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Interactions between polyphenols from *Theobroma cacao* and Lactobacillales to evaluate the potential of a combined strategy for intestinal free-fatty acid removal

Samuel Quiroz-Eraso [a,b], Gina Paola Rodríguez-Castaño [b], Alejandro Acosta-González [b,c,*]

[a] *Maestría en Diseño y Gestión de Procesos, Facultad de Ingeniería, Universidad de la Sabana, Km. 7 Autopista Norte, Chia 25001, Colombia*
[b] *Bioprospection Research Group (GIBP), Facultad de Ingeniería, Universidad de La Sabana, Km. 7 Autopista Norte, Chia 25001, Colombia*
[c] *Unisabana Center for Translational Science, Campus Universitario, Universidad de La Sabana, Km. 7 Autopista Norte, Chia 25001, Colombia*

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ABSTRACT

Reducing the absorption of lipids in the gastrointestinal tract is one approach used to manage caloric intake in the fight against excessive weight. Biocompounds, such as polyphenols and probiotics, have been used in this regard. However, some studies have reported that polyphenols have both inhibitory and stimulatory effects on bacterial growth. This study aimed to investigate the resistance to polyphenol-rich extracts from *Theobroma cacao* L. of Lactobacillales isolated from the human fecal microbiota of lean volunteers (with high saturated fat consumption), to further the knowledge of the potential combination of these bioactive compounds. The strains were selected using an improved and affordable strategy that allowed the rapid screening of strains with fat-removing capacity. Among 1400 isolates, two strains, *Lactobacillus* sp. A1 and *Pediococcus acidilactici* E1, were selected due to their capacity to remove saturated fats from the culture media similar to the reference strain *Lactobacillus* sp. JBD301. Both isolated strains differed in their resistance to cocoa polyphenols: the extract did not affect the growth of strain A1, but reduced the growth of strain E1. However, the extract did not affect the level of *in vitro* fat removal by either strain, confirming the potential use of a combination of bacteria and polyphenols as a promising strategy for the intestinal removal of free fatty acids.

1. Introduction

Fats are present in most foods in the modern diet and often have a desirable effect on sensory properties, enhancing attributes such as color, texture, and flavor (Sikorski and Kolakowska, 2011). As macronutrients, they provide the highest energy density (Rolls et al., 2015). Body mass gain due to energy accumulation in the form of triacylglycerides in adipose tissue results from a caloric intake that exceeds energy expenditure (Paolicelli, 2016). Therefore, strategies for the reduction of excess weight consist of increasing energy expenditure (e.g., exercise) and/or decreasing energy intake (Fonseca et al., 2018). However, energy-dense foods are often more readily available to the average consumer and often have preferred organoleptic properties over their low-calorie counterparts, posing a challenge to maintaining a low-calorie diet (Drewnowski, 2018). Another approach to reducing energy expenditure is to reduce the absorption of lipids in the gastrointestinal tract. In this line, the weight management drug Orlistat, was approved by the FDA in 1999 (Timothy Garvey, 2022). This drug is a lipase inhibitor that prevents lipid hydrolysis and reduces fat absorption by up to 30% (Hill et al., 1999; Yanovski and Yanovski, 2014). However, lipase inhibition results in the presence of undigested lipids in the gastrointestinal tract, causing steatorrhea as a side effect (Tak and Lee, 2021; Yanovski and Yanovski, 2014). Meanwhile, in a study conducted by Chung and collaborators (Chung et al., 2016), the probiotic strain *Lactobacillus* sp. JBD301 showed a strong fat-removing capacity, and its administration to women in a randomized, double-blind, placebo-controlled clinical trial demonstrated its ability to remove intestinal free fatty acids (FFAs) before their absorption by the host, thus showing significant anti-obesity effects with efficacy as high as Orlistat, without side effects.

Bacteria from the genus *Lactobacillus* have been shown to alleviate obesity by removing free fatty acids in the gastrointestinal tract, this

Abbreviations: PRCE, Polyphenol-rich cocoa extract.

* Corresponding author. Bioprospection Research Group (GIBP), Facultad de Ingeniería, Universidad de La Sabana, Km. 7 Autopista Norte, Chia 25001, Colombia.
E-mail address: alejandro.acosta1@unisabana.edu.co (A. Acosta-González).

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effect may be species and strain-specific (Jang et al., 2019). The genus *Lactobacillus* belongs to the order Lactobacillales, which are acid-tolerant and ferment carbohydrates predominantly to lactic acid (Mena and Campos, 2012). This group also includes the genera *Streptococcus, Lactococcus*, and *Pediococcus*, among others (Schoch et al., 2020). A unique feature of bacteria in this order is that they can synthesize their cell membrane phospholipids using only exogenous fatty acids when the fatty acid synthesis pathway FASII is completely suppressed (Yao and Rock, 2017) (e.g., in the presence of triclosan, a trichlorinated diphenyl ether) (Heath and Rock, 2004). Lactobacillales can also change their membrane lipids composition during stress. The mechanisms are strain-dependent, but *Lactobacillus casei*, for example, decreases the unsaturated/saturated ratio of its cytoplasmic membrane during acid stress to increase its rigidity and compactness to counteract proton influx (Broadbent et al., 2010). These capabilities make bacteria of the order Lactobacillales a promising approach to manage fat absorption by the host.

Polyphenols, which are secondary plant metabolites found in fruits and vegetables, may also interact with lipids. Polyphenols decrease the absorption of triglycerides and cholesterol in the gastrointestinal tract and increase total lipids and cholesterol in feces (Chan et al., 1999; Muramatsu et al., 1986). A systematic review conducted by Moorthy and collaborators (Moorthy et al., 2021) found 44 studies reporting a significant reduction in obesity-related parameters associated with polyphenol consumption. Of these studies, 83% showed a reduction in body mass, visceral adiposity, or plasma triacylglycerols. A source of polyphenols with higher phenolic content and antioxidant capacity than two of the most common phenolic-rich beverages (tea and wine) is cocoa and its products (Lee et al., 2003). The polyphenols present in cocoa beans are bioactive compounds responsible for several beneficial effects on human health (Jean-Marie et al., 2021), such as antioxidant (Lee et al., 2003), anti-diabetic (Martin et al., 2017), anti-inflammatory (Goya et al., 2016), anti-carcinogenic (Martin et al., 2013), improved energy expenditure (Rabadan-Chávez et al., 2016), and weight management (Halib et al., 2020).

Phytochemicals in cocoa may have beneficial or antimicrobial effects on certain bacteria (Massot-Cladera et al., 2014; Todorovic et al., 2017). For example, cocoa polyphenols have been associated with an inhibitory effect on the growth of *Staphylococcus*, *Streptococcus,* and *Clostridium* (Massot-Cladera et al., 2012, 2014). As for the *Lactobacillus* genus, there is a discrepancy between the results of different authors, as some of them report an inhibitory effect (Massot-Cladera et al., 2012, 2014), while others report a positive response (Peng et al., 2015; Tzounis et al., 2011), this study aimed to evaluate the effect of a polyphenol-rich cocoa extract (PRCE) on the growth and fat removal capacity of Lactobacillales strains isolated from fecal samples of lean volunteers with high-fat consumption, to establish the potential of combining both, probiotics and polyphenols, in the battle to reduce fat absorption by the host.

2. Materials and methods

2.1. Isolation of fat-removing Lactobacillales strains

2.1.1. Sample collection and processing

Stool samples were obtained between September 2021 and October 2021 from 10 volunteers with a body mass index (BMI) between 18 and 24 who self-reported a frequent consumption of high-fat content snacks (>5 per week). Briefly, participants collected stool samples directly into sterile containers, then samples were kept at ~4 °C until arrival at the processing laboratory within 48 h of collection. The use of fecal microbiota was approved by the Institutional Review Board of the University of La Sabana. Written informed consent was obtained from the participants. This effort did not include the collection of samples from participants in vulnerable populations or from minors.

2.1.2. Isolation of fat-removing strains

Fresh fecal samples were resuspended in phosphate buffer pH 7.4 by vigorous vortexing and used to inoculate culture media at a final concentration of 0.5 mg/ml. Four fecal samples were inoculated individually and the others were inoculated in groups of three. Saturated fats were added to the culture media as needed. Stocks of palmitic and stearic acid solutions (125 mM) were prepared by dissolving 0.32 g of each fat in 10 ml of 96% ethanol, then adding NaOH 1 M until a pH of 10 was reached (measured with pH indicator strips), with constant stirring and heating at 65 °C. After all the ethanol had evaporated, the lipid was dissolved in 10 ml of polyethylene glycol (PEG) 10% w/v (this generates a fat suspension that can be diluted and incorporated in the culture media). The culture media used were a modification of Man-Rogosa-Sharpe (MRS) media (Labyotek) at different concentrations depending on the assay (concentration of solid media was 1× for colony isolation and 0.5× for plate counts) without the addition of tween (HiTek M9369 part A only). One set of MRS with pH adjusted to 4.5 and another set with triclosan added at 0.2 μg/ml were used. Each of these culture media was supplemented with palmitic acid or stearic acid, both to a final concentration of 0.5 mM. Cultures were incubated for 48 h at 37 °C and then colonies were isolated on solid MRS media. Approximately 1400 colonies were purified and screened for their ability to remove palmitic acid from the culture media. Each strain was grown in 300 μL of MRS supplemented with 0.5 mM palmitic acid in deep-well microplates, and then the entire volume of the culture was subjected to lipid quantification. *Lactobacillus* sp. JBD301, which was used as a fat-removing probiotic strain reference in this study, was isolated from the commercial product Ditenar (JINIS Biopharmaceuticals Inc.) (Chung et al., 2016).

2.1.3. Selection of fat-removing strains by colorimetric determination of palmitic acid

The protocol followed was the one described by Wawrik and Harriman (Wawrik and Harriman, 2010), with modifications: (I) as the intention was to measure lipids in the culture media, no saponification or neutralization reagents were used, which was included in the original protocol used to extract lipids from algal membranes. The total volume of culture media (300-200 μl) was mixed with 200 μL of copper reagent (9 vol aq. 1 M triethanolamine, 1 vol 1 N acetic acid, 10 vol 6.45% (w/v) Cu $(NO_3)_2{\cdot}3H_2O$). The samples were then vortexed for 2 min (maximum speed), and 250 μL of chloroform was added and vortexed again for 2 min (maximum speed). Centrifugation at 14,000 *g* for 1 min followed, then the organic phase was transferred to another microcentrifuge tube (~200 μL), and 50 μL was transferred again to a 96-well reading microplate already containing 50 μL of 1% (w/v) sodium diethyldithiocarbamate in 2-butanol (color developing reagent). Absorbance was read at 440 nm, one sample at a time (since chloroform melts the plastic of the microplate). A well containing 100 μL of 2-butanol was used as a blank. Standards were prepared by incorporating palmitic acid solution in MRS broth to obtain concentrations of up to 2.5 mM to construct a calibration curve to calculate the palmitic acid concentration of the samples. Two strains were selected for further characterization based on their ability to remove palmitic acid from the culture media.

2.1.4. Identification of bacterial isolates

MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) Biotyper BRUKER was used for the identification of selected isolates. Purified strains were grown in MRS media and incubated for 48 h at 37 °C. Five single colonies per strain were picked over a MALDI target plate, by direct smear method as a thin layer and allowed to dry at room temperature. Each sample was overlaid with 1 μL of 70% formic acid and then covered with the matrix solution (α-cyano-4-hydroxycinnamic acid—HCCA) and air dried for 3–5 min. Profiles were visualized using FlexControl software (version 3.0) and the MALDI Biotyper RTC. Dendrograms were generated using BioTyper software 3.0. A Bacterial Test Standard (BTS) *Escherichia coli* DH5α protein profile (Bruker Daltonik GmbH, Bremen, Germany) was used for calibration

and as a positive control.

2.2. Removal of palmitic acid and its effect on bacterial growth

2.2.1. Dry weight experiment to measure the effect of palmitic acid on bacterial growth

To compare the growth of the most promising strains (strain E1 and strain A1, and reference JBD301) in MRS 1.5× *vs* MRS 1.5× supplemented with 0.5 mM palmitic acid, the dry weight of the grown cultures was measured. The inoculum was adjusted to $OD_{600} = 0.05$ and culture was incubated at 37 °C at 200 rpm. The experiment was performed in 50 ml Falcon tubes with 5 ml of medium, and time points were 0 and 24 h. Non-inoculated media was used as control. Tubes were centrifuged at 6000 rpm for 10 min, the supernatant was discarded and the pellet was dried at 72 °C for 36 h and the final weight was recorded. Dry weight was determined in triplicate in two independent experiments.

2.2.2. Enzymatic determination of palmitic acid

The fat-removing ability of strains *Pediococcus* sp. E1 (strain E1) and *Lactobacillus* sp. A1 (strain A1), and the reference strain, *Lactobacillus* sp. JBD301 (strain JBD301) was confirmed by an enzymatic assay. Fifty ml of MRS 1.5× medium supplemented with palmitic acid 0.5 mM was inoculated with 1 ml of 10^6 cells/ml (strain E1 optical density [OD_{600}] = 0.05, strains JBD301 and A1 $OD_{600} = 0.01$) of each strain and incubated at 37 °C at 150 rpm. Subsequently, 1 ml samples were taken at 0 and 24 h and free fatty acids were quantified using the EnzyChrom™ Free Fatty Acid Assay Kit according to the manufacturer's instructions, including the addition of isopropanol 5% and Triton X-100 5% and filtration through a 0.45 μm PTFE filter. Each parameter was assayed in duplicate in two independent experiments.

2.3. Characterization of fat-removing strains

2.3.1. Tolerance of fat-removing strains to high osmotic pressure, acid pH, and bile salts

The ability of the selected strains, strains E1 and A1, and JBD301 to tolerate high osmotic pressure was tested as previously described (Collado and Sanz, 2007), with modifications: MRS 1.5× broth was supplemented with different concentrations of NaCl (1%, 2%, 3%, 4%, 5%, 6%, 7%, and 8%) and used for the assay (5 replicates, two independent experiments). Tolerance to highly acid environments was determined as previously described (Hassanzadazar et al., 2012), with modifications: MRS 1.5× broth, that had been adjusted separately to pH 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, and 6 with 1 M HCl, was used for the assay (5 replicates, two independent experiments). Finally, bile tolerance was determined as previously described (El-Naggar, 2004), also with modifications: MRS 1.5× broth supplemented with different concentrations (0.13%, 0.25%, 0.5%, 1.0%, and 1.5%) of bile salts was tested (5 replicates, two independent experiments). In all cases, media was inoculated with 10^6 cells/ml of each strain, incubated aerobically at 37 °C for 48 h, and bacterial growth was monitored by measuring OD at a wavelength of 600 nm. Growth was plotted as a percentage of the growth obtained for control culture media (for acid tolerance, the control media was the one adjusted to pH 6; and for bile salt and high osmotic tolerance, control media had no addition of bile salts or NaCl, respectively).

2.3.2. Effect of fat-removing strains on the induction of TNF-α production by macrophages

To compare the immunomodulatory properties of the selected strains, strains E1 and A1, and JBD301, the production of the pro-inflammatory cytokine TNF-α by macrophages upon bacterial stimulation was measured. One hundred μl of a bacterial suspension (1×10^7 cfu/ml) of each strain was used to expose a cell line differentiated into macrophages. THP-1 cells were obtained from the University of La Sabana Biobank and grown in suspension in RPMI (Sigma-Aldrich) supplemented with 10% (v/v) FBS in a humidified incubator at 37 °C, 5% CO_2. Low passage (passage 15 or less) and high viability (>94%) cells were plated in 24-well flat-bottomed tissue culture plates at a concentration of 1×10^5 cells per ml in the presence of phorbol 12-myristiate-12-acetate (PMA, Sigma-Aldrich). Cells were treated with 200 ng/ml PMA and the PMA was removed after 2 days of treatment. Differentiation and adherence were confirmed by microscopy. The media were changed before stimulation and the cells were then incubated in the presence of the bacterial suspension. Purified lipopolysaccharide (LPS) from *E. coli* (Sigma Chemical Co, Madrid, Spain) was used as a positive control at a concentration of 1 μg/ml. Unstimulated cells were also evaluated as a control for basal cytokine production. Cell culture supernatants were collected and stored at −20 °C until used for cytokine determination. TNF-α was quantified using the ELISA Ready SET Go! Kit (BD Bioscience, San Diego, CA, USA). Each parameter was assayed in triplicate in two independent experiments.

2.3.3. Hemolytic activity of fat-removing strains

The hemolytic activity of the most promising strains, strain E1 and strain A1, and JBD301, was determined as previously described (Foulquié Moreno et al., 2003). Overnight (18–24 h) cultures of the microorganisms were streaked on Columbia blood agar base (Oxoid CM0331) supplemented with 7% v/v human blood and incubated at 37 °C for 48 h. After incubation, the production of a green-colored zone around the colony was classified as α-hemolysis, no effect on the blood agar plates was classified as γ-hemolysis, and lysis of blood around the colony was classified as hemolytic (β-hemolysis).

2.3.4. Antibiotic resistance of fat-removing strains

The antimicrobial resistance of the selected strains, strains E1 and A1, and JBD301, was determined by sensidisk diffusion. Sensidisk (Oxoid) of Clindamycin (CD 2 μg), Piperacillin (TZP 110 μg), Cefuroxime (CXM 30 μg), Ciprofloxacin (CIP 5 μg), Amikacin (AK 30 μg), Cefotaxime (CTX 30 μg), Tetracycline (TE 30 μg), Imipenem (IPM 10 μg), Gentamicin (CN 10 μg), Cephalotin (KF 30 μg), Ampicillin (SAM 20 μg), and Vancomycin (VA 30 μg) were tested on MRS plates in duplicate.

2.3.5. Auto-aggregation ability of fat-removing strains

Auto-aggregation of the selected microorganisms was determined as previously described (Bao et al., 2010). Each strain was inoculated into phosphate-buffered saline (PBS) with an OD of 0.25 at a wavelength of 600 nm using a spectrophotometer (triplicates, two independent experiments). The suspension was then incubated at 37 °C for 20 h, after which the absorbance was measured and recorded. The percentage of autoaggregation (A%) was calculated using the formula: $(A_o - A_t)/A_o \times 100$ where A_o is the absorbance at time 0 and A_t is the absorbance at 20 h.

2.3.6. Antimicrobial assays

The selected strains were tested for antimicrobial activity against three known pathogens: *E. coli*, *Staphylococcus aureus,* and *Klebsiella pneumoniae*. Single colonies of these three pathogens were inoculated on nutrient broth and incubated at 37 °C for 24 h. Bacterial cells were then harvested by centrifugation at 2370 g for 15 min and the supernatant was discarded. Cell pellets were then washed twice, resuspended in sterile PBS, and adjusted to a final OD_{595} of 0.5 (solution referred to as "high" density). This suspension was then diluted tenfold (referred to as "low" density). Aliquots (0.1 ml) of both solutions were added to independent square plates of nutrient agar. Sterile beads were added to each plate to homogenize the sample (by shaking for 10 s in each direction). The beads were then discarded. Agar disks containing the fat-removing strains JBD301, E1, and A1 were placed on each plate so that the tested microorganisms were in contact with the pathogens under investigation (see Fig. S3). Plates were incubated for 24 h at 37 °C and antimicrobial activity was confirmed by measuring a disk-shaped halo around the agar discs (if applied). Each strain was tested in triplicate in two independent experiments.

2.4. Effects of polyphenol-rich cocoa extract on bacterial growth and fat removal

2.4.1. Measurement of total polyphenol content

A cocoa polyphenol extract (total polyphenol content of 254.83 ± 56.85 mg gallic acid equivalents (GAE) per g) obtained from another study (Quiroz-Eraso et al., 2023, submitted) was used. One gram of lyophilized extract was dissolved in 20 ml of Milli-Q water, autoclaved, and added to the culture media as needed. Total polyphenolic content (TPC) was determined by the Folin-Ciocalteu colorimetric method using gallic acid as standard, as previously described (Johnson et al., 2021). Control and sample absorbances were measured at 750 nm and results were expressed as mg of gallic acid equivalents per gram of sample (mg GAE/g). In the case of liquid samples, such as culture media, the density of the medium was considered to express TPC in terms of volume (mg GAE/ml).

2.4.2. Effects of polyphenol-rich cocoa extract on bacterial growth

To determine the susceptibility of the selected strains E1 and A1 and the reference strain JBD301 to PRCE, 10^6 cells/ml of each strain were exposed to culture media supplemented with different extract concentrations using the dilution tube method. Kinetic growth parameters (specific growth rate, **μ,** and generation time, **G**) and final cell viability were measured as response variables. First, an assay was performed in which the three bacterial strains were exposed to two extract concentrations (0 and 15 mg/ml), and cell viability was determined at 0, 4, 8, 12, and 24 h. Then, a second experiment was performed in which the bacterial strains were exposed to five extract concentrations (0, 5, 10, 15, and 20 mg/ml), and cell viability was determined at 0 and 24 h. All cultures were incubated for 48 h at 37 °C under constant agitation (750 rpm). The total polyphenolic content of the culture medium was measured as described previously. Two independent experiments were performed with triplicates for each treatment.

2.4.3. Effects of polyphenol-rich cocoa extract on bacterial palmitic acid removal

To determine the effect of PRCE on fat remotion by the selected strains, each strain was cultured on MRS broth supplemented with palmitic acid and PRCE, and the percentage of fat removal was measured. Different concentrations of PRCE (0, 2, 4, and 6 mg/ml) were evaluated in MRS supplemented with 1 mM palmitic acid. In all cases, incubation was performed for 24 h at 37 °C with constant stirring (750 rpm). The percentage of fat removal was calculated in relation to a control treatment supplemented with 1 mM palmitic acid, to which neither extract nor bacteria were added. The TPC of the culture medium was also measured as previously described. Two independent experiments were performed with triplicates of each treatment.

2.5. Statistical analysis

Statistical analysis was performed with RStudio (2023.03.1.). The *t*-test and one-way analysis of variance (ANOVA) with a minimal significance level of 0.05 were used to evaluate the statistical differences between different cocoa extract concentrations on bacterial kinetic growth parameters, final cell viability, and fat removal. When significant differences were observed, a Tukey's comparison test was performed. Normality, homoscedasticity and independence of the data were confirmed in all cases ($p > 0.05$).

3. Results and discussion

3.1. Selection of fat-removing strains using a rapid, inexpensive colorimetric assay

The effort to isolate fat-removing strains was aided by the recovery of bacteria in a modified standardized medium used for the isolation of Lactobacillus strains (MRS medium) and selection by a rapid, low-cost colorimetric assay. The MRS medium was modified as follows: First, Tween 80 (polyoxyethylene sorbitan mono-oleate) was not added because the oleic acid of Tween 80 is known to be incorporated into membrane lipids of lactic acid bacteria (Nikkilä et al., 1995), and thus potentially interfering with the goal of isolating Lactobacillales that could incorporate palmitic acid, this due to the presence of another lipid in the culture media. Second, the decrease of the pH of the media to 4.5 was used to expose the cells to an environmental stressor, which is likely to exert selective pressure on bacteria that use palmitic acid to alter their membrane lipid composition to increase rigidity and compactness to counteract the stress of excessive proton influx. This mechanism has been observed in *Lactobacillus* species which alter the unsaturated/saturated ratio of their membranes to tolerate acidic stress (Broadbent et al., 2010). Among the 1400 strains tested for their fat-removing capacity, two strains, with the best capacity to remove saturated fats from the culture media were isolated from modified low pH MRS with both saturated fats (palmitic and stearic acid). Both strains were obtained from the inoculated enrichment made with a combination of feces from 3 subjects. Palmitic acid and stearic acid were selected because they are the most abundant saturated fatty acids in Western diets (Ervin et al., 2004). These two saturated fats have pathological effects on the intestinal epithelium, gut microbiota, and inflammatory and lipogenic white adipose tissue profiles (Jamar and Pisani, 2023). Palmitic acid was selected for further experiments because it performed better in the low-cost colorimetric assay for the quantification of lipids in the culture media using copper and sodium diethyldithiocarbamate. This protocol proved to be fast and economical, but because palmitic acid does not dissolve in the culture media, samples must be stirred well before measurements are made. Therefore, the total volume of the culture was used to avoid variability due to heterogeneous sampling.

Two isolates (designated E1 and A1) were selected because they showed removal of the palmitic acid from the culture media comparable to the reference strain. According to MaldiTOF analysis, isolate E1 belongs to the species *P. acidilactici*, and isolate A1 is a strain of the genus *Lactobacillus*. The two isolated strains showed different phenotypic traits, for example, strain E1 grew forming clumps and reached a lower cell density in MRS medium. An increase of the medium concentration of MRS up to 1.5× slightly favored the growth of strain E1, but the strain did not reach the cell density of the reference strain JBD301 and strain A1, E1 was always a one log unit below the other two strains after reaching the exponential phase (data not shown). To determine whether palmitic acid favored the growth of each strain, biomass as dry weight was measured. Under the conditions that were assayed, neither strain was favored by the presence of the fatty acid ($p > 0.05$) (Fig. 1A).

3.2. Enzymatic determination of palmitic acid removal by selected strains

Both selected strains were used for more specific fatty acid determination using the EnzyChrom™ Free Fatty Acid Assay, and the results of fat removal in the MRS medium are shown in Fig. 1B, which shows a statistical difference between the fat removal of the isolated strains ($p > 0.05$) and the reference strain JBD301. Strains E1 and A1 can remove approximately 84% of the palmitic acid removed by strain JBD301. The range of fatty acid removal of these strains is up to 10 times more than reported for other *Lactobacillus* species (Chung et al., 2016).

3.3. Characterization of fat-removing strains

An advantage of probiotic formulations that combine different strains is that these can tolerate different environmental stressors. In the case of strains E1 and A1, each strain may exert its metabolic activity under different stressors in the intestine when used as lipolytic probiotics. Both strains were resistant to low pH (>pH 4) (Fig. 2A), strain E1 was more resistant to high osmotic pressure (NaCl 5 and 6%) (Fig. 2B), and strain A1 was more resistant to bile salts (0.13–0.5%) (Fig. 2C). The

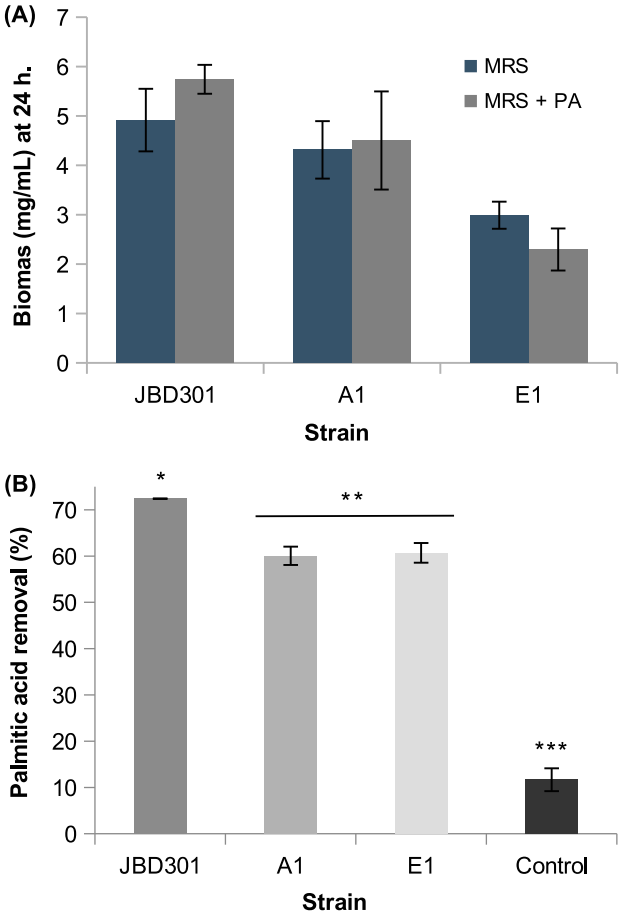

Fig. 1. **(A)** Growth of strains JBD301, A1, and E1 in MRS 1.5× vs MRS 1.5× supplemented with palmitic acid (MRS + PA) at 0 and 24 h, measured by dry weight (error bars represent the standard deviation from two independent experiments, n = 6). Non-significant difference between treatments was determined ($p < 0.05$) **(B)** Percentage of palmitic acid removal from the culture media by strains JBD301, A1, and E1 measured by EnzyChrom™ assay (error bars represent the standard deviation of duplicates from two independent experiments, n = 6). The asterisks above bars indicate clustering due to significant differences between treatments ($p < 0.05$), according to Least Significant Difference (LSD).

strains also showed no significant differences in their stimulation of production of proinflammatory cytokine production by macrophages (Fig. 2D). The strains stimulated the production of TNF-α significantly less than the LPS positive control ($p < 0{,}001$); in contrast, *Bacteroides* strains, including *Bacteroides uniformis* CECT 7771 which ameliorated high-fat diet-induced metabolic and immune dysfunction in obese mice, induced the production of significantly higher amounts of TNF-α than the LPS-stimulated macrophages (Gauffin-Cano et al., 2012); the difference may be that lactobacilli are Gram-positive bacteria, whereas *Bacteroides* spp. are Gram-negative bacteria. LPS is a major component of the outer membrane of Gram-negative bacteria and is also a potent immunogen (Louis and Lambert, 1979).

Apart from this, the phenotypic features of the isolated strains were consistent with the subsequent taxonomic identification. The species *P. acidilactici* has been used as a probiotic for humans in different formulations, is resistant to low pH and the genus presents a high auto-aggregative ability (Olajugbagbe et al., 2020). Accordingly, strain E1 has a significantly higher auto-aggregative ability ($p < 0.005$) than the other two strains (Fig. S1). This characteristic is beneficial for probiotics as it facilitates the adhesion of the strain to the intestinal walls through its surface layer protein (Olajugbagbe et al., 2020).

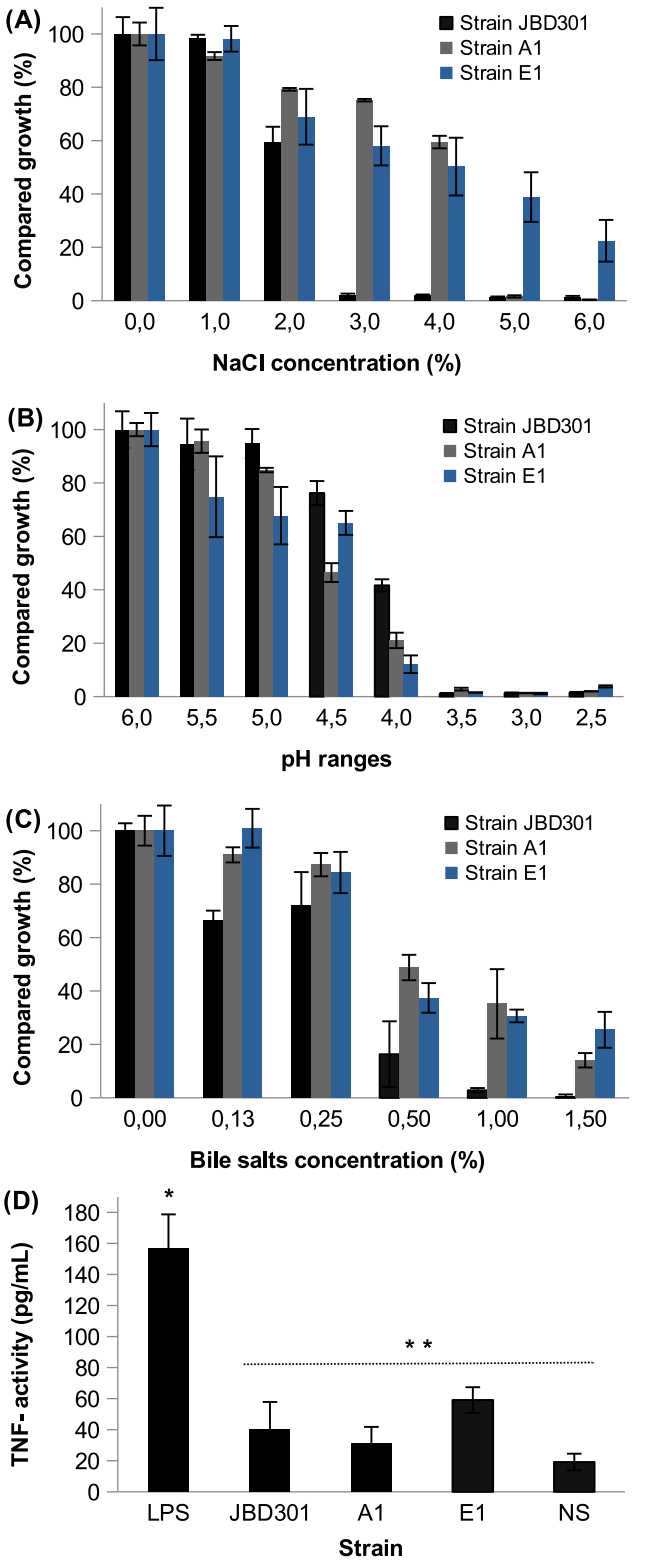

Fig. 2. Growth percentage of fat-removing strains in acid pH **(A)**; high osmotic pressure **(B)**, and bile salts **(C)**, relative to the control media. Error bars represent the standard deviation of 5 replicates from two independent experiments. **(D)** TNF-α production by THP-1 macrophages upon exposure to strains JBD301, A1, and E1; purified LPS from *E. coli* was used as the positive control (LPS) and unstimulated cells (NS) were used as a control for basal TNF-α production. The asterisks above bars indicate clustering due to significant differences between treatments ($p < 0.001$), according to Least Significant Difference (LSD).

Hemolysis was tested to assess the safety of the strains. The guidelines for the evaluation of probiotics in food (Hotel and Cordoba, 2001) state, "If the strain under evaluation belongs to a species with known hemolytic potential, determination of hemolytic activity is required". Strain E1 showed γ-hemolysis (no hemolysis), while strain A1 and the reference strain JBD301 both showed α-hemolysis (Fig. S2). Alpha-hemolysis among the genus *Lactobacillus* is not uncommon (Goldstein et al., 2015), but their use as probiotics is safe only when γ-hemolysis is demonstrated. Meanwhile, among the 12 antibiotics screened, all strains were resistant to three antibiotics, ciprofloxacin, ampicillin, and vancomycin, except for strain JBD301 which was sensitive to ampicillin (Table S1). Finally, antimicrobial activity (Figs. 3 and S3) showed inhibition against pathogens *E. coli*, *K. pneumoniae,* and *S. aureus*. The same assay was carried out by testing the potential probiotic strains against each other to determine a possible antagonism of the microorganisms, but no inhibition was detected.

3.4. Polyphenol-rich cocoa extract effect

To evaluate the effect of PRCE on the growth of strains E1, A1, and JBD301, growth curves were plotted with and without the addition of cocoa extract (Fig. 4A). The growth of strain E1 after 24 h was reduced by more than 1 log(CFU/ml) with the addition of 15 mg/ml cocoa extract, whereas the growth of strains JBD301 and A1 was neither reduced nor increased. To better understand the behavior of strains E1, A1, and JBD301 under the effect of cocoa extract, the growth kinetic parameters were determined. It was observed that the exponential phase was reached between 4 and 12 h of incubation. Between 12 and 24 h, each curve reached the stationary phase. From the exponential phase equation, the generation time (G) values shown in Fig. 4B were determined. The only strain that showed a significant difference in the presence of cocoa extract was strain E1, which had a significantly higher generation time ($p < 0.001$). However, the generation time of strain E1 without cocoa extract is not significantly different from that of strains JBD301 or A1, suggesting that although at the stationary phase strains JBD301 and A1 achieved higher cell viability than strain E1, during the exponential phase they all grew at a similar specific growth rate, if strain E1 is not exposed to cocoa extract; in which case, the growth rate of strain E1 is reduced. Only strains JBD301 and A1 were able to withstand the addition of cocoa extract and maintain similar specific growth rates and generation time values. Similarly, when different extract concentrations were evaluated, *Lactobacillus* spp. strains were able to resist the addition of cocoa extract and the final cell viability of these strains remained almost constant throughout the different concentrations. However, the inhibitory effect of the polyphenolic extract on the bacterial growth of strain E1 was notable, as it was evident that the final population counts decreased with increasing phenolic concentrations, becoming significant at only 5 mg/ml (Fig. 4C). Furthermore, at 20 mg/ml, the cell viability of strain E1 after 24 h is lower than the initial cell viability. A linear regression predicting the final cell viability when strain E1 is exposed to the PRCE indicated that the viability of strain E1 is reduced by 0.7 Log(CFU/ml) for each mg GAE/ml added. However, the effects of PRCE on bacterial fat removal were milder and none of the strain's fat removal activities were affected by the presence of polyphenols. Strain E1 achieved the same fat removal as when there was no addition of the extract (Fig. 4D). A possible explanation for this could be that the PRCE is a stress for strain E1. The cocoa extract contains several polyphenols, including catechins, which are compounds that have been shown to bind to and destabilize the membrane of Gram-positive bacteria (Kajiya et al., 2004). Strain E1 may use palmitic acid for a similar mechanism mentioned above, in which lactobacilli increase the rigidity and compactness of their membranes when exposed to environmental stress such as low pH, which decreases the unsaturated/saturated ratio of their cytoplasmic membrane (Broadbent et al., 2010). This may explain why the lipolytic activity of strain E1 is not affected under stress, but growth is.

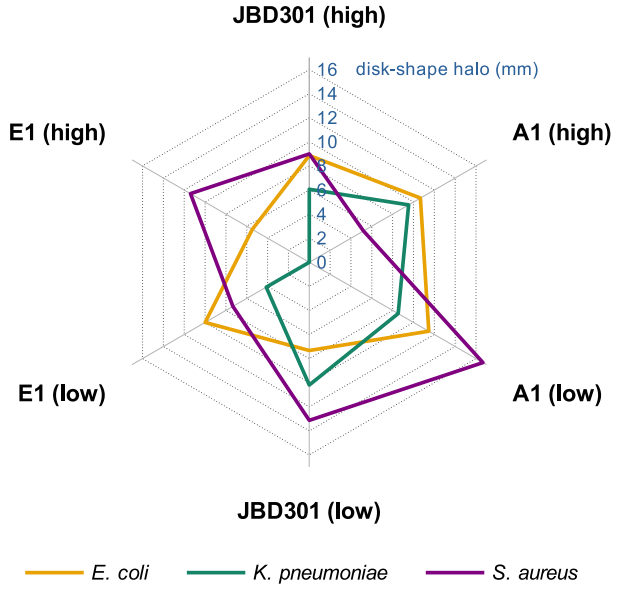

Fig. 3. Antimicrobial activity is represented by the diameter (millimeters) of a disk-shaped halo around the agar discs. The inoculum levels of probiotic strains were denoted as follows: 10^6 ufc/ml (low) and 10^7 ufc/ml (high).

4. Conclusions

In this study, we improved a strategy to isolate fat-removing strains belonging to the order Lactobacillales in a faster and more affordable way than previously described (Chung et al., 2016). For future isolation efforts, including polyphenol-rich extracts in the fecal incubations, is a feasible way to select for strains that can resist the presence of these bioactive compounds, also evaluating probiotic attributes in the presence of μM concentrations of polyphenols, since μM is the quantity they reach in the gastrointestinal tract according to Galleano et al. (2010), can enhance the selection of probiotic strains that can stand the presence of these beneficial biocompounds. Probiotics and polyphenols are scarcely evaluated in combination despite being consumed together regularly (e.g., yogurt and fruits). However, the scientific literature is beginning to evaluate novel formulations of polyphenol-enriched probiotic yogurts (Walter et al., 2021) and nutraceuticals (Sampaio et al., 2023), and there is evidence of the beneficial effect of their combination on conditions (Wang et al., 2023). In this study, we evaluated these biocompounds as a potential strategy for intestinal free-fatty acid removal. However, the combination of these has the potential to provide multiple health benefits. For example, polyphenols have been observed to enhance the adhesion to intestinal mucus of *Lactobacillus* strains (Yuan et al., 2022), both biocompounds have been observed to prevent or restore gut microbiota dysbiosis (Li et al., 2021; Anjana and Tiwari, 2022), and both prevent and manage diabetes (Cao et al., 2018; Tao et al., 2020), among other benefits, thus, more studies that explore if these two biocompounds can act synergistically in different conditions are needed. It is also important to screen for α- and β-hemolysis because it can be presented in bacteria belonging to this order, as was the case for strain A1 and the commercially available reference strain JBD301; the non-antibiotic multi-resistance is another important trait to evaluate and it was low in the screened strains. Compared to other probiotic Lactobacillus strains, A1 and E1 strains were more resistant to environmental conditions such as high osmotic pressure and bile salts, and in the normal range of pH tolerance. Polyphenols had different effects on the viability of fat-removing strains: *Pediococcus* strain E1 is affected by increasing polyphenols concentrations, while on *Lactobacillus* spp.

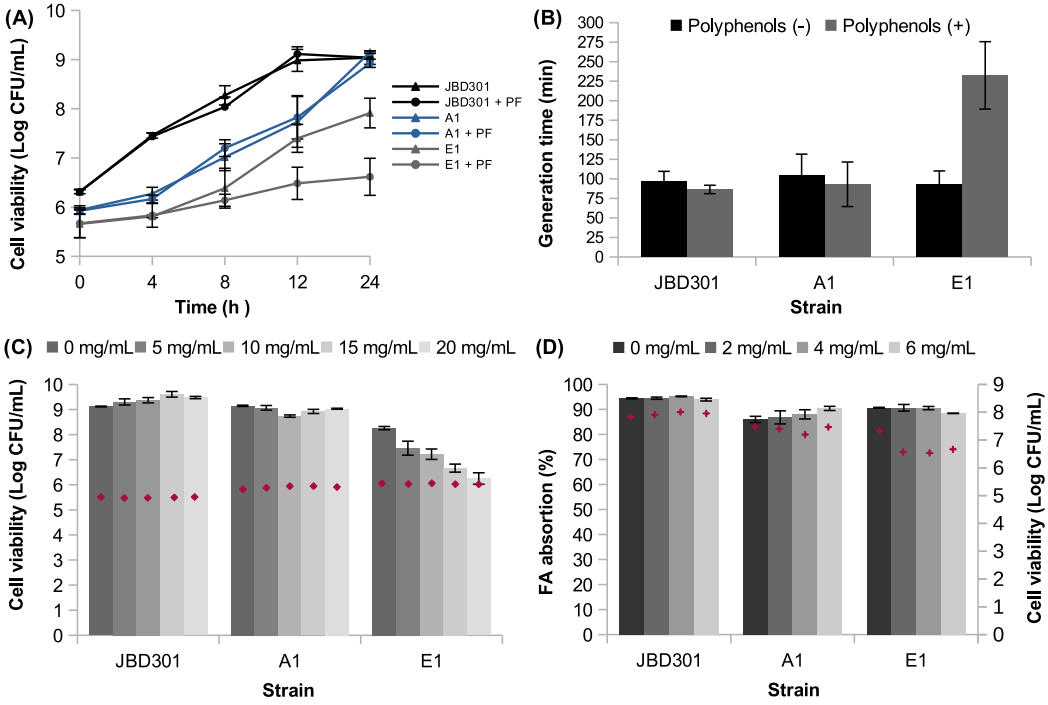

Fig. 4. **(A)** Growth curves of JBD301, E1, and A1, with (15 mg/ml) and without the addition of polyphenol-rich cocoa extract (PRCE) throughout 24 h (error bars represent the standard deviation of duplicates from two independent experiments, n = 6). **(B)** Generation time (G) of JBD301, E1, and A1, with (15 mg/ml) and without the addition of PRCE (error bars represent the standard deviation of triplicates from two independent experiments, n = 6). **(C)** Cell viability of JBD301, E1, and A1 to different PRCE concentrations (error bars represent the standard deviation of triplicates from two independent experiments, n = 6). The red diamonds indicate the initial cell viability. **(D)** Palmitic acid remotion and cell viability [Log(CFU/ml)] of strains JBD301, A1, and E1 under different PRCE concentrations (error bars represent the standard deviation of triplicates from two independent experiments, n = 6). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

strains (A1 and JBD301), polyphenols showed no harmful effect. Despite the effect of polyphenols on the growth of strain E1, *in vitro* fat removal was not affected. Strain E1, which was able to remove saturated fat even in the presence of a polyphenol-rich cocoa extract, did not exhibit hemolysis and belonged to a recognized probiotic species, *P. acidilactici*. This strain has the potential to be evaluated as a probiotic with an anti-obesogenic effect and it can be studied if the presence of polyphenols enhances this effect.

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Institutional board review statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and was approved by the Ethical Committee of La Sabana University. The collection and study used in this research were covered under the permission granted by the Ministerio de Ambiente y Desarrollo Sostenible de Colombia to Universidad de La Sabana, through the contract for access to genetic resources ("Contrato Marco de Acceso a Recursos Genéticos y sus Productos Derivados") No. 325.

Informed consent statement

Written informed consent and verbal assent were obtained from volunteers before participation in the study and data/sample collection. The privacy rights of human subjects were always maintained.

CRediT authorship contribution statement

Samuel Quiroz-Eraso: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization. **Gina Paola Rodríguez-Castaño:** Writing – review & editing, Funding acquisition. **Alejandro Acosta-González:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT to improve readability. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2023.100594.

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