, B, C and D were placed on the basal diet and also challenged with oral gavage of 1 ml of castor oil + 1 ml of 10^8 cfu/ml of lactic acid bacteria isolates A20, A27, A46, A56 respectively. The treatment above was repeated the second day and a post ingestion period of 10 days was observed after the administration of the culture. Prior to the test, rats were fasted for 16 - 18 hours but had access to drinking water.

2.5.3. Blood Collection and Immunological Assays

After the treatment, the rats were allowed to fast overnight (12 hours) and anaesthetised using chloroform and sacrificed. They were dissected and the blood was collected through cardiac puncture. Blood was collected in EDTA tube and dry tube aseptically together with the spleen. The whole blood in the dry tube was centrifuged for 10 minutes at 4000 rpm to obtained serum. The sera were kept at −20˚C for cytokines (TNF-α, IL-1β, IL-6 and IFN-γ) assays. Blood samples collected into EDTA tube was used to determine full blood count (white blood cell, red blood cell, haematocrit, haemoglobin, MCV, MCH, MCHC, neutrophils, eosinophil, basophils, monocytes and lymphocytes) using a haematological analyzer, ADVIA 60 (Bayer Bayer, Germany). The spleens were cleaned in a saline solution (NaCl 0.9%), dried and weighed. The homogenate was prepared at 30% (w/v) in a saline solution and centrifuged at 2500 rpm for 15 min. The spleen protein rates were determined by the Bradford method.

2.6. Statistical Analysis

Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS) version 20.0 statistical software (IBM, SPSS, Inc., Chicago, IL, USA).

Data collected from cytokines assay, proteins analysis and blood cells counting were analysed using a one way analysis of variance (ANOVA) and results were subjected to post hoc test (Tukey test) to separate the means from each other. The data were expressed as Mean± Standard deviation and value of $p < 0.05$ were considered to be significant.

3. Results and Discussion

3.1. Isolation and Biochemical Characterisation of the Isolates

In total, 19 presumptive lactic acid bacteria were isolated on MRS agar. Biochemical characterisation of the 19 isolated LAB was done and their characteristics were obtained. All bacteria isolates were catalase negative and gram positive. All isolates were found to be rod shape-like bacilli with 17 (89.47%) being long rod and 2 (10.53%) short rods.

3.2. Primary Assessment of Probiotic Attributes of Screened Isolates

3.2.1. Acid Tolerance

Out of the 19 isolates tested, 12 (isolates A3, A4, A13, A15, A17, A23, A24, A36, A37, A57, A61) exhibited final counts ≥ 10^3 cfu/ml at low pH for 3 h hence moderate tolerance. A good tolerance (final counts ≥ 6 logcfu/ml) was observed for isolates A11, A20, A27, A31, A42, A46, A51 and A56 under the same conditions (Figure 1).

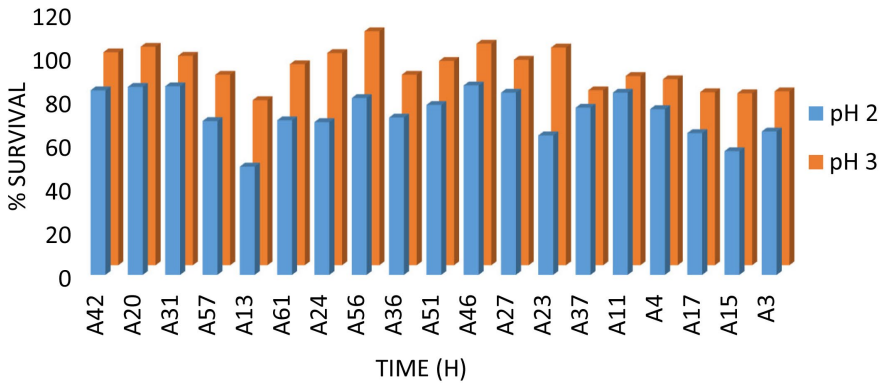


Figure 1. Survival rate of lactic acid bacteria after 3 hours exposure to acidic environment.

3.2.2. Bile Salt Tolerance

Resistance to bile salts is generally considered as an essential property for probiotic strains to survive the conditions in the small intestine. A great variation in the survival percentage was observed in this experiment. All the seven isolates tested could tolerate 0.2% of bile salt concentration withA27, A42, A46, A51 and A56 exhibiting the best bile tolerance. Two isolates A11 and A42 could not tolerate 0.4% bile salt while A20, A24, A27, A46, and A56 could tolerate both concentrations. The survival rate of the isolates after exposure to different concentrations of bile is presented in the figure bellow (Figure 2).

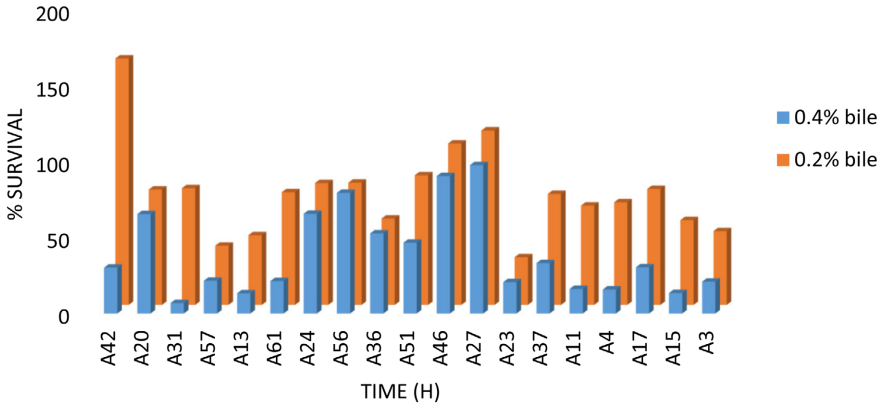
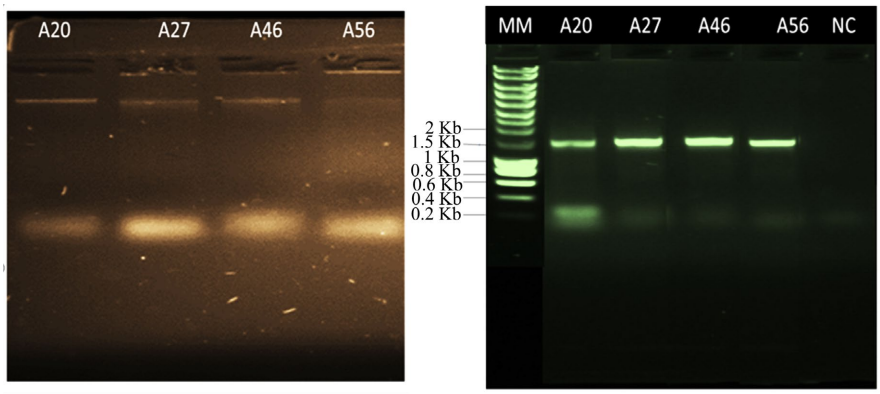


Figure 2. Survival rate of lactic acid bacteria after 4 hours exposure to 0.2 and 0.4% bile salts.

3.3. Genotypic Characterisation and Blasted Sequence of the Isolates

In order to confirm the identity of the isolates previously screened by catalase test and Gram staining, four isolates (A20, A27, A46, A56) with the best acid and bile tolerance were subjected to DNA extraction followed by PCR assay based using the 16S rRNA universal primers. The results obtained showed a specific amplification product of 1.5 kb for all the isolate, confirming them as Lactic acid bacteria (Figure 3).



(a) (b)

Figure 3. Agarose gel electrophoresis of DNA extraction and agarose gel electrophoresis of the PCR products of the selected LAB isolates where: (a) is agarose gel electrophoresis of the DNA extract of the selected probiotic LAB isolates. Lane1: A20, lane: A27, lane: A46, lane: A56; (b) is agarose gel electropheris of the PCR of the selected probiotic LAB isolates. Lane1: 1 kd DNA maker lane2: A20, lane 3: A27, lane4: A46, lane5: A56, lane6: Negative control.

These strains were identified using 16S rRNA gene sequence as strains of *Lacticaseibacillus paracasei* (A20) with 99.87% similarity, *Limosilactobacillus fermentum* (A27) with 98.85% similarity, *Enterococcus faecium* (A46) with 99.5% similarity, *Lactiplantibacillus plantarum* (A56) with 98.75% similarity. These iso-

lates sequences will further be deposited to NCBI GenBank database to have accession number. The four isolates showed best probiotic characteristics *in vitro*, hence were selected for *in vivo* studies.

3.4. Immunomodulatory Properties of the Selected LAB Isolates

3.4.1. Haematological Profile of Rats Dosed with Probiotic LAB

1) Effect of probiotic on full blood count

Full blood count of experimental animals showed an increase in the number of red blood cells in groups that were administered probiotic LAB isolates. There was a significant increase in RBCs of group A (A20) compare to the positive and negative control groups E and F ($p < 0.05$). An increase was observed at the haematocrit level of groups A (A20) and D (A56) compared to the controls E and F though not significant ($p > 0.05$) and there was a strong positive correlation with the red blood cell number. All isolates *in vivo* seemed to reduce circulating white blood cells, with a significant decrease ($p < 0.05$) observed in group A (A20), B (A27) and D (A56). The platelets count of rats dosed with probiotic isolates of group A (A20) and D (A56) was higher and significantly different when compared to the control groups. There was no significant difference between the haemoglobin concentrations of the animal treated with probiotic compare to the controls. The same trend was also observed for the MCV and MCH. However, the MCHC was lower in group A (A20) and significantly different from the control groups (Table 1).

Table 1. Full blood count of rats orally dosed with different probiotic lab.

Parameters	Group A	Group B	Group C	Group D	Group E	Group F	p-value
RBC ($\times 10^6$ μL^{-1})	$6.62^{a} \pm 0.16$	$6.33^{ab} \pm 0.20$	$6.38^{ab} \pm 0.17$	$6.42^{ab} \pm 0.22$	$5.31^{c} \pm 0.13$	$6.26^{b} \pm 0.07$	<0.05
Haemoglobin (g/dl)	$10.69^{a} \pm 0.49$	$11.49^{a} \pm 0.18$	$11.45^{a} \pm 0.09$	$11.60^{a} \pm 0.25$	$11.57^{a} \pm 0.22$	$11.36^{a} \pm 1.08$	0.12
Haematocrit (%)	$38.53^{a} \pm 0.46$	$37.75^{ab} \pm 0.26$	$37.35^{ab} \pm 0.14$	$38.14^{a} \pm 1.93$	$37.80^{ab} \pm 0.29$	$36.42^{b} \pm 0.33$	<0.05
WBC ($\times 10^3$ μL^{-1})	$10.23^{cd} \pm 0.03$	$11.84^{b} \pm 0.97$	$13.46^{a} \pm 0.32$	$9.89^{d} \pm 0.51$	$11.31^{bc} \pm 0.36$	$14.19^{a} \pm 0.62$	<0.05
Platelets ($\times 10^3$ μL^{-1})	$465.8^{ab} \pm 7.98$	$453.8^{abc} \pm 16.89$	$435.0^{bc} \pm 5.48$	$473.0^{a} \pm 13.93$	$428.6^{c} \pm 21.98$	$439.0^{bc} \pm 14.09$	<0.05
MCV (fL)	$58.28^{a} \pm 0.57$	$57.62^{a} \pm 1.72$	$58.72^{a} \pm 0.47$	$58.95^{a} \pm 0.7$	$58.76^{a} \pm 0.8$	$58.1^{a} \pm 0.84$	0.32
MCH (pg)	$17.48^{a} \pm 0.23$	$17.48^{a} \pm 0.30$	$17.42^{a} \pm 0.30$	$17.70^{a} \pm 0.24$	$17.33^{a} \pm 0.23$	$17.35^{a} \pm 0.14$	0.32
MCHC (g/dL)	$29.4^{b} \pm 0.31$	$30.26^{a} \pm 0.20$	$30.61^{a} \pm 0.32$	$30.58^{a} \pm 0.29$	$30.39^{a} \pm 0.25$	$30.39^{a} \pm 0.25$	<0.05

All the results are expressed in term of mean ± standard error, for n = 5 mice per group. Data were analysed by one-way ANOVA followed by Post hoc Tukey test and a statistical significance was set at $p < 0.05$ with a ($p < 0.05$), b ($p < 0.01$) and c ($p < 0$). Group (A - D) Rats respectively dosed with probiotics isolates (A20), (A27), (A46), and (A56) and challenged with castor oil. (E) Rats challenged with castor oil only (positive control). Rats not dosed (negative control).

2) Effect of probiotic on differential white blood cell count

The differential white blood cell count of rats treated with the different probiotic isolates revealed that the rate of neutrophils in treated animals was greater than the negative control group F and significantly different ($p < 0.05$) in group A (A20) and C (A27) except group D (A56) which felt slightly. All were less than the positive control group E which received only castor oil, however the decrease was

only significant ($p < 0.05$) in group D (A56). The eosinophil rate was higher and significantly different ($p < 0.05$) in group B (A27) compare to the treated and controls groups, however no significant difference in the basophil rate was observed in all the treated groups and controls. The lymphocyte counts was higher and significantly different ($p < 0.05$) in groups A (A20) and B (A27) when compare to the positive control. Moreover, there was a non-significant decrease in the lymphocytes rate in group B (A27) and D (A56) compare to the negative control. Isolates A27, A46 and A56 significantly increase ($p < 0.05$) the monocytes count when compare with both controls (Table 2).

Table 2. Differential leucocytes counts of rats orally dosed with different probiotic LAB.

Parameters	Group A	Group B	Group C	Group D	Group E	Group F	p-value
Neutrophils (%)	$22.62^{abc} \pm 0.51$	$20.65^{bcd} \pm 0.64$	$23.00^{ab} \pm 0.22$	$20.33^{cd} \pm 0.33$	$24.76^{a} \pm 2.51$	$20.35^{d} \pm 0.27$	<0.05
Eosinophil (dosed %)	$2.57^{b} \pm 0.26$	$3.29^{a} \pm 0.22$	$2.48^{b} \pm 0.22$	$2.65^{b} \pm 0.22$	$2.74^{b} \pm 0.23$	$2.74^{b} \pm 0.11$	<0.05
Basophils (%)	$0^{a} \pm 0.0$	$0^{a} \pm 0.0$	$0^{a} \pm 0.0$	$0^{a} \pm 0.0$	$0^{a} \pm 0.0$	$0^{a} \pm 0.0$	1
Lymphocytes (%)	$70.8^{a} \pm 2.51$	$65.82^{cd} \pm 0.32$	$68.27^{b} \pm 0.87$	$66.35^{bcd} \pm 0.34$	$65.14^{d} \pm 1.37$	$67.38^{bc} \pm 1.88$	<0.05
Monocytes (%)	$5.6^{b} \pm 0.3$	$6.04^{a} \pm 0.7$	$6.28^{a} \pm 1.6$	$6.25^{a} \pm 0.12$	$5.22^{bc} \pm 0.13$	$5.04^{c} \pm 0.13$	<0.05

All the results are expressed in term of mean ± standard error, for n = 5 mice per group. Data were analysed by one-way ANOVA followed by Post hoc Tukey test and a statistical significance was set at $p < 0.05$ with a ($p < 0.05$), b ($p < 0.01$) and c ($p < 0$). Group (A - D) Rats respectively dosed with probiotics isolates (A20), (A27), (A46), and (A56) and challenged with castor oil. (E) Rats challenged with castor oil alone (positive control). Rats not dosed (negative control).

3) Effect of probiotics on pro-inflammatory cytokines

The pro-inflammatory cytokinesIL-1-β, IL-6, TNF-α and IFN-γ levels were detected in the serum samples after 10 days of experimental challenge. All the groups treated with probiotics lactic acid bacteria showed a decrease in level of pro-inflammatory cytokines when compare with the positive control and an increase when compare with the negative control. All cytokines showed statistically significant differences in some levels among experimental groups. IL-1βlevels were significantly lower in all the groups ($p < 0.05$) treated with probiotics compared to the positive control group E and significantly higher ($p < 0.05$) in group A (A20), B (A27) and D (A56) when compare to the negative control group E ($p < 0.05$). Although there was an increase in the IL-1-βlevel in group C (A46), no significant difference was observed when compare with the negative control ($p > 0.05$) (Figure 4(a)). Significant difference was observed in the IL-6 level of the probiotic application groups B (A27) and D (A56) when compare to positive control groups ($p < 0.05$). IL-6 levels were significantly higher ($p < 0.05$) in probiotic treated group B (A27) and D (A56) when compared with negative control and significantly lower when compare with positive control ($p < 0.05$). However, there was no significant difference between group A (A20) and C (A46) probiotic treatments and negative control F ($p > 0.05$), but a significant decreased was observed when compare with positive control ($p < 0.05$). A similar result can be seen in the levels of TNF-α in the probiotic application groups in which there was a significant difference ($p < 0.05$) between the experimental groups B (A27) and D (A56) and the

control groups (Figure 4(c)). The IFN-γ levels were significantly decreased in all probiotic treated group compare to the positive control and significantly increased in group A (A20) and B (A27) when compare with negative control F ($p < 0.05$) (Figure 4).

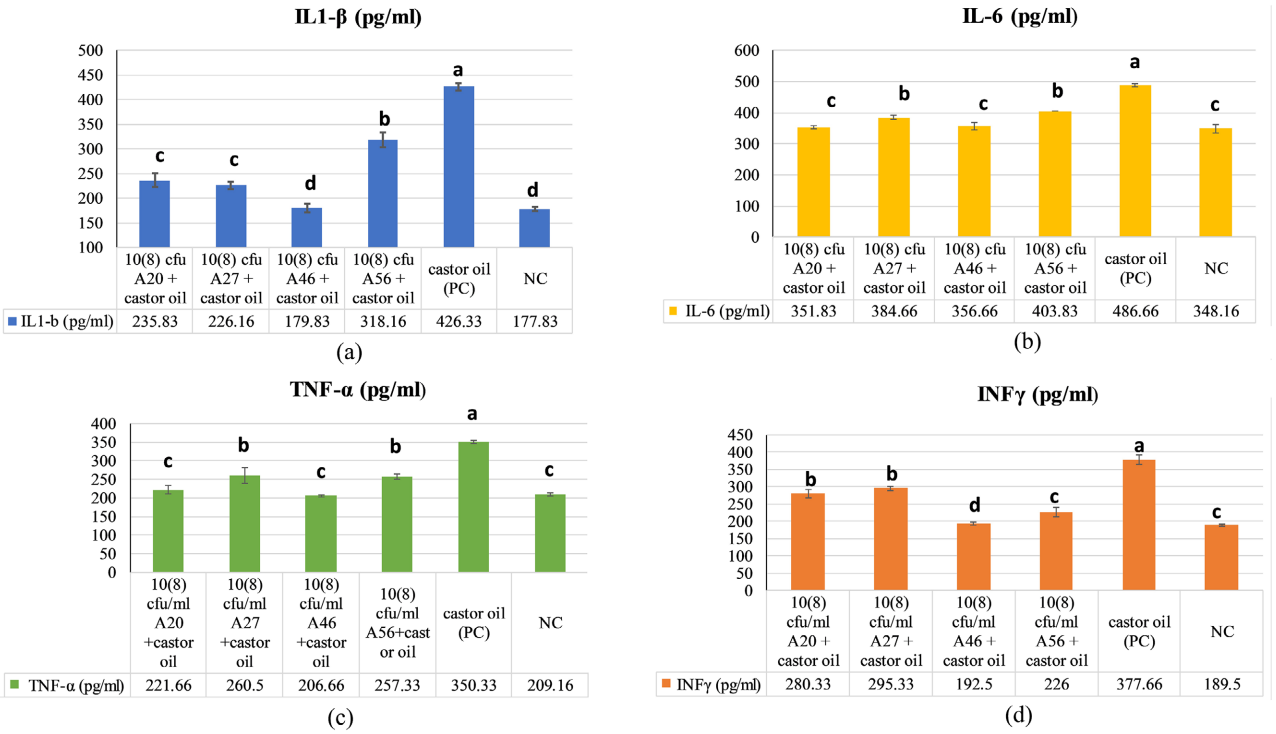


Figure 4. Effect of probiotics on pro-inflammatory cytokines. Levels of IL-1-β, IL-6, TNF-α and IFN-γ of all experimental groups: Bars with different superscript are significantly different ($p < 0.05$). Group ((a) - (d)) Rats dosed respectively with LAB isolates (A20), (A27), (A46), (A56) and challenged with castor oil. Group (E) Rats challenged with castor oil only (positive control). Group (F) Rats not dosed (negative control) where a, b, c, d are the different level of significance. Data were analysed by one-way ANOVA followed by Post hoc Tukey test and a statistical significance was set at a ($p < 0.05$), b ($p < 0.01$), c ($p < 0.001$) and d ($p < 0.0001$).

3.4.2. Effect of Probiotic on Total Spleen Proteins

A non-significant decrease ($p < 0.05$) in the total spleen protein was observed in group C (A46) and D (A56) treated with the probiotic compare to the positive and negative controls. However, group A (A20) and B (A27) showed significant increase in the total spleen protein compare to both control groups ($p < 0.05$) (Table 3).

Table 3. Protein rate of rats dosed with probiotic lab.

Parameters	Group A	Group B	Group C	Group D	Group E	Group F	p-value
Protein rate (mg/ml)	$10.49^{ab} \pm 1.9$	$11.53^{a} \pm 0.88$	$8.97^{ab} \pm 2.17$	$7.69^{b} \pm 1.16$	$9.73^{ab} \pm 1.17$	$9.45^{ab} \pm 1.59$	<0.05

All the results are expressed in term of mean ± standard error, for n = 5 mice per group. Data were analysed by one-way ANOVA followed by Post hoc Tukey test and a statistical significance was set at $p < 0.05$ with a $p < 0.05$, b $p < 0.01$ and c $p < 0.001$. Means along the row with different superscript are significantly different ($p < 0.05$). Group (A - D) Rats were respectively dosed with probiotics isolates (A20), (A27), (A46), and (A56) and challenged with castor oil. (E) Rats challenged with castor oil only (positive control). Rats not dosed (negative control).

4. Discussion

We isolated and characterised LAB from a local traditionally fermented food and investigated their immunomodulatory effects on various parameters of the immune systems. From their cell morphology and biochemical characteristics, all isolates were gram positive, rod shape, non-motile and catalase negative suggesting that all belong to the genus *Lactobacillus*. This study is similar to that of Cai *et al.* [10] who identified Lactobacillus species from yogurt samples based on their morphological characteristics and different biochemical characteristics such as Gram and catalase reaction, motility, sugar fermentation pattern. Microscopically the isolated bacteria were Gram positive, rod shaped, non-motile, and catalase negative. the results of our study corroborate with that of the study lead by Ngwa [11] who successfully isolated lactic acid bacteria from palm wine.

One of the major selection criteria of probiotics is resistance to low pH. Probiotics must remain viable during their passage in the gastrointestinal tract in population levels of 10^6 - 10^7 cfu/ml in order to deliver their health benefits [12]. The acid environment of the stomach and the inhibitory effects of bile salts secreted in the duodenum are the major obstacles against probiotic survival. The *in-vitro* evaluation of the survival of the potential probiotic strains in simulated GI tract conditions may only be necessary in predicting the actual *in vivo* survival of a strain when consumed in a non-protected way [13]. In the current study, pH value of 2.5 was used, in order to select potential probiotic strains. Such low pH value is very selective and although it is not the most common pH value encountered in the stomach, it guaranties the isolation of the acid-tolerant strains [14]. All the isolates obtained were more or less tolerant at pH 2.5. Among the 19 isolates, 9 (A11, A20, A27, A31, A36, A42, A46, A51, A56) showed a good tolerance at pH 2.5 after 3 h of incubation period, suggesting that these LAB strains could survive the gastrointestinal tract and function effectively. The resistance to acid could be due to the fact that LAB are usually faced with stable low pH or sudden and transient acid stress [15] [16]. Several mechanisms are involved in the acid resistance regulation of LAB, including central metabolic pathways, proton pump, changes of cell membrane composition and cell density, DNA and protein damage repair, as well as neutralization processes [15] [17]. [13] [18] [19] isolated 90 strains of *Lactobacillus fermentum* from traditional dairy products, among them 35 strains showed tolerance against low pH, which directly indicate the similarity with our findings. A similar research was also conducted with 13 strains of *Lactobacillus plantarum*, and 7 strains were highly tolerant against low pH [20].

Tolerance to bile is one of the most essential attributes for probiotic bacteria, as it ascertains their ability to survive in the small intestine, and accordingly their ability to play a functional role as probiotics [21]. Bile response is a complex phenomenon, involved a variety of processes. Active efflux of bile salts/acids, bile salt hydrolysis and changes in the design/composition of cell membrane and cell wall, seem to be the most basic bile-specific mechanisms for resistance in Lactobacillus species [22]. Majority of the strains could survive 0.2% bile concentration after 4

h incubation period, suggesting a potential recovery of the initial levels during the passage through the small intestine. However the poor tolerance (poor survival) observed at 0.4% bile for isolates A11, A42 and A51 could be explained by the fact that after bacterial exposure to this bile salt concentration, disruption of cellular homeostasis occurred that caused the dissociation of lipid bilayer and integral proteins of their cell membranes, resulting in bacteria content leakage and finally death of the cell [23]. Previous studies carried out by Kingwatee *et al.* [24] showed that *Lactobacillus casei* 01 strain was very sensible on different bile salt concentrations (0.3%, 0.5%, and 1%), after 30 min of incubation of cells with simulated intestinal juice, there were no viable cells. In contrast, Jothi *et al.* [25] showed that the strains Lactic acid bacteria VJ15 and LAB VJ 32 were sensitive at 1% and 2% bile concentration under 12 h incubation period. Furthermore, studies point out the huge variability in bile resistance that can be encountered within a species or genus [26] revealing that bile tolerance is a strain-dependent feature and tolerances of species cannot be universal [23].

The results of the haematological parameters revealed that rats dosed with LAB isolates showed signs of better health based on their haematological status. All the isolates induce an increase of red blood cells. However, the increase was significant in group A (A20) suggesting that this isolate has the capacity to stimulate red blood cells production in the host. There was a direct relationship among some haematological indices. For example, increase in RBCs count led to the increase in haematocrit level. The probiotics did not have an effect in the MCV, MCH and haemoglobin level of the animals since there was no significant difference between the test groups and Controls. The decrease in white blood cell (WBC) count observed in the probiotic treated groups, could be considered as clinical physiology related to the presence of a disease [27]. The significant increase observed in the platelet counts especially in group D (A56) may indicate possible effect on blood flow, since platelets are determinants of blood viscosity which correlates positively to blood pressure. These results are parallel with a study carried out by Tiepma *et al.* [9] in which they studied the immunostimulatory effect of lactobacillus from raffia wine and observed an increase in red blood cells and a decrease in white blood cells count in rats fed with probiotics when compare with positive and negative controls. Indices of white blood cells, such as the differential leucocytes, are the principal haematological parameters which confers immunity. They defend the body against foreign proteins. Circulating blood leukocytes are required to migrate to sites of tissue injury and infection with the principal aim of eliminating the primary inflammatory trigger and contributing to tissue repair. In innate immunity, this process is largely initiated by pathogen-associated molecular patterns (PAMPs), released by invading microorganisms, and damage associated molecular patterns (DAMPs), derived from damaged and/or dead-cells, or in response to tissue and/or cellular stress [28]. In addition, antigens, largely through activation of resident memory T cells, can trigger recruitment of leukocytes via secretion of various primary inflammatory cytokines. Tissue sentinel cells, including mast

cells, macrophages, and dendritic cells (DCs), play a key role in detection of such danger signals and can release a wide range of pro-inflammatory mediators to promote leukocyte recruitment. Isolates A20 and A27 significantly increase lymphocytes production in the host therefore would enhance the immune system as they occur to immune cell-mediated responses. This significant increase of lymphocytes could be an indicative of their abilities to boost the immune system in the host against invaders. The significant increase in the rate observed in monocytes of group B (A27), C (A46) and D (A56) compare to positive and negative controls group could be attributed to the recognition of a foreign body or a foreign substance by the host because the circulating blood monocytes are the precursors of macrophages [29]. Maldonado Galdeano *et al.* [30] had previously observed that lactic acid bacteria of yoghurt could increase activity of lymphocytes and macrophages. Like the resident flora, probiotics can interfere with the immune system of the host. The walls of gram (+) bacteria stimulate monocytes (macrophages). In fact, there is an increase in macrophage response in the presence of lipoteichoic acids, which is a component of the membranes of gram (+) [29]. This significant increase obtained is in agreement with the postulation that the success of *lactobacillus spp*. as an immunomodulator is linked to its ability to bind to PRR expressed on immune cells and the cascade of other events that follows. [31]. Similar stimulation results have been obtained on the clinical studies conducted in humans which converge to suggest modulation of innate immunity (activation of phagocytosis and lymphocytes) by oral administration of various strains of lactobacilli and bifidobacteria [32]. The rate of neutrophils corroborates with the white blood cells, an increase in those two factors could mean the presence of infection. The high rate of eosinophil's observed in group B (A27) suggest that this isolate could stimulate the immune system by a mechanism involving phagocytosis. No change in the rate of basophils across all the groups could mean that probiotic isolates could not significantly boost the increase or decrease of the latter also involved in phagocytosis.

The increase of spleen proteins in the animals of groups A20 and A27 (in which there were an increase of red blood cells and an increase of lymphocytes) suggest a stimulation of the immune system because the spleen is an immune organ. [31] we believe, the increase of proteins level in this organ would be linked to an increase of antibodies.

It is widely recognized that castor oil is metabolized into ricinoleic acid in the gut, which in turn irritates and causes inflammation in the intestinal mucosa, resulting in the release of inflammatory mediators. It is generally believed that the inflammation process is regulated by pro-inflammatory and anti-inflammatory cytokines. Today, increasingly prevalent and intensive studies on probiotics show the effects of direct or indirect stimulation of the endogenous flora and the immune system [33]. Animal models and human clinical trials show that probiotics affect cytokine release and reduce inflammation. It has recently been shown that the gut microbiota induces macrophages and dendritic cells (DCs) to produce IL-

6 and IL-1-β, which both trigger Th17 differentiation. The same cytokines also trigger the differentiation of IL10-producing B regulatory cells. TNF-α and IL-6 play important roles in some immune functions and metabolic disorders. Some studies have claimed that probiotic application can lead to an increase in the TNF-α and IL-6 levels, while others state otherwise. For example, Morita *et al.* performed a study testing several probiotic bacteria, and they reported that all of the tested bacteria increased the production of IL-6, IL-12, and TNF-α. Moreover, Kaur *et al.* [34] reported that Lactobacillus and Bifidobacterium induced the production of TNF-α and one study reported that the application of *L. casei* led to decreases in the TNF-α and IL-6 levels. On the other hand, in another study, the probiotics reduced the expression of TNF-α, IL-6, beta-defensin 2, and Toll-Like Receptor-2. The differences between the scientific studies with regard to the levels of the cytokines could be explained by the fact that the effects/activities of probiotics are strain specific, and that a combination of probiotics could be beneficial. Probiotics have proven to be effective in inflammation. Recent reports have suggested that the administration of probiotic may have beneficial anti-inflammatory activity. In experimental studies, probiotic supplementation was found to have a potential to reduce TNF-α expression [34]. In another studies, Ayob *et al.* [35] and Maia *et al.* [36] showed that probiotic usage can reduce the IFN-γ, IL-1β, TNF-α and IL-8 levels. We also reported in the current study that the probiotic isolates reduced TNF-α, IFN-γ, IL1-β and IL-6 levels in treated groups when compared with the positive control group. This decrease could be as a result of the probiotics inducing the production of anti-inflammatory molecules such as IL-10 which is produced mainly by monocytes, T cells, B cells, natural killer cells, macrophages, and dendritic cells, and regulates the inflammatory process by inhibiting the over expression of many pro-inflammatory cytokines, chemokines, and chemokine receptors. Thus, Probiotic possesses anti-inflammatory properties, which may account for its efficacy against acute inflammatory conditions and diarrhoea induced by castor oil in rats.

The results of the effect of the isolates on the respective aspects of the immune system may now give us an insight and possible scientific explanation on the robust health of the "mbororo" people. The result clearly showed that the isolates had a marked effect on innate and cellular immunity. These isolates stimulated both innate and cell mediated immune response in rats and prevented the incidence of diarrhoea associated with purgative components. They also suppress excessive release of pro-inflammatory cytokines, which plays an important role in the onset of the inflammatory process during any antigenic challenge. IsolatesA20 and A27 stimulated mostly cell-mediated immune response whereas isolates A46 and A56 mostly innate immune response. These isolates may offer exciting opportunities in food and dairy sector for their use to produce probiotic fermented products with functional properties. They may also serve as novel human probiotic organisms which may help in protection from various diseases such as diarrhoea, acute and chronic inflammatory conditions.

5. Conclusions

Cameroonian traditionally fermented cow milk is a rich source of LAB isolates as revealed by preliminary identification and amplification of the 16S rRNA. These isolates could withstand the acidic pH of the stomach and the bile salt content of the intestine *in vitro* and therefore could survive the harsh environment of the gastrointestinal tract and exert their functions as probiotics. Oral administration of these live probiotic lactic acid bacteria stimulated the immune system and down-regulated excessive release of pro-inflammatory cytokines which play an important role in the onset of inflammatory process.

This study had some limitations: the antibiotic susceptibility and the virulence screening tests of the selected isolates were not done to ascertain their safeness, particularly for Enterococcus faecium. Also, the sequences of the selected isolates were not deposited to NCBI GenBank database to have accession number.

Acknowledgements

We are grateful to all who participated in this research.

Authors' Contributions

TBF, conceived and designed the study: TBF, ABTV and TTLL implement the study: TBF and MEE supervised the study. TBF, TTLL and ABTV conducted data analysis: TBF, ABTV and TTLL interpreted study results: TTLL wrote the first draft of the manuscript, DDF and TB reviewed and corrected the manuscript. All authors approved the final copy.

Ethical Approval

The investigations were performed in accordance with the institutional protocols established by the University of Buea Institutional Animal Care and Use Committee (UB-IACUC), ethical clearance permit number: UB-IACUC no. 015/2019.

Conflicts of Interest

The authors declare no competing interests.

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Definitions, Acronyms, Abbreviations

LAB	Lactic Acid Bacteria
IBDs	Intestinal Bowel Diseases
NEC	Necrotizing Enterocolitis
TNF	Tumour Necrosis Factor
HCL	Hydrochloric Acid
NaOH	Sodium Hydroxide
EDTA	Ethylenediamine Tetraacetic Acid
DNA	Deoxyribonucleic Acid
PCR	Polymerase Chain Reaction
BLAST	Basic Local Alignment Search Tool
BLASTN	Basic Local Alignment Search Tool for Nucleotide
UB-IACUC	University of Buea Institutional Animal Care and Use Committee
CFU	Cell Forming Unit
ELISA Kit	Enzyme-Linked Immunoassay kit
IL-1β	Interleukin 1 Beta
SEM	Standard Error of the Mean
ANOVA	One-way Analysis of Variance
CI	Confidence Interval
NCG	Negative Control Group
NGpbs	Normal Group
APC	Antigen Presenting Cell
ATR	Adaptive Tolerance Response
CFU	Colony Forming Units
DC	Dendritic Cell
MAMPs	Microbe-Associated Molecular Patterns
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Volume