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Article

Preliminary Assessment of the *In Vitro* Immunological Activity of Native *Lactobacillus* Strains from the Ecuadorian Amazon

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ABSTRACT

The isolation and characterization of new *Lactobacillus* strains from fermented traditional foods is a global trend as it enhances the potential for discovering novel probiotic foods. The fermented cocoa (*Theobroma cacao*) mucilage is an intriguing substrate for isolating lactic acid bacteria involved in fermentation. This study represents a preliminary investigation into the isolation, quantification, characterization, and immunological activity of *Lactobacillus* strains derived from cocoa beans sampled from two farmer associations (Kallari and Wiñak) in the Ecuadorian Amazon region. A mother culture was prepared using fermented cocoa pulp, cultivated on selective MRS media. After growth, the isolates were morphologically characterized. A significantly higher bacterial concentration was recorded in Kallari Association samples if compared with Wiñac. A total of 25 strains were isolated, eight of which were rod-shaped and positive to catalase tests and were characterized as *Lactobacillus*. *In vitro,* immunological activity was performed on differentiated THP-1 cell lines. Cells were treated with bacterial concentrates, and immunological activity was determined through interleukin-10 expression. Results indicated that W6 strain showed the highest immunological activity. These results indicated that *Lactobacillus* strains isolated from fermented cocoa pulp in the Ecuadorian Amazon show promise as a new source of probiotics.

Keywords: cocoa; lactic acid bacteria; biological activity; isolation; cocoa fermentation; probiotics**;** cocoa pulp

INTRODUCTION

The human body balances gastrointestinal microbiota and the immune system [1,2]. Disruption of this microbiota can lead to diseases, discomfort during food consumption, and difficulties in nutrient absorption[3]. Probiotics and derivatives of beneficial bacteria help promote immune system balance by inducing the secretion of anti-inflammatory cytokines and other factors that reduce non-specific immune responses and the reactivity of macrophages and lymphocytes [4,5]. Bacteria from the *Lactobacillus* genus and others have been shown to support gastrointestinal homeostasis [6]. Many *Lactobacillus* species can thrive during the fermentation of fruit

beverages. Evidence suggests that once isolated and tested *in vitro*, *in vivo*, and during human consumption, these bacteria could favorably influence the microbiota and immune system equilibrium [7,8].

The isolation and characterization of novel *Lactobacillus* strains from traditionally fermented foods is a rapidly expanding field worldwide. This approach holds significant potential for the development of new probiotic products using native bacterial strains that are specifically adapted to local conditions [9-11].

Among such fermented foods, cocoa (*Theobroma cacao*) mucilage is a relevant growth substrate for bacteria with potential probiotic properties. The cocoa plant is native to Ecuador, as evidenced by archeological findings of theobromine alkaloid in the oldest organic cocoa matter discovered in Zamora Chinchipe province [12]. Today, cocoa cultivation remains a cornerstone of economic sustainability for Amazonian communities in Ecuador, where, for many, the sale of cocoa beans is the primary source of income [13]. Furthermore, cocoa cultivation is critical in protecting the rainforest against slash-and-burn practices, a significant driver of agricultural expansion that threatens the fragile Amazonian ecosystem. While factors such as genotype, kernel composition, soil type, and plantation age influence the development of cocoa flavors [14], post-harvest practices like bean fermentation are essential for reaching the quality standards demanded by the market [15].

The specific microbial inoculum determines the flavor compounds generated during cocoa fermentation. These microbes drive the biochemical processes that form free amino acids, peptides, and sugars at the end of fermentation, ultimately shaping the desired quality of fermented cocoa beans [12,16]. Lactic acid bacteria (LAB), particularly species belonging to the genus *Lactobacillus*, are essential for achieving optimal results. These bacteria contribute to cocoa flavor development and hold potential as a foundation for functional probiotic foods [17], potentially enhancing the value of Amazonian cocoa products. To our knowledge, a significant gap exists in understanding Ecuador's microbial communities involved in cocoa bean fermentation. Although these local microorganisms have the potential to act as probiotics, their applications remain largely unexplored. Delving into this understudied niche offers a valuable opportunity to impact the Ecuadorian cocoa industry positively.

The isolation, characterization, and evaluation of these microorganisms could lead to the development of novel fermentation techniques that enhance the quality of Ecuadorian chocolate while introducing health benefits. Therefore, studies on native *Lactobacillus* strains represent a significant opportunity for the Ecuadorian Amazon. If these native microorganisms exhibit probiotic properties, they could offer multiple advantages, including producing new functional foods, increased economic opportunities for local communities, and potential improvement in the immune system of the region's inhabitants, who face the highest malnutrition rates in the country [18].

No studies have been reported to elucidate the immunological function of native LAB strains or species with probiotic potential in the Ecuadorian Amazon region. This is why this preliminary research becomes the first approach to the isolation, characterization, and determination of initial immunological properties of bacteria of the *Lactobacillus* genus from fermentation processes of cocoa seeds collected from Napo, Ecuador.

MATERIALS AND METHODS

Sampling, isolation, and counting of Lactic Acid Bacteria (LAB)

Cocoa bean samples were collected from fermentation boxes belonging to the Kallari (K) and Wiñak (W) farmer associations in Napo province, Ecuador. The microbial communities involved in the fermentation of cocoa mucilage in all Amazonian regions of Ecuador, including Napo, remain largely unexplored. This study aims to address this knowledge gap by investigating the microbial ecology of cocoa fermentation in the Napo region. Given the presence of well-established cacao producer associations, Kallari and Wiñiak, with the necessary infrastructure and expertise, their involvement was crucial to ensure the successful collection and analysis of samples.

Yeasts dominate the initial stages of cacao mucilage fermentation. Their growth creates optimal conditions (anaerobic environment) for the development of LAB, which begins to proliferate significantly within 24-72 hours [19, 20, 21]. For this reason, samples for this study were collected on the second day of fermentation. Four samples (each 200 grams) were taken from each fermentation box. Each sample consisted of five subsamples collected at a depth of 30 cm from five different locations within box [19]. Samples were assigned alphanumeric codes: "K" or "W" for Kallari or Wiñak association, respectively, and "S" followed by a number for the subsample (e.g., W1S1, W1S2, W2S1, W2S2, K1S1, K1S2, K2S1, K2S2). Seeds were stored in airtight zip-lock bags and refrigerated at 4°C for transport and further laboratory processing.
Isolation of LAB was carried out following the method described by Camu et al. [22]. Twenty grams of cocoa seeds, from each sample, were mixed with 180 ml of peptone water and shaken vigorously for 3 minutes to create a homogenous solution containing pulp particles. One milliliter of this solution was used for serial dilutions. Aliquots (0.1 ml) containing an estimated 10^5 to 10^7 CFU were inoculated into three Petri dishes containing Man-Rogosa-Sharpe (MRS) agar. The plates were incubated for 72h at 37°C to observe colony growth.

Characterization of *Lactobacillus* strains

After incubation, colony-forming units were counted for each sample and dilution. Biochemical tests such as Gram stain, catalase test, and growth parameters (color, shape, elevation, borders) were used to identify the genus. Only colonies exhibiting morphological characteristics consistent with the genus *Lactobacillus*, such as rod-shaped, Gram-positive, non-pigmented, catalase-negative, and anaerobic, were considered [23]. Catalase test was carried out following MacFaddin's method [24]. Each strain was transferred to a slide, and a drop of 3% hydrogen peroxide was added. The reaction was visualized under a 40x magnifying optical microscope. The presence or absence of bubbles indicates catalase activity: no bubbles indicate a negative reaction (no catalase enzyme to break down hydrogen peroxide), while bubble formation indicates a positive reaction. Despite advancements in molecular techniques, biochemical tests remain a cornerstone of taxonomic identification for the genus *Lactobacillus* [25, 26, 27].
Each strain was assigned a unique alphanumeric code consisting of a letter representing the farmer association ("K" for Kallari or "W" for Wiñak) followed by a number denoting the isolated strain. Finally, all isolates displaying morpho-cultural characteristics of LAB were cryopreserved in 50% (v/v) glycerol solution.

In vitro immunological activity of *Lactobacillus*

LAB isolated from cocoa mucilage that presented morphological and biochemical characteristics belonging to the genus *Lactobacillus* (Gram + bacilli, catalase-negative) were studied to determine their immunological activity. The assay was performed on the human acute leukemia monocyte cell line THP-1 from the American Type Culture Collection (ATCC) at different stages of differentiation (M0, M1, M2). The THP-1 cell line is one of the most used models to test the immune regulatory properties of probiotics derivate from bacteria [1,2,6]. THP-1 cells were transformed into macrophages by activation using PMA (Phorbol 12-myristate 13-acetate). Once differentiated into an M0 macrophage state, they are incubated with IL-3 and IL-4 to obtain M2 macrophages or with IFN-gamma and lipopolysaccharide (LPS) for macrophage activation (M1) [28]. In this study, 106 cells/ml concentration was seeded in six-well plates and exposed to 50 nm phorbol myristate acetate (PMA) to induce differentiation for 24 h. Cells were then washed and exposed to a wash water bath. Subsequently, cells were maintained without stimuli for 24 h and then subjected to the effect of the bacteria under study. The assay was duplicated using 1 mg/ml pellet of the bacterial strains. The expression of interleukin-10 (IL10) and tumor necrosis factors (TNFα), measured by qPCR and ELISA, was evaluated in differentiated THP-116 macrophages.

Statistical analysis

A comparison of means for data obtained from bacterial quantification was processed using the SPSS v. 24 statistical package. Simple ANOVA with Tukey HSD test and significance level $p<0.05$ was used for both inter-sample and inter-locality differences.

Statistical analysis of the immunological activity data was performed using GraphPad Prism 6. A Kolmogorov-Smirnov normality test was conducted to determine the use of parametric or non-parametric analysis. Then, non-parametric ANOVA and Mann-Whitney rank test were used to analyze differences between conditions, using an alpha value of 0.05 (* $p < 0.05$, ** $p < 0.01$).

RESULTS

Isolation and quantification of LAB

Figure 1 presents the quantification of LAB isolates following a 72-hour incubation period on Petri dishes. Analysis of LAB quantification revealed a more uniform distribution of colony-forming units (CFUs) in samples collected from Wiñak (panel A), with no significant differences observed. In contrast, Kallari samples (panel B) displayed a higher overall CFU count than Wiñak, and considerable variations were evident between the collected subsamples. This finding underscores the relevance of collecting and analyzing composite samples, to ensure a representative picture of the LAB population within different fermentation boxes at each sampling site. Mixed bacterial cultures were observed in the samples analyzed (Figure 2).

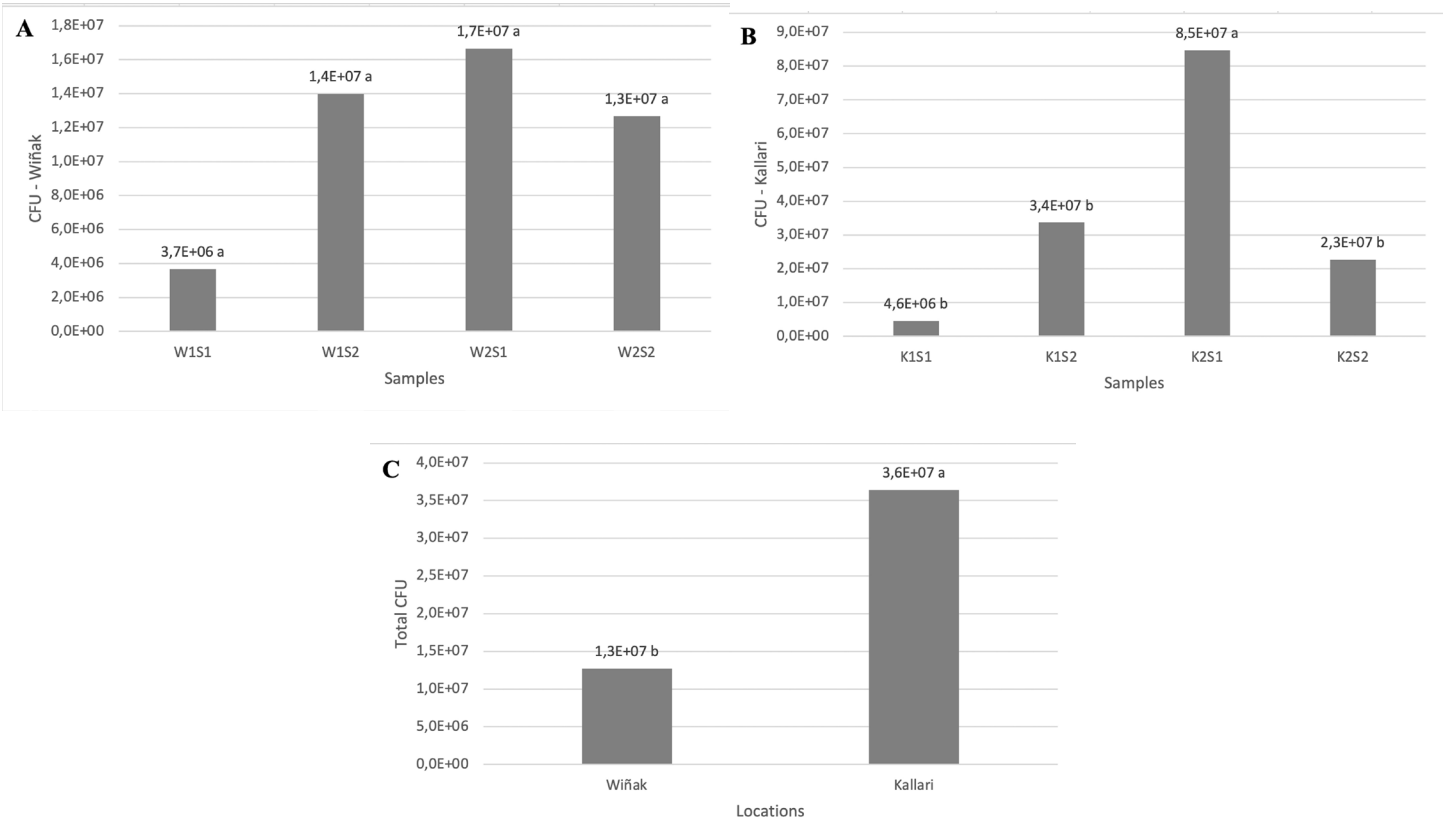

Figure 1. Bacterial quantification in fermenting cocoa bean samples from Wiñak (A) and Kallari (B) and the average of samples in both locations (C). Different letters in the bars represent significant differences $p<0.05$, Tukey HSD.

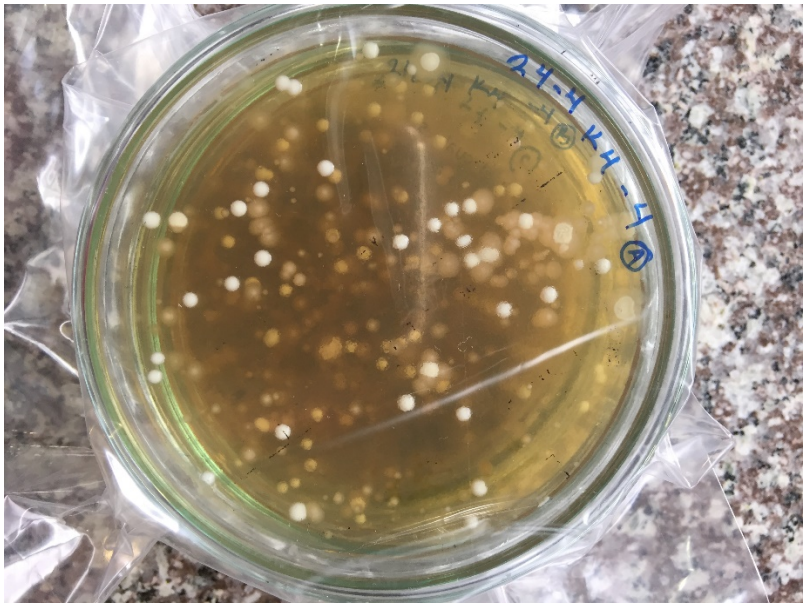

Figure 2. Mixed culture of LAB on MRS agar, isolated from mother cultures of fermenting cocoa beans from Kallari association

Characterization of *Lactobacillus* strains

Bacterial isolates exhibiting morpho-cultural characteristics consistent with LAB were selected for further characterization. Table 1 presents the 25 isolates identified that showed distinct morpho-cultural features.

	Color[1]	Elevation[2]	Edge[3]	Consistency[4]	Development[5]	Gram[6]	Shape	[7]Catalase
W1	1	C	E	+	++	+	rod	-
W2	2	C	E	++	+	+	rod	-
W4	1	C	E	++	+++	+	rod	-
W5	4	P	I	+	+++	+	rod	+
W6	1	C	E	++	++	+	rod	-
W7	2	C	E	++	++	+	rod	-
W8	5	C	E	+++	+++	+	rod	+
W9	4	C	E	+++	+++	+	rod	+
W10	5	C	E	++	+++	-	rod	+
W11	3	P	E	-	+	+	rod	+
W12	1	C	E	+	++	+	rod	+
W13	3	P	E	-	++	+	rod	+
K1	3	P	E	+	+	+	circular	+
K2	4	C	E	++	+	+	circular	-
K3	3	P	E	-	+++	-	rod	+
K4	2	C	E	-	++	-	circular	+
K5	1	C	E	-	+++	-	circular	-
K6	4	C	E	-	++	+	rod	-
K7	2	P	E	-	+++	+	circular	-
K8	2	P	E	-	+++	-	circular	-
K9	2	C	E	-	+++	-	rod	-
K10	2	P	E	-	+++	+	rod	-
K11	3	P	E	-	+	+	circular	+

[1]Color: 1-yellow, 2-light yellow, 3-opaque, 4-white, 5-pinkish; [2]Elevation: C-convex, P-flat; [3]Margin: E-entire, I-irregular; [4]Consistency: -none slime, +light slime, ++moderate slime, +++ abundant slime; [5]Growth: -none, +light, ++moderate, +++ abundant; [6]Gram: -negative, +positive; [7]Catalase: -negative, +positive.

Table 1. Morpho-cultural and biochemical characteristics of bacteria isolated from cocoa bean mucilage at Kallari and Wiñak associations

Gram staining, microscopic observation, and catalase test allowed the identification of eight isolates (W1, W2, W4, W6, W7, K6, K10, K13) showing a bacillus shape, belonging to the genus *Lactobacillus* (Table 1, Figure 3).

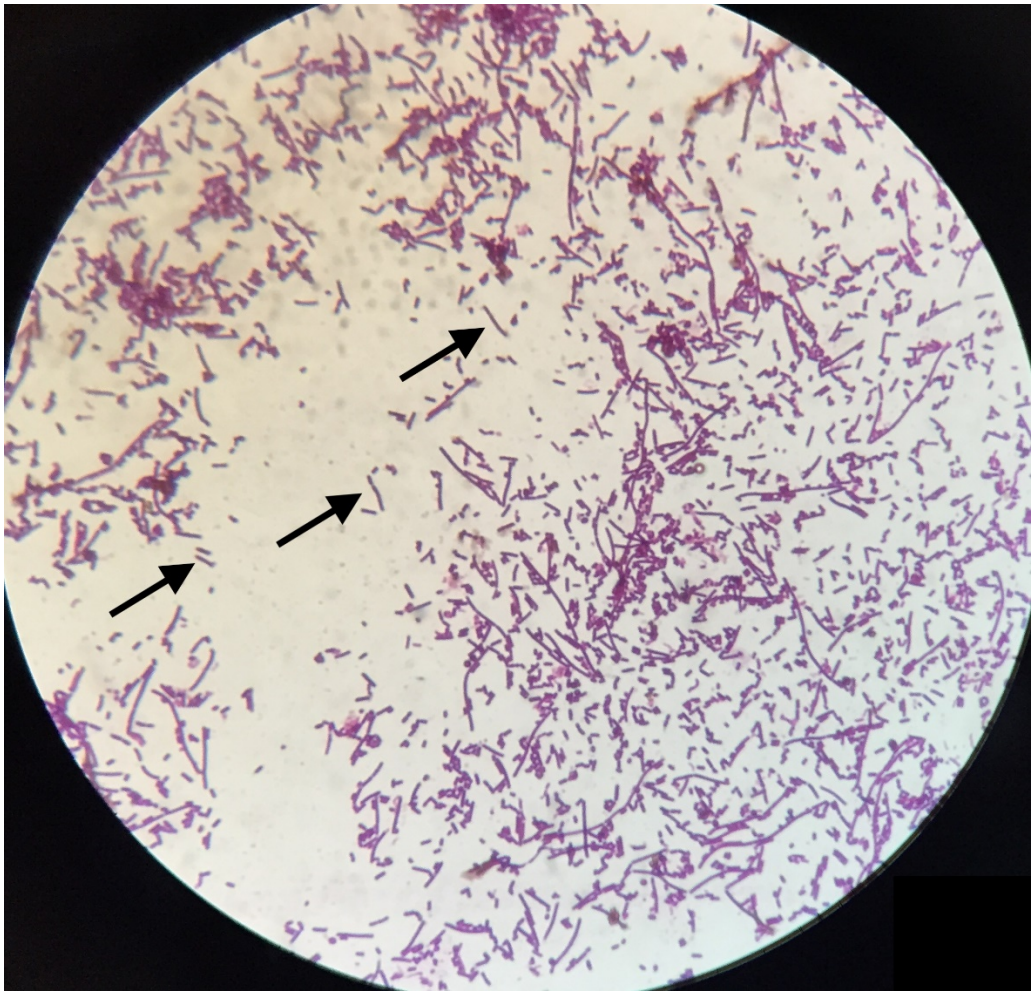

Figure 3. *Lactobacillus* spp. Observed at optical microscope, isolated from Kallari sample (see black arrows).

Preliminary *in vitro* immunological activity of *Lactobacillus* isolates

To assess the immunological activity of *Lactobacillus* isolates, they were co-incubated with TPH-1 cell lines. Figure 4 illustrates the effects of different strains on TNFα and IL-10 expression. Panel B shows that W6 strain revealed the highest significant levels of IL-10 secretion by macrophages as measured by ELISA. This finding was further corroborated by the qPCR analysis of IL-10 expression (panel D).

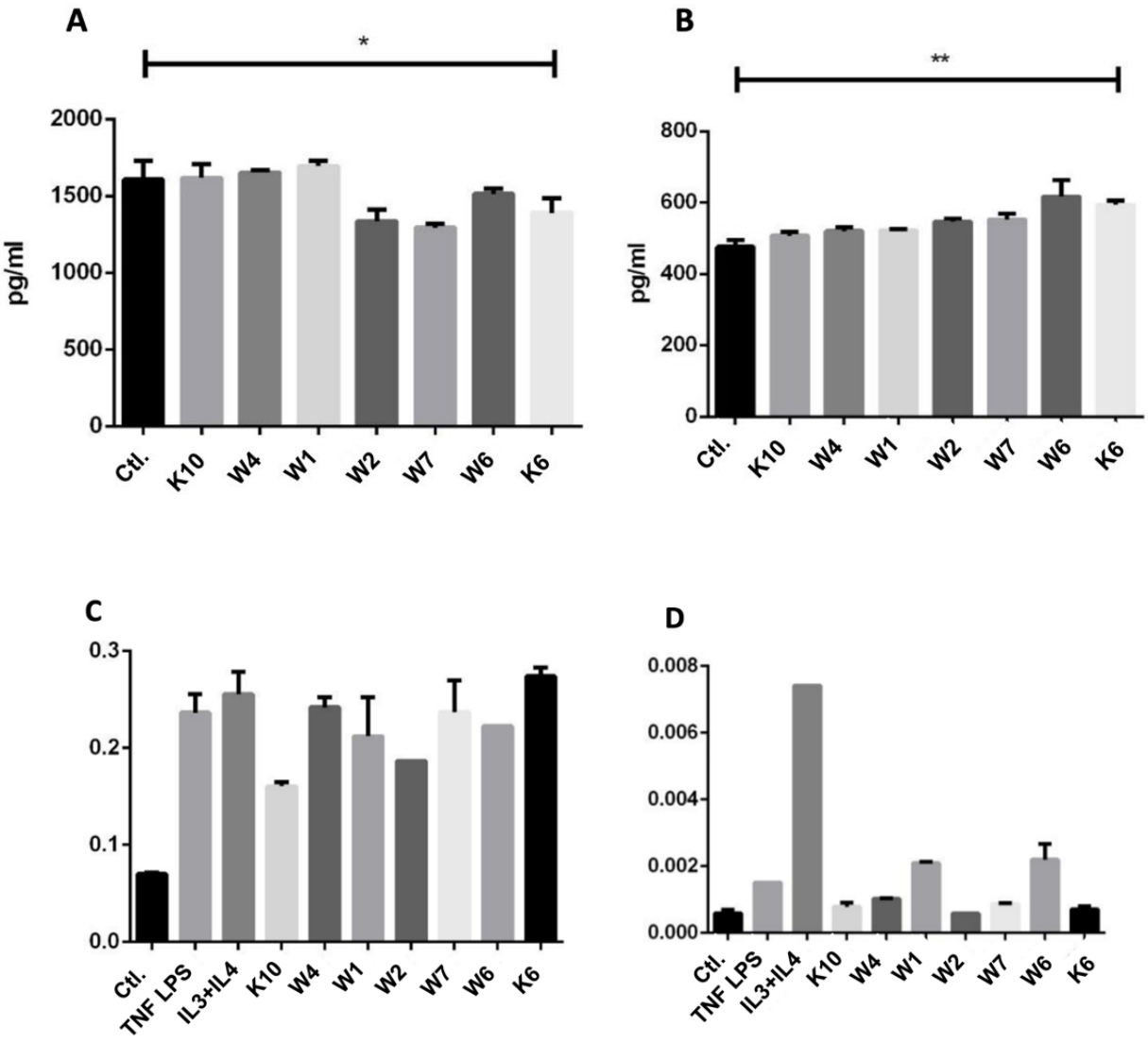

Figure 4. Immunological activity of *Lactobacillus* isolates. A: TNFα expression by ELISA; B: IL-10 expression by ELISA; C: TNF expression by qPCR; D: IL-10 expression by qPCR.

DISCUSSION

Temperature, incubation time, and concentration of acetic and lactic acids affect the cocoa fermentation process. Those factors highly influence the production of metabolites essential for developing aroma, flavor, and high-quality chocolate precursors [12,29,30]. The LAB populations within fermentation boxes revealed differences in bacterial concentration, depending on the geographical proceeding of the cocoa bean sample. As reported in other studies, this situation suggests adapting the microorganisms to the environment they originated in [31]. The analysis of the samples showed the presence of mixed microbial cultures, as other studies showed. Cocoa fermentation is a spontaneous process that naturally occurs thanks to microbes already present in the environment. These microbes feed on the bean pulp, reducing oxygen and creating ideal conditions for them to thrive. Several microbes include yeasts, lactic acid, and acetic bacteria.

The initial fermentation stage is characterized by a reduction in oxygen diffusion within the bean matrix due to the surrounding pulp volume, which creates an anaerobic environment. During this phase, a sequential

microbial dominance occurs. Yeasts are first developed by consuming sugars and producing ethanol. The alcoholic fermentation produces an alkaline environment that, together with alcohol and oxygen, inhibits yeast activity and prevents lactic bacteria development. In the second fermentation phase, more oxygen enters the matrix, creating adequate conditions for lactic bacteria growth that converts pulp sugars into lactic acid.
Unlike other fermented foods, cocoa relies heavily on the beans' enzymes to develop flavor. Without fermentation, cocoa beans would be tasteless. Microbes break down the pulp and create essential flavor compounds 12,19 during this process.
The highest LAB concentration is typically recorded on the second day of fermentation, including for the *Lactobacillus* genus. Gram staining, microscopy, and the catalase test confirmed the presence of bacteria with bacillary morphology, characteristic to *Lactobacillus* genus. These results align with those reported by previous research [12,30,32,33]. While recent studies increasingly employ molecular analyses for specific LAB strain identification, several authors suggest that morphological, cultural, and biochemical characterization is enough to determine *Lactobacillus* presence in various samples [23,34,35].
The W6 strain recorded the highest significant levels of IL-10 secretion by macrophages. THP-1 differentiated macrophages generally exhibit a pro-inflammatory signature characterized by high TNFα expression under controlled conditions [4,36]. However, according to some authors, IL-10 expression is the most crucial indicator of an anti-inflammatory and homeostatic macrophage lineage, a key characteristic of probiotics [37]. The ability of the W6 strain to stimulate IL-10 secretion suggests its potential probiotic properties.

CONCLUSIONS

Given the recent discovery that the cocoa plant is endemic to the Ecuadorian Amazon [38], research into this species has increased importance for local economic development and future studies that could lead to additional quality certifications. This study is distinguished by its focus on the probiotic potential of mucilage derived from cocoa cultivated in the region of Napo, located in Amazonian Ecuador.
Analysis of LAB isolates from cocoa mucilage samples collected in Napo, under the fermentation process, revealed a rich diversity based on morphology, culture, and biochemistry. Kallari samples displayed higher LAB concentrations, potentially influencing cocoa bean quality and processing. Furthermore, *in vitro* immunological studies identified W6 strain as the most promising candidate due to its ability to induce IL-10 secretion in macrophages, suggesting its potential probiotic properties. In vitro experiments were decided to provide preliminary scientific results for future research. Data obtained in the present study provide sufficient justification to carry on with this research, focusing on (i) performing assays on *in vivo* models, (ii) studying other types of anti-inflammatory cytokines, as well as (iii) the evaluation of additional probiotic activities as survivability under simulated gastrointestinal conditions, adhesion to intestinal epithelial cells and antimicrobial activity against pathogens.
These findings emerged in this preliminary research and highlight the probiotic potential of LAB isolated from Amazonian cocoa fermenters.

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