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Research Article

Keywords: Anacardium occidentale, Attalea speciosa, Cytokine, Lactobacillus casei, Mice, Nitric Oxide

Posted Date: November 20th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11272167/v1

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Additional Declarations: No competing interests reported.

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Abstract

Sepsis triggers an exacerbated systemic inflammatory response associated with the disseminate infection. A promising adjuvant approach in sepsis management is the use of probiotics, which can modulate the intestinal microbiota, improve immune responses, and attenuate inflammation. In this context, the present study aimed to characterize the physicochemical properties of a mixed fermented mixed beverage (PBC) composed with babassu almond milk, cashew apple fermented juice (13,9%) enriched with *Lactobacillus casei* by assessing its phytochemical characteristics, antioxidant activity, and to evaluate the immunomodulatory and antimicrobial effects of prophylactic consumption on septic mice,. Physicochemical analysis determined total phenolic compounds and flavonoids. The *in vitro* analysis investigated the cytotoxicity, antioxidant activity, and anti-inflammatory potential. Subsequently, for the *in vivo* evaluation Swiss female mice received BMF for 15 days, After this period the sublethal sepsis was induced in by cecal ligation and puncture (CLP) . The animals were allocated into three groups: Sham - non-infected and untreated; CLP - infected and untreated and CLPPBC -

infected and treated with the fermented beverage containing babassu and cashew extracts. It was investigate the bacterial load by colony-forming units, the blood cellularity, and production of inflammatory mediators as nitric oxide and cytokine levels. The results demonstrated that the babassu-cashew mixed beverage exhibited significant antioxidant activity. Moreover, beverage consumption significantly reduced bacterial dissemination to the bloodstream ($p<0.0001$) and abdominal cavity ($p<0.0001$), attenuated pulmonary infiltration ($p<0.0054$), and decreased serum levels of pro-inflammatory cytokines IL-6, TNF-alpha, Interferon gamma ($p<0.0135$) and IL-17A ($p<0.0293$), which are key factors in sepsis progression and severity. PBC also increased IL-10, showing an anti-inflammatory effect. Altogether, the results indicate that our product has probiotic properties and is able to reduce the systemic damage caused by sepsis in mice.
Key words: *Anacardium occidentale, Attalea speciosa*, Cytokine, *Lactobacillus casei*, Mice, Nitric Oxide,

1. INTRODUCTION

Alternative strategies to conventional sepsis manegment gained increasing relevance, as this syndrome remains one of the major global public health challenges [1]. Sepsis has an estimated global incidence of 276 to 678 cases per 100,000 people per year and remains a major cause of morbidity and mortality, with a particularly significant impact among children and neonates [2]. Importantly, sepsis is characterized by an exacerbated systemic inflammatory response triggered by an underlying infectious process [3].

Sepis cause an inflammatory dysregulation that leads to severe functional and structural alterations in multiple organs [3], culminating in acute lung and kidney injury (ALI) [4, 5], and liver failure [6]. Moreover, sepsis induces splanchnic hypoperfusion and intestinal microbiome dysbiosis, increasing intestinal permeability. This disruption allows luminal contents to interact with resident immune cells, thereby influencing systemic immune response [7], The impairment of gastrointestinal barrier integrity contributes significantly to sepsis progression, severity and may result in death [8].

Current therapeutic approaches for sepsis remain insufficient, as they primarily focus on pathogen eradication and supportive care [9], often neglecting the need to correct immune system imbalances and restore the patient's intestinal microbiota [7,10]. This limitation underscores the urgent need to explore novel therapeutic strategies [11, 9]. In this context, a promising adjuvant approach to sepsis management involves the use of probiotics, which can modulate the intestinal microbiota, enhance immune responses, and attenuate inflammatory processes even when used prophylactically [12, 13].

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer health benefits to the host [14], [15]. The most commonly used probiotic genera include *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and *Saccharomyces* [16]. Lactic acid–producing bacteria, particularly those belonging to the genus *Lactobacillus*, exhibit significant antioxidant activity, which is mediated both by viable microbial cells and by the cell-free culture supernatant [17]. Moreover, these microorganisms produce bioactive molecules, such as bacteriocins, that inhibit the growth of pathogenic bacteria, enhance epithelial barrier function by increasing IgA (immunoglobulin A) production, and stimulate dendritic cells activation within the intestinal epithelium [18].

The primary benefit of probiotic intake is the restoration of the intestinal microbiome [19, 20]. Moreover, previous studies have highlighted the role of probiotics in infection prevention and their capacity to modulate the immune system, thereby contributing to host immune homeostasis [21- 25].

Probiotics have been incorporated into dairy-based foods [26],[27]. However, the increasing prevalence of lactose intolerance and milk allergies, adherence to vegan and vegetarian diets, the rising incidence of hypercholesterolemia, and the environmental impacts associated with dairy production have collectively driven

demand for non-dairy alternatives [28- 30]. The food matrix can directly influence microbial viability and adhesion at the site of action, regulate their release throughout the gastrointestinal tract, interact with the resident microbiota, and enhance both the nutritional and functional properties of the final product [31, 32].

Probiotics are increasingly incorporated into plant-based food matrices [32], including babassu (*Attalea speciosa Mart. ex Spreng; Arecaceae*), a palm tree species native to the Amazonian transition region with substantial economic and cultural relevance to local populations [33]. The water-soluble extract obtained from babassu almonds, popularly called babassu milk, is rich in fatty acids, carbohydrates, and dietary fiber, has high economic potential, and making it a favorable substrate for probiotic growth [34]. Similarly, *Anacardium occidentale* (cashew), belonging to the *Anacardiaceae* family, produces the cashew nut, which is widely consumed worldwide, and the cashew apple, a by-product representing approximately 90% of the fruit mass. Although underutilized, the cashew apple displays remarkable nutritional potential [35]. Cashew apple is rich in phenolic compounds, vitamin C, carotenoids, anacardic acids, tannins, and reducing sugars, which collectively contribute to its pronounced antioxidant [36], antimicrobial [37], and antibiofilm properties [38].

The use of plant-based probiotics in sepsis has gained prominence due to the urgent need for adjuvant therapeutic strategies for sepsis control, exerting antimicrobial, and modulatory effects on the exacerbated inflammatory response and restoring the gastrointestinal microbiota. This study investigated the functional properties of a mixed probiotic beverage formulated from babassu milk, cashew apple, and enriched with *Lactobacillus casei*. Specifically, the study aimed to characterize its physicochemical properties, assess its antioxidant activity, and explore the *in vivo* impact of prophylactic consumption in controlling microbial load as well as its effectiveness as immunomodulatory in septic mice, .

2. MATERIALS AND METHODS

2.1 Preparation of the mixed probiotic beverage based on extracts from babassu coconut almond and cashew apple.

The production process of the fermented mixed beverage (PBC) was previously described by Madeira et al. [39]. Briefly, to obtain the water-soluble babassu coconut extract, fresh babassu almonds sourced from the Coquelândia community in the municipality of Imperatriz, Maranhão, Brazil, were combined with water at a 1:2 (w/v) ratio. The water-soluble extract was prepared using a multifunctional food processor (Vegan Milk Machine), which includes a specific “milk” function designed for the production of crude aqueous extracts. The resulting extract was filtered to remove solid residues and subsequently cooled to room temperature (25 °C).

For the preparation of the mixed beverage, cashew pulp purchased from local markets in the city of Imperatriz, Maranhão, Brazil, was added at a concentration of 13.9%, according to previously established growth conditions. Fermentation was initiated by inoculation with activated *Lacticaseibacillus casei* NRRL B-442. The inoculum was prepared by transferring a glycerol stock culture of *L. casei* B-442 into sterile MRS broth. The culture was incubated until the optical density at 600 nm (OD600) reached 0.600, corresponding to 9.00 log CFU/mL The fermentation process was carried out in an incubator under aerobic conditions at 30 °C for 24 h, as previously described [39]. Viable cell counts were subsequently determined to evaluate bacterial growth and fermentation performance.

The viability of the probiotic in the babassu–cashew mixed beverage was determined by serial dilution. Aliquots of 0.1 mL from the appropriate dilutions were plated onto MRS agar (De Man, Rogosa and Sharpe agar). The plates were incubated at 37 °C for 72 hours, as previously described [40].

2.2 pH measurement

The pH of the probiotic beverage from babassu and cashew (PBC) was measured using a potentiometer equipped with a combined electrode by direct reading, according to previously described procedures [41].

2.3 Acidity determination

Acidity was determined by measuring the lactic acid content in 1 mL of the sample diluted in distilled water. Titration was performed with NaOH, using three drops of phenolphthalein as an indicator, as described by [42].

2.4 Phytochemical analysis

2.4.1 Determination of total phenolic content

Total phenolic content was determined using the Folin–Ciocalteu reagent. For quantification of phenolic compounds, 100 µL of the undiluted supernatant from the fermented beverage was mixed with 1 mL of a 20% sodium carbonate solution and 100 µL of the Folin–Ciocalteu reagent (*Vetec Química Fina*, Brazil). Distilled water was then added to obtain a final volume of 4 mL per tube. Samples were prepared in triplicate, homogenized using a vortex mixer, and incubated in the dark for 2 hours. Absorbance was measured at 760 nm using a UV–Vis spectrophotometer (Gehaka UV-340G). The concentration of phenolic compounds was determined from a gallic acid standard curve and expressed as gallic acid equivalents per gram of sample [43].

2.4.2 Determination of flavonoid content

Flavonoid content was assessed using the aluminum chloride colorimetric method. Briefly, 500 µL of the beverage supernatant obtained by centrifugation was mixed with 500 µL of a 5% aluminum chloride hexahydrate solution and 3 mL of methanol. The samples were homogenized using a vortex mixer and incubated in the dark for 30 minutes. After the reaction period, samples were centrifuged at 2,000 rpm, and absorbance was measured at 425 nm using a UV–Vis spectrophotometer (Gehaka UV-340G). All analyses were performed in triplicate using a quercetin standard curve (Sigma-Aldrich, São Paulo, SP, Brazil), and results were expressed as quercetin equivalents per gram of sample [44].

2.5 Cell culture

Murine macrophages (RAW 264.7) and human fibroblasts (GM07492A) were used for cytotoxicity assays. Cells were maintained in sterile cell culture flasks containing Dulbecco's Modified Eagle Medium (DMEM; Gibco™) supplemented with penicillin (100 µg/mL), streptomycin (100 U/mL), amphotericin B (0.25 µg/mL), and 10% fetal bovine serum. In addition, RAW 264.7 macrophages were supplemented with 1% sodium pyruvate. Cell cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.6 Cytotoxicity assay

An MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was performed to assess cell viability in two cell lines: RAW 264.7 and GM07492A. Cells were treated for 24 hours with PBC supernatant filtered through a 0.22 µm bacteriological filter and diluted in DMEM at ratios of 1:2, 1:4, 1:8, 1:16, and 1:32, or with culture medium alone as the control. Subsequently, the supernatant was replaced with fresh culture medium, and 10 µL of MTT solution was added. The plates were incubated for an additional 3 hours in the dark. After incubation, the medium was removed and 100 µL of dimethyl sulfoxide was added to solubilize the formazan crystals. Plates were gently shaken for 5 minutes, and absorbance was measured at 540 nm using a BioTek® microplate reader, as previously described [41].

2.7 Antioxidant activity assessment

Only concentrations that did not exhibit significant cytotoxicity were selected for antioxidant activity assays.

2.7.1 DPPH radical scavenging assay

The antioxidant capacity of the mixed beverage was assessed by its ability to scavenge DPPH radicals (2,2-diphenyl-1-picrylhydrazyl). The supernatant was obtained by centrifugation at 9,700 rpm for 10 minutes and subsequently mixed with a methanolic DPPH solution at dilutions of 1:4, 1:8, 1:16, and 1:32. The reaction mixtures were incubated for 30 minutes at room temperature in the dark. After incubation, samples were centrifuged at 2,000 rpm, and absorbance was measured at 517 nm using a spectrophotometer. A methanol-DPPH mixture was used as the negative control, whereas a solution containing the fermented beverage supernatant and methanol served as the blank. An ascorbic acid solution (56.8 µM; equivalent to 10 µg/mL) was used as the positive control. , as previously described [45].

2.7.2 Assessment of antioxidant activity by lipid peroxidation

A lipid peroxidation assay was employed to assess the antioxidant capacity of PBC. In the first step, 25 µL of distilled water, 125 µL of beverage supernatant diluted in distilled water (at ratios of 1:4, 1:8, 1:16, and 1:32), and 250 µL of lipid substrate (5% egg yolk solution) were mixed. Subsequently, 25 µL of the Fenton reagent prepared at a 1:1 ratio using 0.14 M ferrous sulfate ($FeSO_4$) and 0.14 M hydrogen peroxide (H_2O_2) was added, and the mixtures were incubated for 30 minutes in the dark.

Thereafter, 750 µL of thiobarbituric acid, 750 µL of acetic acid, and 25 µL of trichloroacetic acid were added. The reaction mixtures were then incubated in a water bath at 95 °C for 60 minutes. After heating, 3 mL of butyl alcohol was added, followed by centrifugation at 3,000 rpm for 15 minutes. The upper butanol layer was collected, and its absorbance was measured at 532 nm using a spectrophotometer. A Trolox solution (59.9 µM; equivalent to 15 µg/mL) was used as the positive control, as previously described [46].

2.8 Nitric oxide quantification

RAW 264.7 macrophages were activated by incubation with lipopolysaccharide (LPS; 1 µg/mL). A cell suspension was prepared in 96-well microplates at a density of 1×10^5 macrophages per well and incubated overnight. The beverage supernatant was serially diluted (1:4, 1:8, 1:16, and 1:32) in DMEM and added to the cells 24 hours prior to LPS stimulation. Cells were then maintained under incubation for an additional 24 hours following LPS exposure. Subsequently, 50 µL of supernatant from each well was transferred to a new plate and mixed with 50 µL of Griess reagent. The reaction mixtures were incubated for 10 minutes in the dark, and absorbance was measured at 550 nm using a BioTek® microplate reader, as previously described [47].

2.9 Animals

In this study, 2-month-old female Swiss mice (average weight: 28 g) were used. The animals were obtained from the Central Bioterium of *Universidade Federal do Maranhão* (São Luiz, Brazil), where they were maintained at 26 ± 2°C and 44%–56% relative humidity under a 12-hour light–dark cycle and with free access to sterile food and water. The animals were kept in cages with dimensions $41 \times 34 \times 18\ cm^3$ (length × depth × height) with a removable grid. The study protocol was approved by the Animal Use Ethics Committee of *Universidade Federal do Maranhão* (Protocol: 23115.031277/2019-19.). All surgeries were performed under anesthesia, and efforts were made to minimize suffering.

2.10 Sepsis induction by cecal ligation and puncture (CLP)

Polymicrobial sepsis was induced by CLP as described previously [48, 49]. with minor modifications. Briefly, the mice were anesthetized with a mixture containing of ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (10 mg/kg), by intraperitoneal route. During the laparotomy the cecum was mobilized, ligated below the cecal valve, and punctured five times using an 18-gauge needle to induce sublethal sepsis. The cecum was then returned to the peritoneal cavity, and the abdomen was closed in two layers. Saline (0.5 mL/10 g body weight) was administered subcutaneously for fluid resuscitation. For analgesia, tramadol 10 mg/kg was administered subcutaneously 30 minutes before sepsis induction. After 24 hours, the animals were euthanized by an overdose

of anesthetics for subsequent analyses, (150 mg/kg ketamine hydrochloride and 120 mg/kg xylazine hydrochloride) [50, 51].

2.11 Experimental design

A total of five mice were randomly assigned to each experimental group: the Sham group, in which laparotomy was performed without cecal ligation or puncture; the CLP group, in which the cecum was ligated and punctured; and the CLP treated with PBC (PBC) group, in which mice were subjected to CLP-induced sepsis and treated with a probiotic beverage produced from fermented babassu nut extract and cashew pulp. The experiments involved the assessment of cellular, immunological, and functional parameters. Biological samples were collected after euthanasia to assess antimicrobial, anti-inflammatory, and immunological properties [49], [52].

2.12 Colony-forming unit determination

For bacterial quantification in blood and peritoneal fluid, the samples (10 µL) were diluted in 96-well plates containing 90 µL of phosphate-buffered saline (PBS) per well. Subsequently, 100 µL aliquots of these dilutions were plated on Mueller-Hinton. The plates were incubated at 37 °C for 24 hours to allow bacterial colony growth, followed by colony counting and CFU determination. The results are expressed as CFU/mL [49].

2.13 Spleen, bone marrow, lymph node, and bronchoalveolar cell counting

After euthanasia, the femur, spleen, and mesenteric lymph nodes were excised. The femur was flushed with 1 mL of PBS to isolate bone marrow cells. The mesenteric lymph node was crushed with 1 mL of PBS, while the spleen was washed with 5 mL of PBS and passed through a silk mesh. To obtain bronchoalveolar fluid, the trachea was exposed and cannulated with a catheter, followed by instillation of 1 mL of cold PBS. Cells were stained with 0.05% crystal violet (in 30% acetic acid) and counted using a bright-line Neubauer chamber (Sigma–Aldrich) under a standard light microscope with 400× magnification [54].

2.14 Cytokine quantification

The cytometric bead array (BD™ Cytometric Bead Array (CBA) Mouse Inflammation Kit (Cat. No. 552364)) technique was performed to quantify tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-17A, IL-10, IL-2, and IL-4, and interferon (IFN)-γ in the serum, as described previously using a mouse inflammatory cytokine kit (Becton Dickinson Biosciences, San Jose, CA, USA) [54].

2.15 Statistical analysis

Results are expressed as mean ± standard error and standard deviation and subjected to normality tests, followed by analysis using appropriate statistical tests. GraphPad Prism 8.0 software was used for all tests with a significance level of 5%. The Shapiro-Wilk test was used to assess the data distribution. For parametric samples, ANOVA followed by Tukey's post-hoc test was employed and t test, and for non-parametric tests, Kruskal-Wallis followed by Dunn's post-hoc test was used [55].

3. RESULTS

3.1 Enumeration of viable probiotic cells

PBC proved to be suitable for the growth and maintenance of the viability of the inoculated probiotics, exhibiting a cell count of 8.64 ± 0.04 log CFU/mL.

3.2 Physicochemical analysis, evaluation of pH and acidity, and determination of total phenolic and flavonoid contents.

Following fermentation of the food matrix, an increase in organic acid content was observed, accompanied by a corresponding decrease in pH, as shown in Table 1. In parallel, total phenolic compounds and flavonoids were detected in the fermented beverage.

Table 1. Physicochemical Analysis: pH, Acidity, and Phenolic and Flavonoid Contents of the Mixed Beverage.

pH Value	Acidity	C. Phenolics (mg EAG g -1)	Flavonoids (mg EQ g −1)
3.86 ± 0.03*	3.24 ± 0.18	8.8 ± 0.02	0.152 ± 0.005

(*) Results are expressed as mean ± SD, from triplicate

3.3 PBC showed low toxicity

In the cytotoxicity assay, the highest concentration of the fermented beverages (1:2) exhibited significant toxicity. In contrast, at the other concentrations, cell viability remained above 80%. Moreover, increasedcell proliferation was observed at the 1:32 dilution, as shown in Figure 1.

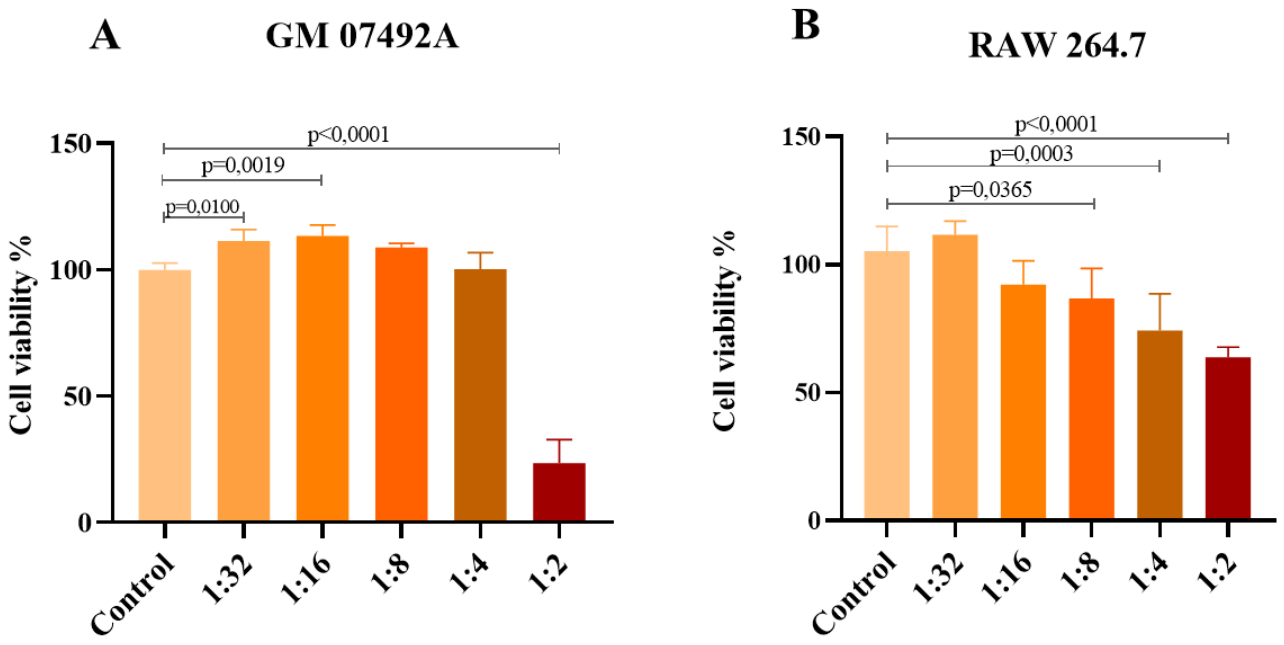


Figure 1.Cytotoxic effect of PBC (Probiotic Babassu and Cashew Beverage) in. (**A**) RAW 264.7 macrophages (1×10^5 cells/well) and (**B**) GM07492A fibroblasts (5×10^4 cells/well), The cells were incubated for 24 h with serial dilutions of the fermented beverage supernatant (1:2, 1:4, 1:8, 1:16, and 1:32). Cell viability was assessed by by adding 10 µL of MTT solution, followed by incubation for 3 h. Absorbance was measured at 540 nm using a microplate reader. The results are expressed as relative cell viability compared with the untreated control. Differences among groups were determined using the parametric ANOVA test. Data represent mean ± SEM of triplicate samples and are representative of two independent experiments.

3.4 PBC show Antioxidant activity

The antioxidant activity of the beverage was evaluated using the DPPH radical scavenging assay (Figure 2A). The positive control (ascorbic acid) exhibited a strong free radical–neutralizing capacity, differing significantly from the negative controlThe high levels of the PBC demonstrated antioxidant activity, with values comparable to the positive control. Only the lowest concentration showed a significant reduction in antioxidant activity compared with ascorbic acid. In the lipid peroxidation assay (Figure 2B), a significant difference was observed between the negative control and the positive control (Trolox), highlighting the effectiveness of the latter in inhibiting the oxidative process. The highest beverage concentration exhibited antioxidant activity comparable to that of Trolox, indicating a strong capacity to protect against lipid peroxidation. The remaining concentrations displayed a dose-dependent response, with a gradual decrease in inhibitory activity as the beverage concentration was reduced.

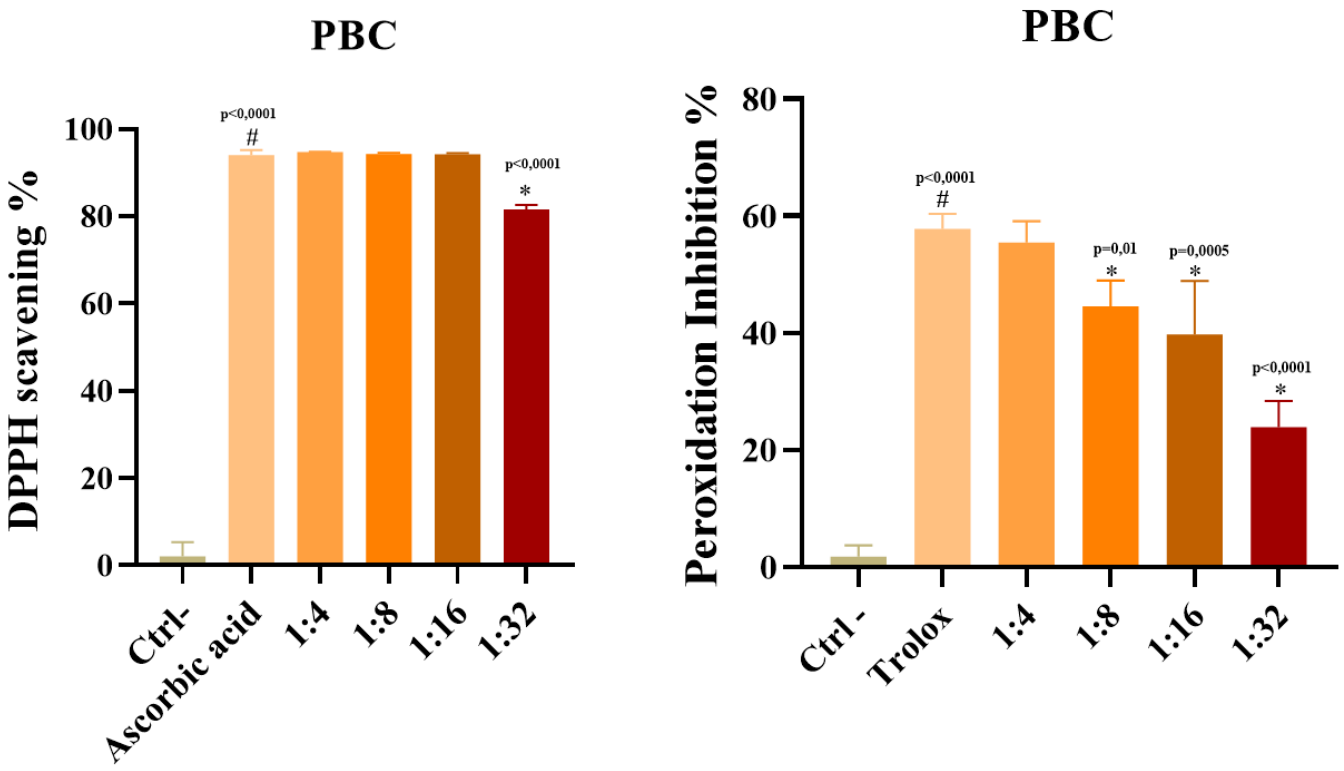


Figure 2. Antioxidant activity of PBC (Probiotic Babassu and Cashew Beverage) evaluated by. (**A**) DPPH radical scavenging activity and (**B**) Inhibition of lipid peroxidation. For DPPH assay PBC supernatants were serially diluted (1:4, 1:8, 1:16, and 1:32) and the samples were incubated for 30 min, in presence of DPPH. Ascorbic acid was used as positive control. For the analyses the samples were centrifuged, and quantifeid by spectrophotometry at 517 nm. For the lipid peroxidation assay PBCwas incubated with a lipid substrate and Fenton reagent as the oxidative inducer. After incubation the supernatants were removed and the thiobarbituric acid–malondialdehyde (TBA–MDA) complex was extracted with butanol and quantified by spectrophotometry at 532 nm. Differences among groups were determined using the parametric ANOVA test. Data are presented as mean ± SEM of triplicate samples and the results are representative of two independent experiments. (#) Significant difference compared with the negative control (Ctrl−), (*) Significant difference compared with the positive control (A: ascorbic acid; B: Trolox).

3.5 Nitric oxide measurement

The mixed probiotic beverage did not affect nitric oxide (NO) production when incubated simultaneously with LPS. However, after 24 hours of pre-incubation, the beverage increased NO levels at the two lowest concentrations

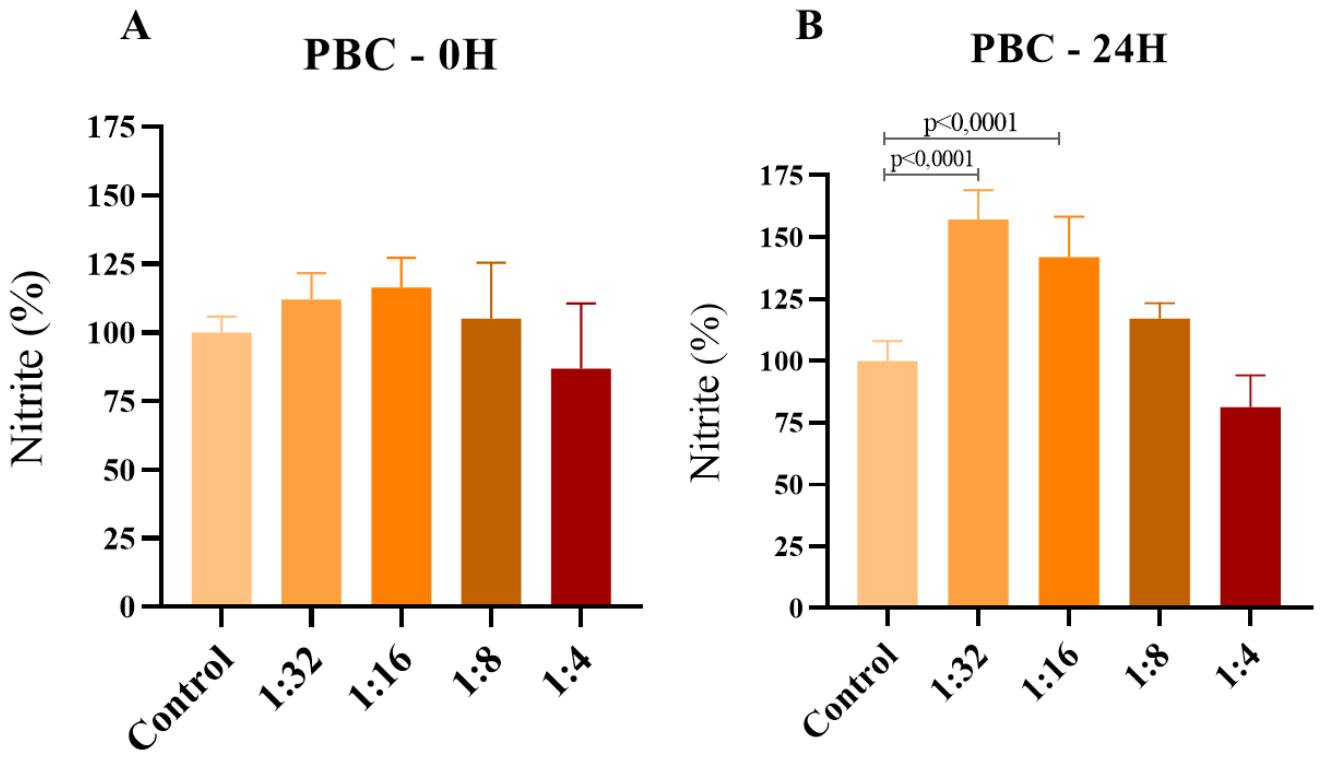
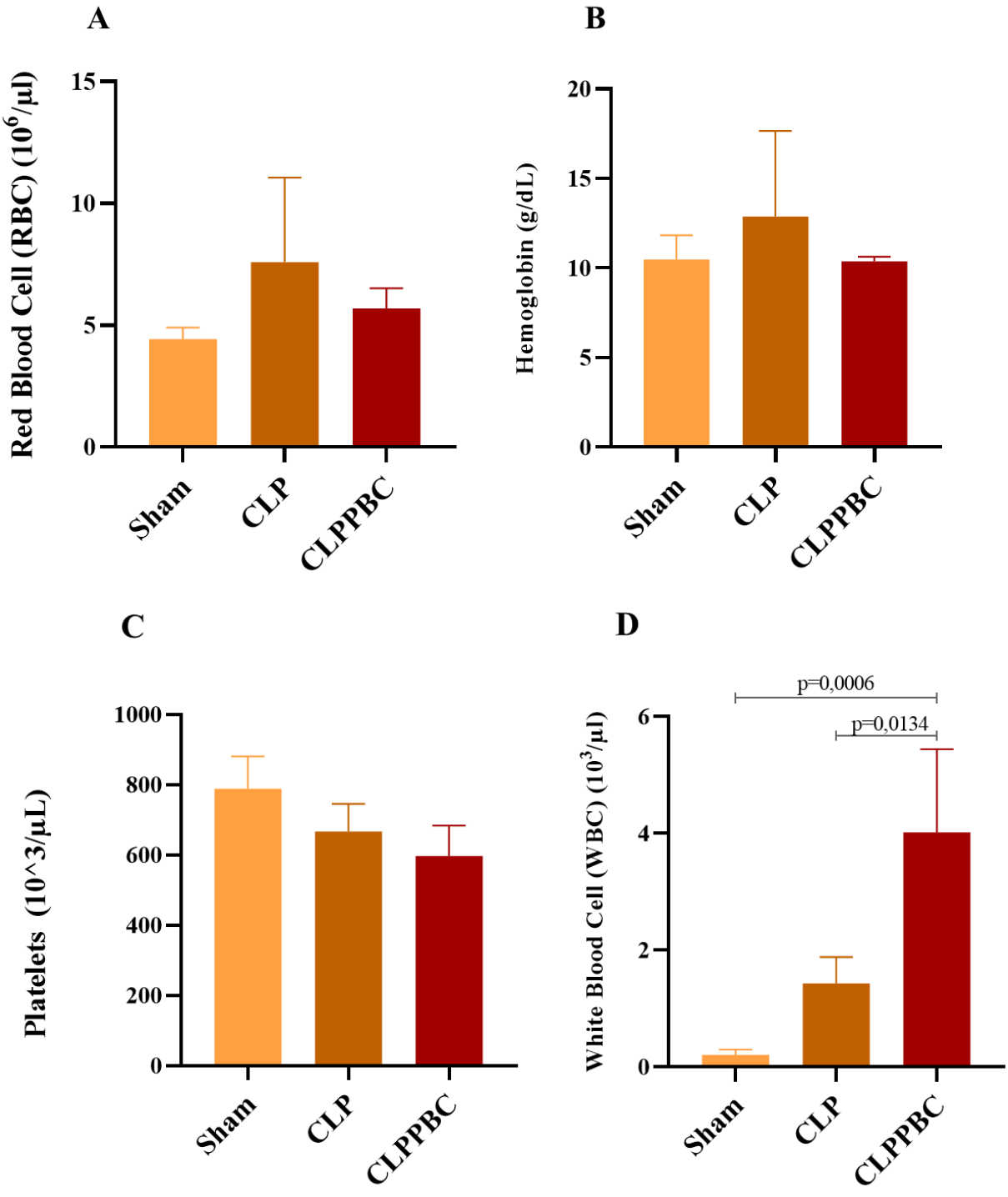


Figure 3. A macrophage activation assay was performed using RAW 264.7 cells stimulated with LPS. The probiotic beverage was tested at various dilutions (1:4, 1:8, 1:16, and 1:32) either as a simultaneous treatment (0 h) or as a pre-treatment (24 h). Nitric oxide (NO) production, an inflammatory marker, was quantified indirectly by measuring nitrite using the Griess reaction, followed by spectrophotometric reading at 550 nm. Differences between groups were analyzed using parametric ANOVA. Data are presented as mean ± SEM from triplicate wells and are representative of two independent experiments.

3.6 In vivo results in mice with CLP sepsis

3.6.1 Systemic effects PBC

Assessment of erythrocyte-related hematimetric indices revealed no statistically significant differences between treated and untreated groups (Figure 4A, B). Similarly, probiotic treatment did not affect platelet counts (Figure 4C) compared with either the CLP or Sham groups. In contrast, a significant increase in total leukocyte counts was observed in the group subjected to prophylactic treatment with PBC relative to both the Sham and CLP groups (Figure 4D). Consistent with this finding, a concomitant increase was also observed in the differential lymphocyte count (Figure 4E). No statistically significant differences were observed in monocyte, or granulocyte counts among the assessed groups (Figure 4F–G).


A
Red Blood Cell (RBC) (10^6/µl)
15
10
5
0
Sham
CLP
CLPPBC
B
Hemoglobin (g/dL)
20
15
10
5
0
Sham
CLP
CLPPBC
C
Platelets (10^3/µL)
1000
800
600
400
200
0
Sham
CLP
CLPPBC
D
White Blood Cell (WBC) (10^3/µl)
p=0,0006
p=0,0134
6
4
2
0
Sham
CLP
CLPPBC

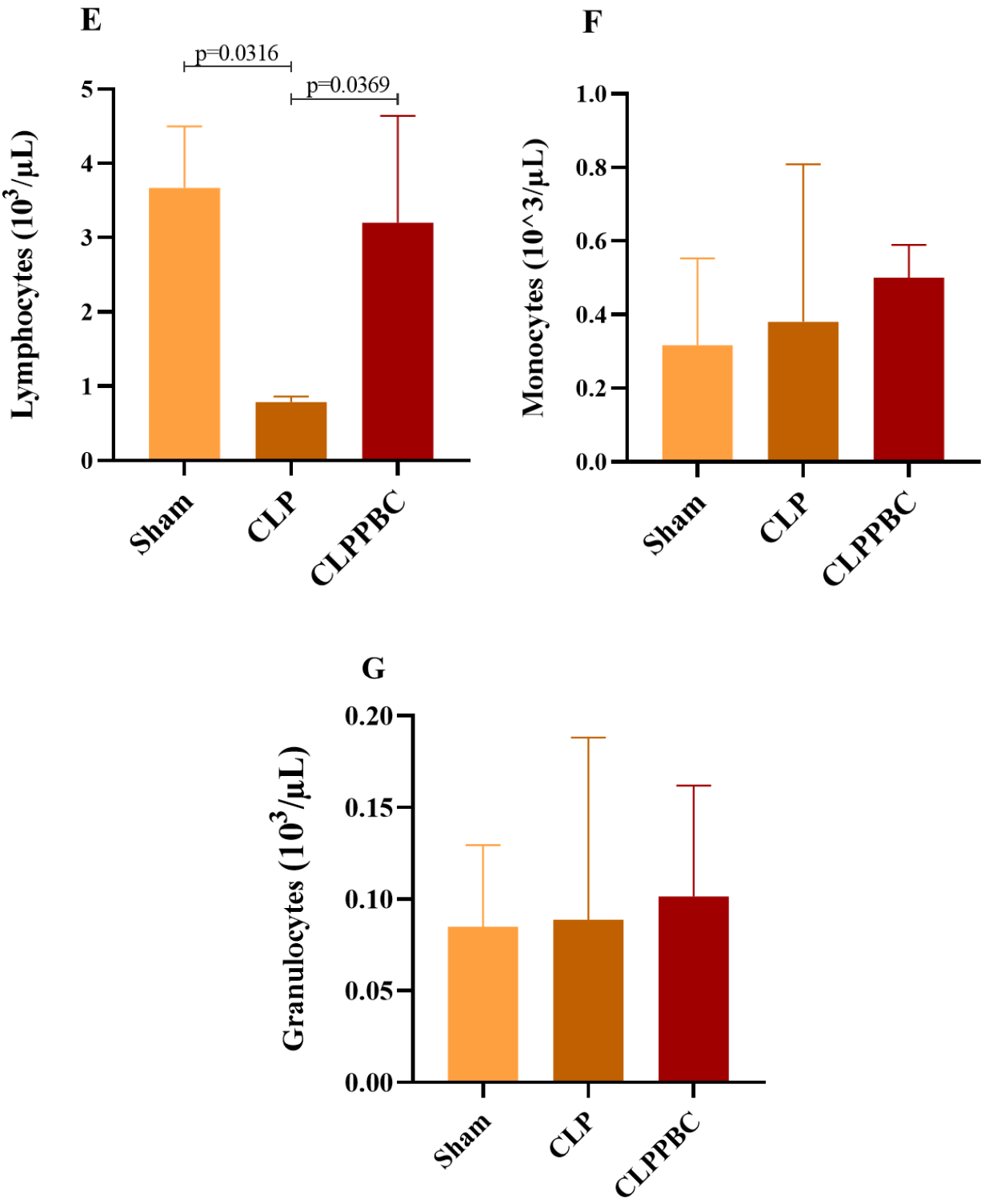
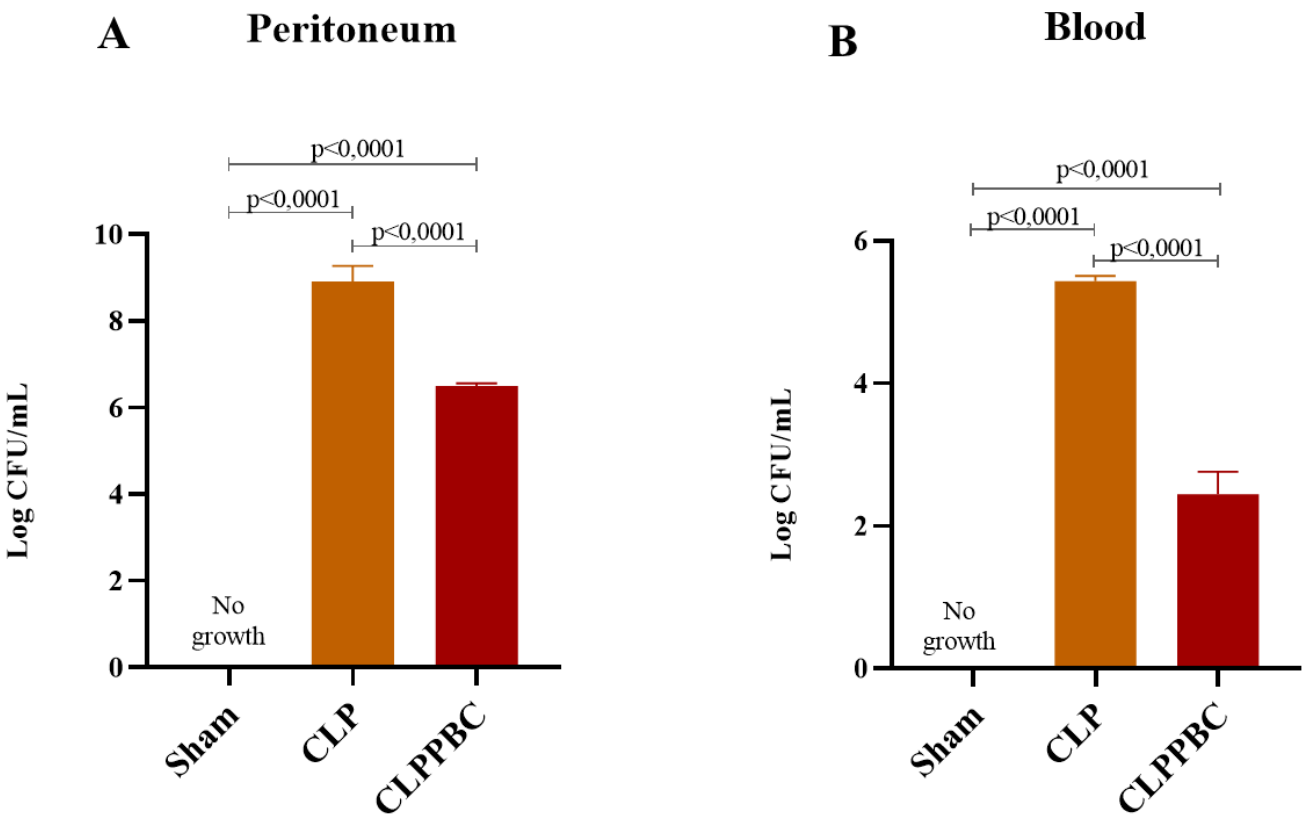


Figure 4. Systemic circulation. To evaluate the hematological profile, blood samples were collected from the animals and analyzed for the following parameters: (A) red blood cells (RBC); (B) hemoglobin; (C) platelets; (D) white blood cells (WBC); (E) lymphocytes; (F) monocytes; and (G) granulocytes. The experimental groups were as follows: uninfected and untreated (Sham); infected and untreated (CLP); and infected and treated with the fermented babassu–cashew extract beverage (CLPPBC).

3.6.2 PBC reduced the microbial dissemination during CLP sepsis

The establishment of infection was confirmed by significant CFU growth in the CLP group, with a marked difference compared with the uninfected Sham group. In the CLP group, mean log CFU/mL values reached 8.9 in the peritoneal cavity (Figure 5A) and 5.4 in the bloodstream (Figure 5B). In contrast, prophylactic treatment with PBC significantly reduced CFUs ($p < 0.0001$) in both the peritoneal cavity (Figure 5A) and the systemic circulation (Figure 5B).



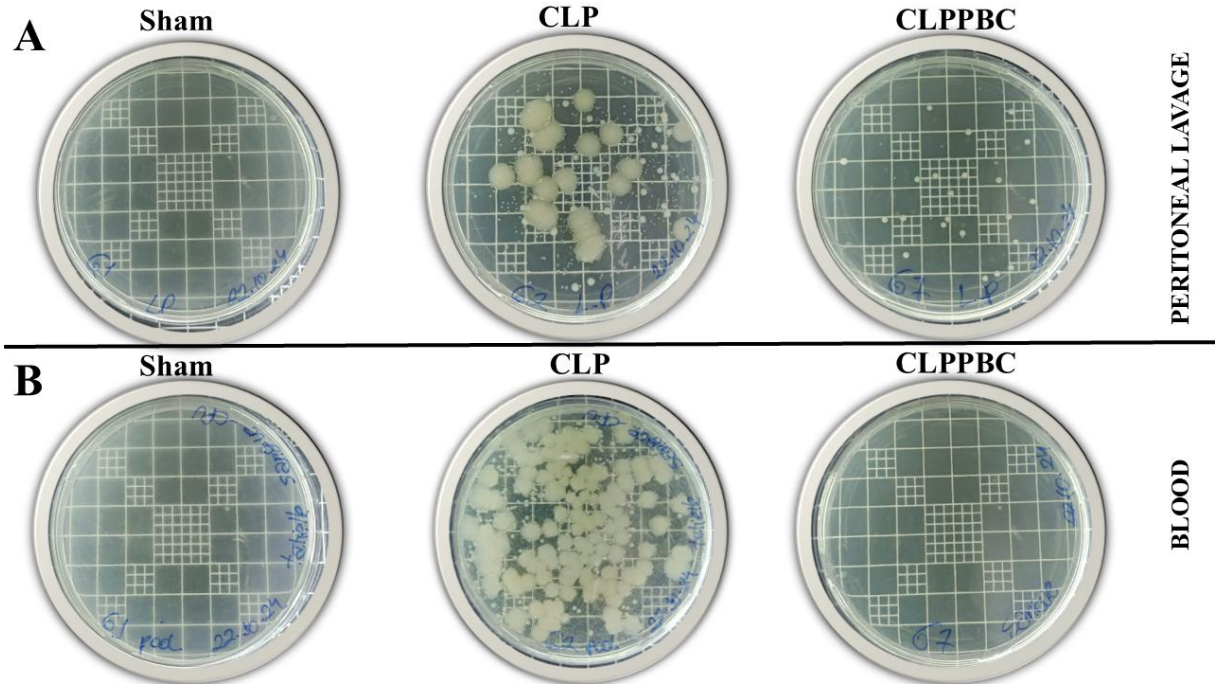


Figure 5 Colony-forming units (CFU)/mL on Mueller–Hinton agar. To quantify the microbial burden in the peritoneal lavage and blood, samples were collected, serially diluted, and plated onto Mueller–Hinton agar, followed by incubation at 37 °C for 24 h. After incubation, colonies were counted and data were expressed as log CFU/mL. The experimental groups were uninfected and untreated (Sham), infected and untreated (CLP), and infected and treated with the babassu–cashew extract beverage (CLPPBC). (A) Peritoneal lavage; (B) Blood. Results are expressed as mean ± SD.

3.6.3 Treatment with PBC increase the cell recruitment to lymphoid organs

Treatment with PBC significantly increased the number of splenic cells compared with the CLP group ($p < 0.05$) (Figure 6A). Similarly, an increased number of cells was observed in the lymph nodes relative to the CLP group ($p < 0.05$) (Figure 6B). Notably, this increase was also significant when compared with the Sham group, suggesting that PBC exerts an immunomodulatory effect even in the absence of established infection

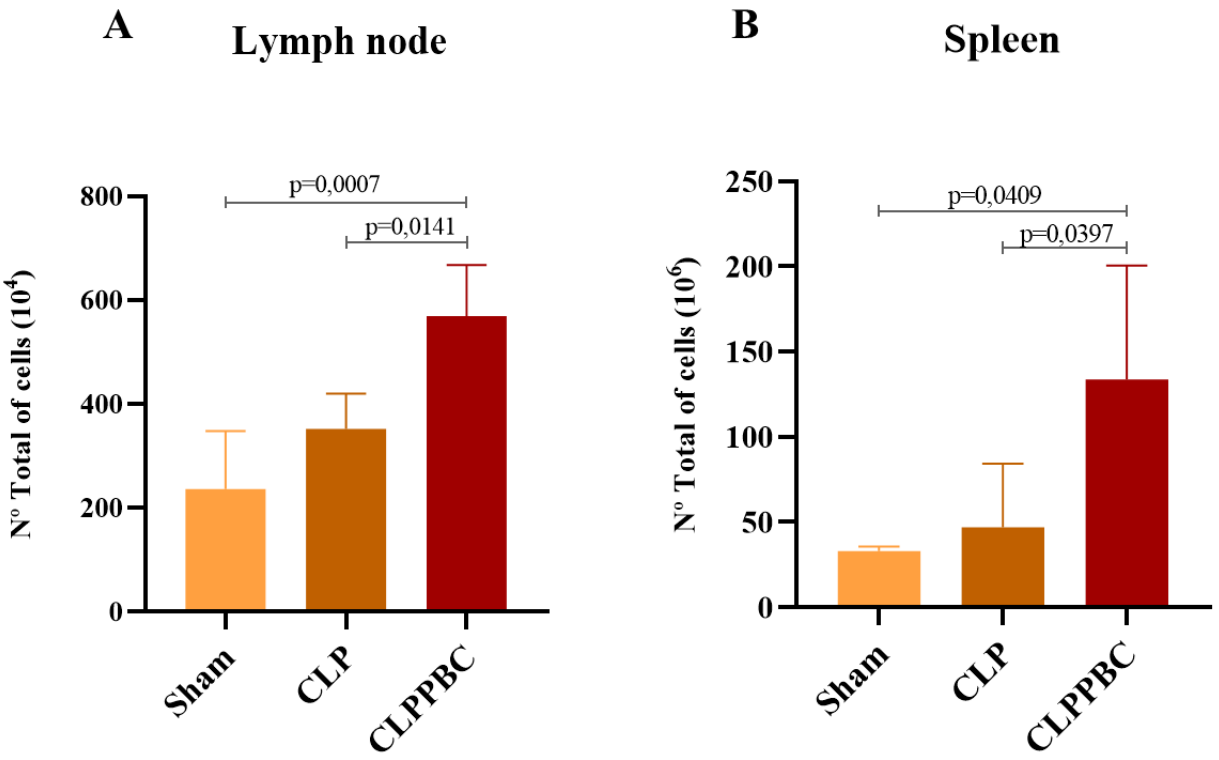


Figure 6. Cellular recruitment to peripheral lymphoid organs. To determine the total number of immune cells in tissue homogenates, cell suspensions were prepared and counted using a Neubauer chamber under light microscopy. The experimental groups were uninfected and untreated (Sham), infected and untreated (CLP), and infected and treated with the babassu–cashew extract beverage (CLPPBC).

3.6.4 PBC prophylactic treatment reduced the pulmonary inflammation

The CLP group exhibited a significant increase in pulmonary inflammatory infiltrate compared with the Sham group, confirming the establishment of the characteristic inflammatory response associated with sepsis. However, prophylactic treatment with PBC markedly attenuated this process, significantly reducing inflammatory infiltrate relative to the CLP group (Figure 7). These findings suggest a protective effect of PBC against sepsis-induced pulmonary inflammation.

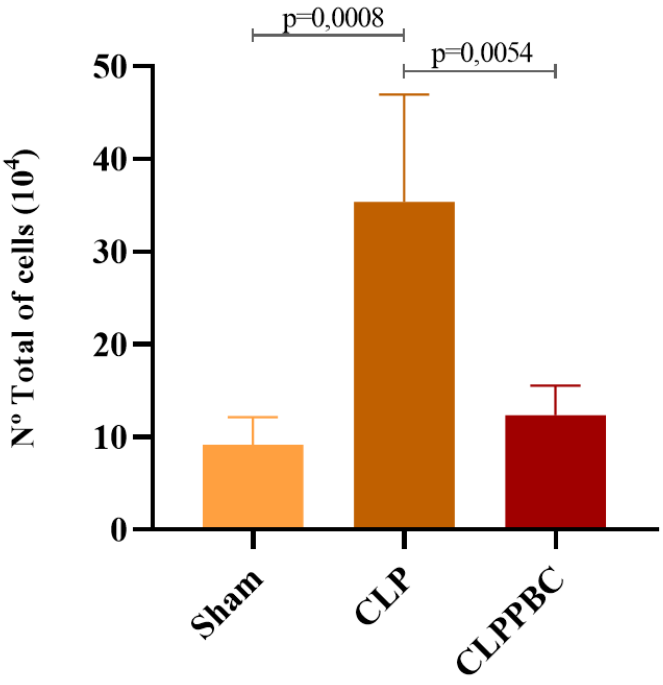
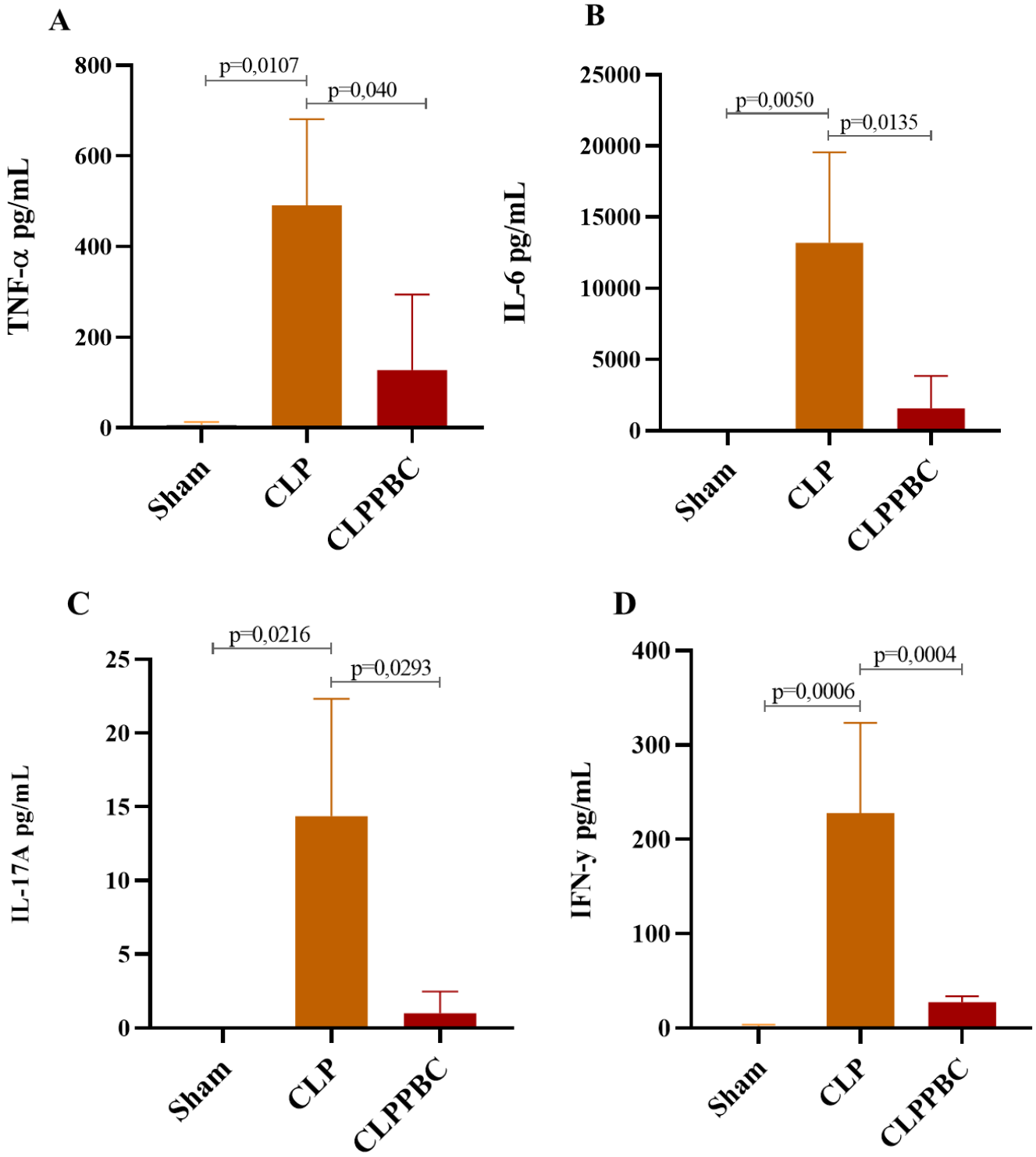


Figure 7 Total cell counts in bronchoalveolar fluid (BAL). To determine the total number of immune cells in the bronchoalveolar lavage, cell suspensions were prepared and counted using a Neubauer chamber under light microscopy. The experimental groups were uninfected and untreated (Sham), infected and untreated (CLP), and infected and treated with the babassu–cashew extract beverage (CLPPBC).

3.6.5 Treatment with PBC decrease the systemic inflammatory cytokines levels

Cytokine production was analyzed in serum. Levels of the pro-inflammatory cytokines TNF-α, IL-6, IL-17A, and IFN-γ were significantly elevated in CLP group compared with the Sham, confirming that sepsis increases these inflammatory mediators. In contrast, the treatment with PBC decrease the inflammatory cytokines levels when compared with the CLP group (Figure 8A–D).. Notably, treatment withPBC increased IL-10 levels in relation to either CLP or Sham groups (Figure 8E). Sepsis increased IL-4 and IL-2 levels relative to the Sham group; however, no significant differences were observed between PBC and CLP groups (Figure 8F–G).


A
TNF-α pg/mL
800
600
400
200
0
p=0,0107
p=0,040
Sham
CLP
CLPPBC
B
IL-6 pg/mL
25000
20000
15000
10000
5000
0
p=0,0050
p=0,0135
Sham
CLP
CLPPBC
C
IL-17A pg/mL
25
20
15
10
5
0
p=0,0216
p=0,0293
Sham
CLP
CLPPBC
D
IFN-y pg/mL
400
300
200
100
0
p=0,0004
p=0,0006
Sham
CLP
CLPPBC

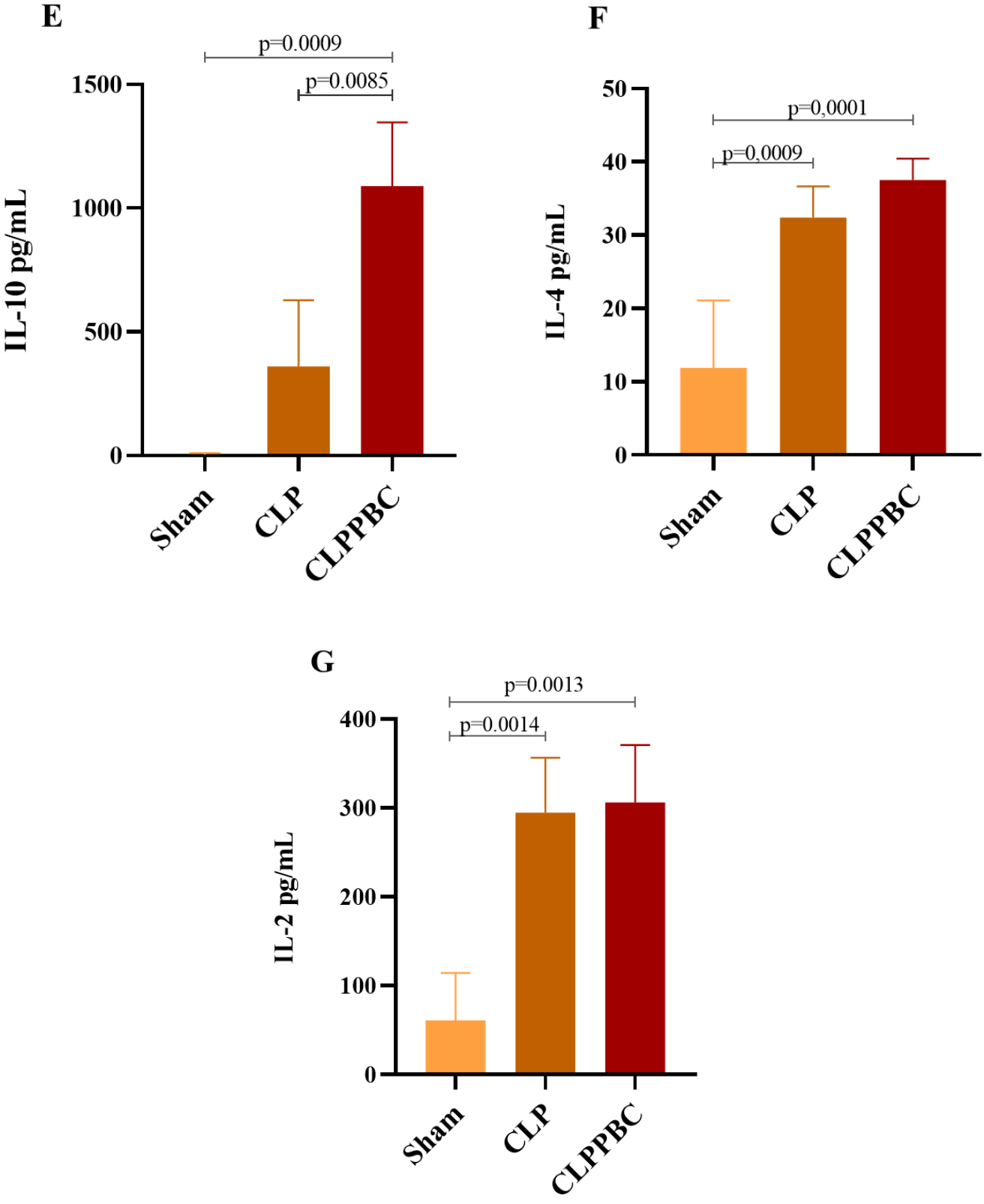


Figure8 Serum cytokine quantification. Animal serum samples were used to determine the concentrations of TNF-α, IL-6, IL-17A, IFN-γ, IL-10, IL-4, and IL-2 using the Cytometric Bead Array (CBA) cytokine kit, according to the manufacturer's instructions (BD Biosciences). The experimental groups comprised infected, untreated animals

(CLP) and infected animals treated with the fermented babassu and cashew extract beverage (CLPPBC). Data are presented as mean ± SEM (n = 4 animals per group).

4. Discussion

The present study evaluated the biological effects of a plant-based beverage formulated with babassu (*Attalea speciosa*) kernel milk, cashew apple (*Anacardium occidentale*), and *Lactobacillus casei* (PBC) in experimental sepsis. The beverage showed *in vitro* antioxidant activity, with redox-regulating mechanisms. Prophylactic administration of PBC reduced bacterial dissemination, attenuated pulmonary inflammation, decreased inflammatory cytokines, and increased IL-10 levels, protecting animals from death by sepsis.

The PBC formulation supported *L. casei* viability, indicating that the plant-based matrix may be suitable for incorporating probiotic microorganisms. This matters because maintaining viable probiotic cells is a key characteristic of functional fermented beverages. However, the effective concentration of probiotic bacteria depends on the strain and the intended biological effect [26]. Plant-based substrates can challenge probiotic survival because of their physicochemical characteristics. However, both babassu- and cashew-derived matrices contain carbohydrates and other components that may enhance microbial metabolism [35, 36, 52, 56–58]. The present findings therefore support the potential of PBC as a plant-based vehicle for *L. casei*.

The in vitro cytotoxicity *evaluation* further supported the safety of the PBC formulation, as all tested concentrations maintained high RAW 264.7 cell viability, with even the highest concentration (1:2) maintaining viability around 70%. The high-dose cytotoxicity may be related, at least in part, to an increased lactic acid production by *L. casei,* since this effect can lower pH. Importantly, this possibility was not directly investigated and should therefore be considered a hypothesis rather than a demonstrated mechanism.

At lower concentrations, PBC maintained cell viability and, in some cases, increased cellular proliferation, which may reflect the contribution of bioactive compounds present in the plant matrices [60, 61]. These data are consistent with previous observations in plant-based probiotic beverages [62]. In addition, the absence of significant changes in erythrocyte counts or hemoglobin levels in treated animals supports the absence of evident hematological toxicity under the experimental conditions used here.

The PBC antioxidant effect may be related to the phenolic composition of its plant-derived ingredients. Cashew is rich in phenolic compounds, tannins, and ascorbic acid, whereas babassu contains phenolics and proanthocyanidins that may contribute to antioxidant activity [65,66]. Consistent with this composition, PBC efficiently scavenged DPPH radicals and reduced lipid peroxidation *in vitro* [67,68]. Although DPPH activity does not directly demonstrate antioxidant activity in *vivo*, it indicates the presence of compounds with free-radical-scavenging capacity. This finding is relevant to sepsis because oxidative stress and lipid peroxidation contribute to inflammatory amplification and tissue injury [71–75]. Thus, PBC's antioxidant properties may have contributed to the protective effects observed in septic animals.

The *in vivo* results on blood cellularity confirmed PBC's low toxicity, as hematological parameters remained stable in all groups, showing that PBC did not improve outcomes or increase cytotoxic risk.

The reduction in bacterial CFUs observed in the peritoneal cavity and bloodstream in PBC treatment may reflect improved host control of infection. This reduction in bacterial burden is particularly relevant because bacterial translocation and systemic dissemination contribute to sepsis progression, organ injury and increase the

risk of death. Sepsis promotes severe intestinal barrier dysfunction and alterations in the gut microbiota, which may facilitate the passage of microorganisms and microbial products into the circulation [83–91].

Probiotics contribute to intestinal homeostasis through several mechanisms, including increasing beneficial bacteria that control the proliferation of pathogenic microorganisms, producing antimicrobial compounds and modulating the intestinal environment [92–94]. However, even without assessing gut microbiota composition or intestinal barrier integrity, PBC may act via the gut microbiota, since reduced bacterial dissemination was accompanied by pulmonary protection. The PBC-treated animals showed reduced inflammatory cell counts in BAL fluid, suggesting attenuation of sepsis-associated pulmonary inflammation. However, future research should prioritize investigating changes in gut microbiota and barrier function to better clarify the mechanisms underlying the observed effects.

Pulmonary injury is a major complication of systemic inflammation during sepsis, as the excessive leukocyte recruitment contributes to disruption of the alveolar–capillary barrier and subsequent lung injury [95–97]. Therefore, the reduced cellular recruitment observed in the PBC group may reflect a reduced anti-inflammatory response in the lungs. This effect may relate to the lower systemic bacterial burden and cytokine production observed in treated animals.

PBC demonstrated immunomodulatory effects by increasing IL-10 and reducing TNF-α, IL-6, IL-17A, and IFN-γ following CLP induction. These cytokines are associated with the sepsis response and tissue injury [101–105]. The reduction of inflammatory cytokines observed after PBC treatment is consistent with previous studies demonstrating anti-inflammatory effects of fermented beverages and probiotic microorganisms in experimental models of systemic inflammation [13,18]. In particular, the reduction in TNF-α and IL-6 may reflect attenuation of endothelial and tissue inflammatory responses during sepsis and indicate that PBC may modulate multiple components of the inflammatory response rather than acting exclusively on a single cytokine pathway.

The results showed an apparent preservation of cellularity in secondary lymphoid organs after PBC treatment. Thus, the increased cellularity observed in PBC-treated animals may indicate preservation of immune responses during sepsis. However, increased cellularity should not be interpreted directly as evidence of enhanced cellular migration, because changes in cell proliferation, survival, retention, or redistribution could produce a similar phenotype, especially if we consider that sepsis is characterized by marked immunological dysfunction, including depletion of lymphoid populations in secondary lymphoid organs [107–110].

Increased IL-10 can reinforce the immunomodulatory profile of PBC, as IL-10 is an important regulator of inflammatory responses and can limit excessive innate immune activation [111, 112]. Previous studies have demonstrated that increased IL-10 availability can attenuate inflammatory cytokine production and improve outcomes in experimental sepsis [113,114]. These findings support our hypothesis, as previous results show that babassu [33] and *A. occidentale* [52] can improve survival in lethal experimental sepsis, an effect associated with an imbalance between pro- and anti-inflammatory cytokines, among other factors. More recently, Barroqueiro et al [76] showed that an aqueous babassu extract reduced bacterial CFUs in blood and peritoneal fluid and increase IL-10 levels in mice with MRSA sepsis. This evidence supports the possibility that bioactive constituents of babassu contributed to the antimicrobial and immunomodulatory effects observed in the present study.

Cashew apple may additionally contribute. Oliveira et al. [52] reported that *Anacardium occidentale* treatment in a murine model of sepsis reduced IL-6, MCP-1, and TNF-α levels and decreased bacterial CFUs in

the blood. The similarities between those findings and the present results suggest that bioactive compounds derived from cashew may additionally contribute to PBC's protective effects. Together, these observations support the hypothesis that the beverage's biological activity results from the combined contribution of its plant-derived components and *L. casei*.

However, a main limitation of the present study is that the individual contribution of each formulation component cannot be determined. Because the experimental design did not separately evaluate babassu milk, cashew apple, *L. casei*, or the non-fermented formulation, it was not possible to establish whether the observed effects result predominantly from one component, the fermentation process, or interactions among the different components. To address this in future studies, it would be important to clarify the respective and potentially synergistic contributions of each ingredient and the fermentation process on gut microbiota modulation and intestinal barrier function.

The present findings showed that prophylactic administration of PBC produced a varied protective response in experimental sepsis, characterized by reduced bacterial dissemination, attenuated pulmonary inflammation, modulation of pro-inflammatory cytokines, increased IL-10, and preserved immune cellularity.

Proposed pathways of PBC activity include inhibiting pathogen growth and dissemination, immunomodulatory effects by enhancing anti-inflammatory cytokine production, suppressing pro-inflammatory mediators, and reducing oxidative stress by neutralizing free radicals. Based on this, it is reasonable to propose that PBC shows probiotic bioactivity.

Taken together, the present results indicate that the prophylactic administration of PBC exerts multifaceted protective effects during experimental sepsis, characterized by reduced bacterial dissemination, attenuation of pulmonary and systemic inflammation, modulation of cytokine production, and preservation of immune responses. These mechanisms are thought to act in concert to protect against sepsis-associated inflammation and pulmonary injury. In addition, the PBC effects may result from the combined actions of bioactive compounds in babassu and cashew apple and the metabolic activity of *L. casei*.

Although the mechanisms remain to be established, these data support the potential of this plant-based fermented beverage as a functional formulation. These results indicate that PBC could be explored as an adjunct to conventional therapies in preclinical models and can also be considered in early-phase clinical studies aimed at preventing or attenuating sepsis-associated inflammation and organ dysfunction.

Therefore, further investigation is necessary to clarify whether modulation of the gut microbiota and intestinal barrier contributes to the observed reduction in bacterial dissemination, as these mechanisms were not directly evaluated in the present study. Preclinical requirements must be addressed before clinical studies can focus on safety and tolerability, then evaluate potential effects on inflammatory biomarkers and sepsis-related outcomes. Overall, the present results should be considered proof-of-concept evidence supporting further translational development, rather than evidence of clinical efficacy.

5. Conclusions

The present study demonstrated that PBC supports the growth of probiotic microorganisms, promotes favorable physicochemical changes, and exhibits significant antioxidant activity. Under the experimental conditions, the formulation reduced polymicrobial dissemination. It contributed to host immunomodulation. In

addition, PBC shows characteristics that support its potential as a nutraceutical product and a plant-based alternative to probiotic beverages. Furthermore, the ingredients used in the formulation help valorize regional food resources. Nevertheless, additional clinical studies are required to confirm these effects and to establish the functional applicability of this formulation in humans.

All authors contributed

to the conception and design of the study. T.G.D. was responsible for methodology, validation, formal analysis, investigation, data curation, writing of the original draft, manuscript review and editing, visualization, and project administration. P.M.M. contributed to methodology, validation, formal analysis, data curation, manuscript review and editing, and visualization. J.R.F. participated in methodology, validation, formal analysis, and investigation. R.N.M.G. contributed to investigation, manuscript review and editing, and visualization. V.M.-N. was responsible for data curation, manuscript review and editing, and funding acquisition. A.A.M.V. contributed to methodology, data curation, and manuscript review and editing. R.A.G. Oliveira participated in investigation, manuscript review and editing, and visualization. F.F.C. contributed to methodology, validation, formal analysis, investigation, data curation, writing of the original draft, and manuscript review and editing. D.S.S.M. participated in methodology, validation, formal analysis, and investigation. M.F.C. contributed to investigation, manuscript review and editing, and visualization. A.L.F.P. participated in methodology, validation, formal analysis, and investigation. A.S.R. contributed to methodology, validation, formal analysis, data curation, writing of the original draft, manuscript review and editing, visualization, supervision, and project administration. M.C.G. Maciel contributed to methodology, validation, formal analysis, data curation, writing of the original draft, manuscript review and editing, visualization, supervision, and project administration. All authors read and approved the final version of the manuscript.

Funding: This research was funded by the Maranhão Research Foundation (FAPEMA), Brazil (BEPP-03289/23; INFRA-15460/25; APP-01415/25); the Brazilian National Council for Scientific and Technological Development (CNPq), Brazil (Pró-Amazônia/CNPq 445615/2024-9; PPBio 441189/2023-7); the Coordination for the Improvement of Higher Education Personnel (CAPES), Brazil (Finance Code 001); Fund for Economic, Scientific, Technological and Innovation Development (FUNDECI) of Banco do Nordeste do Brasil (BNB), Brazil and the Studies and Projects Funding Agency (FINEP), Brazil (2362/24; 1449/24; 0721/24). Maranhão Research Foundation (FAPEMA), Process No. ACT05691/21, within the framework of the Technical Cooperation Agreement for Support to the Development of the Brazilian Semiarid Region (FAPEMA/CAPES), Biological Sciences/Microbiology. Education Personnel (CAPES), Brazil (Finance Code 001; BM-02164/23).

Data Availability The data sets used in this study are available from the corresponding author upon request

Declarations

Ethics Approval The study protocol was approved by the Animal Use Ethics Committee of the Universidade Federal do Maranhão (Protocol: 23115.031277/2019-19.). All surgeries were performed under anesthesia, and efforts were made to minimize suffering

During the preparation of this manuscript, the authors used ChatGPT (OpenAI, San Francisco, CA, USA) to improve the language and readability of the manuscript. After using this tool, the authors reviewed and edited the content as needed and took full responsibility for the content of the publication

Consent for Publication All authors consent to publication.

Consent to Participate Not applicable.

Competing interests The authors declare no competing interests.

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