LUCAS MARQUES • CARLOS ALBERTO GUERRA • ANDRÉ FIORNTE GUERRA

BIOCONSERV

The Revolution in Scientific Conservation

The Science Behind Bioconserv:
Ensuring Safety and Extended Shelf Life

BRC

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The Revolution in Scientific Conservation

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BRC

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Introduction

About BRC:
More than a seasoning factory, BRC is a research, development, and innovation center focused on solutions in the food industry. Since its foundation in 2011, BRC has excelled in the production and commercialization of ingredients that enhance the taste and quality of foods. Today, the company goes beyond these activities, creating advanced technologies to meet the specific demands of the meat and sausage market.

With a comprehensive and high-quality portfolio, BRC stands out for its innovative work in research and development. Our infrastructure includes state-of-the-art equipment and laboratories, and our team consists of highly qualified professionals. These pillars are fundamental to our leadership role in food science and technology.

Key Achievements and Scientific Partnerships:
BRC has excelled on various scientific fronts, conducting research and publishing in renowned journals such as "Meat Science," "Fermentation," and "Sustainability." We participate in important international conferences, such as the "Applied Microbiology and Benefits Microbes" in Paris (2021), the "2nd Global Summit on Food Science and Technology" in Rome (2023), and the "69th International Congress of Meat Science and Technology" in Padua (2023), reaffirming our commitment to innovation and excellence.

In 2022, BRC achieved second place in the Most Innovative Ingredient category at the Fi Innovation Awards, a prestigious award that celebrates innovation in the South American food, beverage, and ingredient sector. This recognition was achieved with Bioconserv, our bioaroma with antimicrobial and probiotic properties.

Bioconserv:
Bioconserv is a revolutionary ingredient with antimicrobial and probiotic properties that not only enhances the sensory and visual attributes of foods but also has about 10 times less sodium compared to conventional products and approximately five times greater effectiveness. This ensures tastier, safer, and healthier foods.

This eBook details the scientific evidence supporting the effectiveness of Bioconserv, highlighting its ability to transform the reality of food manufacturers. Our goal is to provide a comprehensive overview of how Bioconserv can be an innovative and effective solution for food preservation, backed by rigorous scientific data.

Bioconserv

Product Description
BioConserv is an innovative biopreservative designed to enhance food safety and extend the shelf life of various products. It is a clear to light yellow aqueous solution with a viscosity close to pure water and a characteristic odor of lactic acid bacteria-fermented beverages. The product has a hydrogen ion concentration (pH) of less than 3.5 and a low sodium content, making it a health-conscious choice for food preservation. The manufacturing process involves precise microbial activation and fermentation steps, ensuring the consistency and effectiveness of the biopreservative.

BioConserv is produced using a meticulous fermentation process involving *Lacticaseibacillus* strains. The bacteria are activated, cultivated, and fermented in an optimized medium, ensuring maximum efficacy. The product is pasteurized to eliminate any unwanted microorganisms while retaining the beneficial

metabolites produced by the probiotics. The result is a highly effective preservative that can be used across a wide range of food products, from meats and sausages to dairy and ready-to-eat meals.

The development of BioConserv involves advanced biotechnological methods to ensure the highest quality and safety standards. The product is manufactured in state-of-the-art facilities equipped with bioreactors that provide controlled fermentation conditions. These conditions are critical for producing consistent batches of BioConserv, each meeting stringent quality control parameters. The rigorous production process also includes calibration steps to ensure the correct concentration of active microbial cultures, which are essential for the biopreservative's effectiveness.

Antimicrobial and Probiotic Properties
BioConserv exhibits robust antimicrobial properties due to the metabolites produced by probiotics during fermentation. These properties include:

- **Antimicrobial:** BioConserv effectively inhibits the growth of harmful bacteria, thereby ensuring food safety and reducing the risk of foodborne illnesses.
- **Anti-inflammatory:** The product helps mitigate inflammation, contributing to overall health.
- **Immunomodulatory:** It supports the immune system, enhancing the body's natural defenses.
- **Antiproliferative:** BioConserv can inhibit the proliferation of unwanted microorganisms, further extending the shelf life of food products.
- **Antioxidant:** The product contains compounds that prevent oxidative damage, maintaining the quality and nutritional value of the food.
- **Anxiolytic and Antidepressant:** The presence of serotonin-like compounds contributes to mental well-being, highlighting the holistic benefits of BioConserv.

Benefits Compared to Conventional Products
BioConserv offers several advantages over conventional food preservatives:

- **Health Benefits:** Unlike traditional preservatives that often contain high levels of sodium and synthetic chemicals, BioConserv is a natural product with low sodium content and no synthetic additives. Its postbiotic properties promote gut health and overall well-being.
- **Extended Shelf Life:** BioConserv significantly extends the shelf life of food products, reducing the rate of spoilage and the risk of microbial contamination. This extension of shelf life not only ensures safer food consumption but also minimizes product returns, loss of products, and consequential financial setbacks for industries.
- **Environmental Impact:** The production process of BioConserv is environmentally friendly, with 100% yield and no effluent generation. It utilizes natural and organic sources, aligning with sustainable practices.
- **Regulatory Compliance:** BioConserv meets stringent food safety standards, including Halal and FSSC 22000 certifications. These certifications assure consumers of the product's safety, quality, and compliance with Islamic dietary laws, enhancing marketability in diverse regions.
- **Reduction in Food Waste:** By extending the shelf life and ensuring the safety of food products, BioConserv helps reduce food waste significantly. This reduction in waste translates to better food security and economic savings.

- **Consumer Trust:** The use of natural ingredients and the absence of synthetic chemicals build consumer trust. The product's certifications further enhance its credibility, making it a preferred choice for health-conscious consumers.

In summary, BioConserv not only provides superior antimicrobial and probiotic benefits but also addresses critical issues related to health, environmental sustainability, and economic efficiency. Its natural formulation and extensive certifications make it an outstanding alternative to conventional food preservatives, ensuring safer and healthier food options for consumers.

Recognition and Awards

Fi Innovation Awards
The Fi Innovation Awards represent a pinnacle of recognition in the food and beverage industry, celebrating innovation and excellence in ingredients, products, and solutions that impact both industry practices and consumer experiences. These prestigious awards, along with the Start-Up Innovation Challenge, highlight advancements that push boundaries and enhance sustainability across the sector.

BRC's BioConserv has garnered significant acclaim at the Fi Innovation Awards, underscoring its role as a natural flavoring agent derived from natural raw materials and ingredients. It stands out for its postbiotic properties, which include beneficial effects on the gut-brain axis, anti-inflammatory and immunomodulatory actions, as well as antioxidant and antimicrobial capabilities. These attributes not only contribute to enhancing food quality and safety but also align with sustainability goals by reducing food waste and promoting healthier dietary practices.

The awards' distinguished judging panel, comprising C-Level professionals and esteemed researchers, reinforces the credibility and impact of BRC's innovations in advancing food science and technology. By addressing critical issues such as food preservation, BioConserv exemplifies a sustainable solution that supports economic efficiency, environmental stewardship, and social responsibility in the food industry. This recognition underscores BRC's commitment to driving positive change through cutting-edge research and development, ultimately benefiting both industry stakeholders and consumers worldwide.

Awards and recognitions
In addition to the notable achievement at the Fi Innovation Awards, BRC Ingredientes has been honored with several other prestigious industry awards and recognitions, showcasing its commitment to innovation, quality, and sustainability in the food sector.

- **Halal Certification**

Achieving Halal certification is another testament to BRC's dedication to maintaining high standards in food safety and ethical production. This certification confirms that BRC's products and manufacturing processes comply with Islamic principles and values. Importantly, it also validates the company's robust food safety management system, ensuring the safe consumption of its products by both Muslim and non-Muslim consumers. BRC's certification audit resulted in zero non-conformities, highlighting the company's commitment to providing safe and high-quality food products.

- **FSSC 22000 Certification**

In 2021, BRC Ingredientes earned the FSSC 22000 certification, version 5.1. This certification, which includes ISO 22000, ISO TS/22002-1, and additional requirements established by the certification scheme, is recognized by the Global Food Safety Initiative (GFSI). The FSSC 22000 certification aids organizations in managing food safety across the production chain, ensuring compliance with regulatory, legal, and industry standards. By achieving this certification, BRC demonstrates its ethical responsibility, reliability,

and commitment to its customers and partners. The certification enhances the company's reputation and assures clients of the integrity and quality of its management, production, and supply systems.

- **Scientific Contributions and Conference Participation**

BRC's role in advancing food science and technology is further evidenced by its active engagement with the scientific community. The company has formed valuable partnerships with national and international scientific institutions and universities, resulting in significant research and publications in esteemed journals such as "Meat Science," "Fermentation," and "Sustainability." BRC has also participated in prominent international conferences, including "Applied Microbiology and Benefits Microbes" (Paris 2021), the "2nd Global Summit on Food Science and Technology" (Rome 2023), and the "69th International Congress of Meat Science and Technology" (Padova 2023). These contributions highlight BRC's leadership and ongoing commitment to innovation in the food industry.

These awards and recognitions collectively demonstrate BRC Ingredientes' excellence in innovation, food safety, and sustainability, solidifying its reputation as a leading company in the food ingredients sector.

Introduction: Potentially Postbiotic-Containing Preservative to Extend the Use-By Date of Raw Chicken Sausages and Semifinished Chicken Products

The challenge of extending the shelf life of perishable foods, such as raw chicken sausages and semifinished chicken products, has long been addressed through various preservation methods. Traditionally, synthetic preservatives have dominated this field due to their cost-effectiveness and efficacy. However, growing consumer demand for natural and healthier alternatives has spurred interest in biopreservatives derived from Generally Recognized as Safe (GRAS) microorganisms.

Postbiotics, a term encompassing non-viable microbial products with potential health benefits, have emerged as promising candidates for food preservation. These include metabolic by-products and cell extracts known to influence physiological functions beneficially without the risks associated with live microorganisms. In this context, the present study explores the use of a potentially postbiotic-containing preservative (PPCP) produced through semiculture fermentation involving *Lacticaseibacillus paracasei* and *Saccharomyces cerevisiae*.

Raw chicken sausages and semifinished chicken products, notorious for their perishability due to high biological activity, served as the test subjects. The study evaluated PPCP's effectiveness in inhibiting spoilage microorganisms under different incubation temperatures and time periods. Results from in vitro trials indicated significant microbial inhibition at concentrations above 3.0% PPCP, albeit with cost implications.

Moreover, the research considered practical applications by testing PPCP in situ, revealing its potential to extend product shelf life under simulated distribution and market conditions. This approach aligns with current trends favoring natural preservation methods that enhance food safety and quality while meeting regulatory standards.

In conclusion, leveraging the metabolic products of GRAS microorganisms like *L. paracasei* and *S. cerevisiae* presents a promising avenue for developing biopreservatives that address both consumer demand for natural food additives and industry requirements for effective, economically viable solutions in food preservation.

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Article

Potentially Postbiotic-Containing Preservative to Extend the Use-By Date of Raw Chicken Sausages and Semifinished Chicken Products

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Abstract: This study aimed to evaluate the use of potentially postbiotic-containing preservative (PPCP), produced in a semiculture fermentation system with *Lacticaseibacillus paracasei* DTA 83 and *Saccharomyces cerevisiae* var. *boulardii* 17, to extend the use-by date of raw chicken sausages and semifinished chicken products. Microorganisms associated with the spoilage of chicken products were stimulated to grow by pair incubation of the products at two different temperatures and with collection at different times. The turbidity method was performed to evaluate the microbial susceptibility to PPCP. PPCP was added in chicken products to obtain an in situ partial inhibitory effect on spoilage microorganisms to extend the use-by date. The in vitro trial showed total inhibition of the microbial growth by adding above 3.0% of PPCP. Although this concentration showed a remarkable inhibitory potential, its addition can severely impact the formulation cost. Thus, the application of doses with partial microbial inhibition may be a suitable strategy for the use of PPCP in chicken products. The results revealed that cold chain management and couse of PPCP in chicken products extended the proposed use-by date, suggesting an alternative food preservation technology for the use of naturally derived compounds.

Keywords: biopreservative; biocontrol; metabiotic; beneficial; compounds

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1. Introduction

The potential of microorganisms and/or their metabolic products to extend the shelf life and enhance the safety of foods dates back centuries [1]. While biocontrol of food using Generally Recognized as Safe (GRAS) microorganisms was already reported in the literature, studies concerning the use on industrial scale is still scarce [2,3]. The cost to purchase available biocins in the commerce is determinant to discourage the use [4]. As a result, food operators prefer chemically synthesized preservatives [5,6]. Thus, precultured medium with GRAS microorganisms may be a low-cost alternative regarding biopreservatives in foods.

Raw meat, fresh poultry meat, and poultry products, such as raw chicken sausages and semifinished chicken products, are highly perishable foods due to their biological composition. Therefore, the high consumption of poultry products leads to concerns about product safety, shelf life, quality, and desirable sensory characteristics [7]. Regulatory agencies prescribe for "raw chicken or seasoned meat, cold or frozen stored" a microbiological limit that separates good quality from marginally acceptable quality (m = 5 log cfu/g) [8–10]. Thus, the theory of barriers, as the use of preservatives to slow microbial growth, is often applied to extend shelf life.

Over the past decades as consumer awareness of the impact of food on health grew, alternative technologies for food preservation based on naturally derived compounds emerged. *Lacticaseibacillus paracasei* DTA 83 and *Saccharomyces cerevisiae* var. *boulardii* 17 were reported as candidate strains to deliver probiotics in food matrices. Moreover, probiotic strains were extensively used in the meat poultry chain, increasing consumers' interest in functional foods. The high capacity of probiotics to provide beneficial health effects in the host attracted scientific and commercial interests, highlighting microbial administration as a health-promoting strategy [11,12].

Some rigorous processes and analyses precede the commercialization of probiotic-containing functional foods to guarantee their safety for consumption [13]. However, there are also many restrictions related to the consumption of live microbes: systemic infections due to translocation, particularly in vulnerable patients such as pregnant and pediatric and geriatric populations, acquisition of antibiotic resistance gene, and interference with gut colonization in neonates [14,15]. Therefore, delivering probiotics to health-impaired individuals or when medical institutions are considered is still a matter of discussion. Indeed, the postbiotic effect derived from GRAS microorganisms can be safe in all circumstances. As a result, the production of products containing nonviable microorganisms or microbial cell extracts to provide beneficial effects in the host, such as probiotics, received considerable attention in recent years [16].

Postbiotic or synonymous, such as parabiotics, metabiotics, ghostbiotic, and heat-inactivated microorganisms, refers to inactivated or produced compounds by microorganisms with a known chemical structure that can optimize host-specific physiological functions and regulate metabolic and/or behavior reactions connected with the activity of host natural microbiota [17,18]. To date, these terms were not endorsed by regulatory agencies; however, they were extensively studied and reported in the current literature [19–21].

In this background, this study aimed to produce potentially postbiotic-containing preservative (PPCP) in a semiculture fermentation system with *L. paracasei* DTA 83 and *S. boulardii* 17 to extend the use-by date of raw chicken sausages and semifinished chicken products. In addition, three logistic distribution routes (R), including distribution centers (DC) and sale disposal of the products in the markets (M), were drafted to evaluate the impact of the cold chain management on the use-by date of the products.

2. Materials and Methods

2.1. Method Design

The products (raw chicken sausages and semifinished chicken products) were manufactured on industrial scale at a meat industry located in the state of Paraná, Brazil. The study was performed in two phases. During Phase 1, microorganisms associated with the spoilage of chicken products were collected after pair incubation of the products at lower (3 °C) and higher (25 °C) temperatures, with collection within four days. Different culture media were obtained from HiMedia (Mumbai, India). Brain–heart infusion (BHI), casoy, de-Man, Rogosa, and Sharp (MRS), and yeast-peptone-dextrose extract (YPD) broth were used to collect major group of microorganisms, including Gram-positive and negative bacteria and yeasts. The susceptibility of the target microbiota to PPCP was assayed by turbidity method. During Phase 2, PPCP was produced on a pilot-industrial scale semicultured fermentation system and tested in situ in a controlled blind design, adding 1.0% and 1.5% of PPCP. The standard formulation of each group was produced under the same conditions

to serve as a control [22]. Five packages of each sample group (totaling 45 packages) were addressed to the laboratory for shelf-life validation. A durability study was performed by a microbial growth predictor, named MicroLab_ShelfLife® (Appendix A), under a realistic temperature profile recorded by an electronic device in three R, totaling 27 simulations. Borderline of 5 log cfu/g was entered in the predictive modeling to indicate the use-by date of the products according to regulatory agencies [8–10].

2.2. Microbial Collection

L. paracasei DTA 83 was isolated from stools of infants aged two weeks old at Rio de Janeiro (Brazil) in selective modified MRS agar medium (Lawvab) [23]. A protocol of Fernandes Figueira Institute (FIOCRUZ) was rigorously applied to collect and transport the samples. The strain was firstly identified by sequencing of the 16S rDNA. Then, the complete genome was drafted and deposited in GenBank under the accession number QRBH00000000, [23–25]. *L. paracasei* DTA 83 was classified as a candidate probiotic by in vitro and in vivo trials [26,27]. This strain was considered to carry out food bioprocesses as reported by Guerra et al., Silva et al., and Oliveira et al. [23,28,29]. *S. boulardii* 17 (FLORATIL-200, Merck, France) was acquired as freeze-dried culture sachets.

The cultures were registered in the self-declared system of the Brazilian genetic heritage (SISGEN): *L. paracasei* DTA 83 and *S. boulardii* 17 (FLORATIL-200, Merck, Paris, France).

2.3. PPCP Production

PPCP was produced in a semiseparated coculture system at BRC Ingredientes Ltd.a, located in the state of São Paulo, Brazil. *L. paracasei* DTA 83 and *S. boulardii* 17 cultures were thawed at 7 °C for approximately 4 h and centrifuged at 6000× *g* for 5 min (2K15, Sigma Laborzentrifugen, Osterode am Harz, Germany) for pellet separation. The liquid fraction (culture medium and glycerol) was discarded. Then, the remaining cell pellet was reconstituted with MRS or YPD, followed by overnight incubation at 36 and 30 °C for the growth of *L. paracasei* DTA 83 and *S. boulardii* 17, respectively. To obtain sufficient biomass to produce PPCP on a pilot-industrial scale, the cultures were scaled up 1/10 (*v/v*) in axenic cultivation in a sterile culture medium with 0.05 M soy protein, 0.1 M glucose, and 0.005 M phosphate. A cylindrical bioreactor (300 L), made of stainless steel equipped with a stirring system and with a domed top and bottom, was used to produce PPCP. About 70% of the nominal capacity of the vessel was loaded with culture medium under a slight agitation (about 84 rpm) performed axially using a mechanical stirrer with a four-blade propeller (50 × 15 mm, length × width) and a 45° pitch coupled to the bioreactor. The heat treatment (75 °C/2 h) was carried out by the electrical activation of three resistors (3 kw), which were equidistant installed around the circumference of the vessel and positioned at $\frac{1}{4}$ the height of the tank bottom. After that, the temperature was reduced to 36 °C by adding 20 kg of drinking ice. Semi-separated co-culture system was performed by inoculating *L. paracasei* DTA 83 to obtain a final cell concentration of ca. 7 log cfu/mL. After 30 h, the pH decreased to around 4.8 and the temperature of the medium was reduced to 30 °C at a rate of 0.5 °C/min. Then, *S. boulardii* 17 was inoculated to obtain a final cell concentration of ca. 6.0 log cfu/mL. After three days of fermentation coupled with pH decay to around 4.0, the product was heated at 90 °C for 10 min (heating rate of 1.2 °C for minute) to obtain PPCP. Variables such as pH and temperature were continuously monitored over the process by a portable digital pH meter (AK40, Akrom, São Paulo, Brazil) equipped with automatic temperature compensation. The cultures were enumerated on selective agar medium as reported by Oliveira et al. [30,31] PPCP was hot bottled in polypropylene containers of 20 L. The presence of remaining cells of *L. paracasei* DTA 83 and *S. boulardii* 17 in PPCP after the heating treatment was assessed by plate counting on MRS and WL agar medium as previously described. Plates were examined for the presence of typical colonies of each culture.

2.4. Determination of Kinetic Fermentation Parameters

Initial (X_0), maximum (X_{max}) and viable cell concentration (log10 cell/mL) during the time (t) (X), specific maximum growth rate (μ_{max}), and Lag phase period (λ) were normalized according to modified Gompertz's mathematical model (Equations (1) and (2)) or Baranyi's model (Equations (3) and (4)). To evaluate the adequacy of mathematical models, coefficient of determination (R^2) obtained by DMFit software version 3.5 (Institute of Food Research, Norwich, UK), root mean square error (RMSE), bias factor (Bf), and accuracy factor (Af) were determined (Equations (6)–(8) [32–34]:

$$y(t) = X_{max} \cdot exp(-exp((\mu_{max} \cdot e / X_{max}) \cdot (\lambda - t)) + 1) \quad (1)$$

$$y(t) = \ln(X / X_0) \quad (2)$$

$$y(t) = y_0 + \mu_{max} \cdot X_{max}(t) - (1/m) \cdot \ln\left(1\left(\left(e^{(m \cdot \mu_{max} \cdot X_{max}(t))} - 1\right) / e^{(m \cdot (y_{max} - y_0))}\right)\right) \quad (3)$$

$$X_{max}(t) = t + (1/\mu_{max}) \cdot \ln\left(e^{(-\mu_{max} \cdot t)} + e^{(-h_0)} - e^{((-\mu_{max} \cdot t) - h_0)}\right) \quad (4)$$

$$h_0 = -\ln \alpha_0 = \ln(1 + (1/q_0)) = \mu_{max} \cdot \lambda \quad (5)$$

$$RMSE = \sqrt{\sum \left(value_{predicted} - value_{observed}\right)^2 / n} \quad (6)$$

$$A_f = 10^{((\sum log|value_{predicted} / value_{observed}|)/n)} \quad (7)$$

$$B_f = 10^{((\sum log(value_{predicted} / value_{observed}))/n)} \quad (8)$$

where, X—viable cell concentration (cell/mL) on the time (t); X_0—initial viable cell concentration (cell/mL); X_{max}—maximum viable cell population (ln cell/mL); $y(t)$—viable cell concentration (ln cell/mL) on the time (t); y_0—initial viable cell concentration (ln cell/mL), y_{max}—maximum viable cell concentration (ln cell/mL); m—parameter related to the curving profile between the log and the stationary phase; n—number of experimental points taken over the experiment.

2.5. In Vitro Trial

Spoilage Microbial Obtention and Inoculum Preparation

Potentially food spoilage microorganisms were obtained from raw chicken sausages and semifinished chicken parts (seasoned chicken slit back and thigh) produced on industrial scale at a meat industry located in the state of Paraná, Brazil. Microorganisms were stimulated to grow in five packages per sample group incubated at 3 °C and 25 °C following the MicroLab_ShelfLife® method. A package per group was analyzed immediately after receiving the samples in the laboratory. Biological oxygen demand (BOD) incubators were used to incubate the samples with withdrawal on days 2 and 4 (3 °C) and on days 1 and 3 (25 °C). A decimal suspension (1/10) was prepared by weighing 25 g of the product into 225 mL of PB. An aliquot (100 µL) was transferred with a micropipette and sterile tip to screw-cap tubes with enrichment culture broth medium (BHI, casoy, MRS, and YPD) for growth of Gram-positive and negative bacteria and yeasts (all media were obtained from HiMedia, Mumbai, India). The tubes were incubated at 30 °C for 24 h. Then, those with expressive microbial growth represented by an absorbance value above 0.2 at 620 nm (Biospectro, SP-2000UV, São Paulo, Brazil), were used to prepare the inoculum. Tubes absent of growth were incubated for more 24 h and re-evaluated. Remaining the absence, the tubes were eliminated from the test.

An aliquot (1 mL) from each tube with expressive microbial growth grouped per culture medium was transferred to an empty sterile screw-cap tube. Washed out biomass cell pellet was obtained as described in Section 2.3. The turbidity of the inoculum tube was adjusted to achieve 0.5 McFarland standard (ca. 8.0 log cfu/mL of *L. paracasei* DTA 83 and ca. 6.5 log cfu/mL of *S. boulardii* 17). To perform this step accurately, a spectrophotometric device (Biospectro, SP-2000UV, São Paulo, Brazil) was used to compare the inoculum

turbidity and the 0.5 McFarland standard. The microbial suspension was used within 30 min.

2.6. Microbial Susceptibility to PPCP

The turbidity method was performed to evaluate PPCP doses that achieved microbial control regarding spoilage microorganisms. Thus, PPCP was randomly outlined ranging concentrations from 0.5 to 3.5% in BHI broth medium, raising up 0.5% from tube to tube. The inoculum was prepared as described in Section 2.2. Tubes absent of PPCP and absent of inoculum were included as control and blank, respectively. The tubes were incubated at 30 °C in a stirred thermostatically water bath and at regular 6 h time intervals the turbidity was measured in a spectrophotometer device (Spectrum SP-2000UV/2000UVPC, Shanghai, China). The external surface of the tubes was dried with a paper towel and the absorbance was directly measured in the tubes, dispensing the use of cuvettes. The blank tube was used to calibrate the photometer device before measurements. Potentially dosage to achieve microbial control was expressed considering three categories: (i) totally inhibit, a category that implies the absence of growth above that dosage (absorbance value very close to the blank); (ii) partially inhibit, a category that implies a reduction in the growth with that dosage (with absorbance value lesser than the positive control); (iii) not inhibit, a category that implies a normal growth below that dosage (with absorbance value equal to the positive control).

2.7. In Situ Trial

Poultry Products Processing

Broiler chickens of about seven weeks of breeding were obtained from the meat industry suppliers located in the state of Paraná (Brazil) and used to manufacture raw chicken sausages and semifinished chicken products on a pilot industrial scale. Birds were transported to the processing plant and slaughtered according to the welfare protocol for Broilers [35]. Trained workers ensured that each bird was properly slaughtered before feather removal, evisceration, and cleaning. Carcasses were prepared for further production by removal of feathers, internal organs, and feet. Then, they were thoroughly washed and chilled to 4 °C within 4 h to reduce any possible foodborne pathogen growth. Carcasses were trussed after chilling in a leg dressing machine (Linco Food Systems, Trige, Denmark). Dorsal-blade part of poultry carcasses, breast, legs, and wing, which was divided into drumette, wingette (midsection) and tips, were mechanical separately in a portion cutting equipment (Linco Food Systems, Denmark) to shape desired end-products. Chicken slit back and thigh were passed by the seasoning stage with spices into a spinning drum (Incomaf, Salvador, Brazil) for 15 min. Three batches of each part were prepared: control (no addition of PPCP), T1 (1.0% of PPCP), and T2 (1.5% of PPCP) (Table 1).

A stainless-steel digital thermometer was used to monitor the temperature of the batter to maintain the temperature below 7 °C throughout the process. After tumbling, the products were cold storage in a cold chamber (Gelopar, Chapada Araucária, Brazil) to achieve temperatures below 4 °C. Valuable poultry meat remaining in carcasses were separated in a meat harvesting machine (607-513, Baader, Rugby, UK) and further used to prepare raw chicken sausages according to the standard formulation showed in Table 1. Chicken meat was minced in an electric grinding machine (CPG119, Cozzini, EUA) by using a stainless-steel plate disc knife with 10 mm hole diameter and mixed in an automatic mixer (MJ35, Jamar, Sao Paulo, Brazil) for 90 s. Then, the other ingredients were added, and the mixture was mixed for more 90 s. Three batches of sausage were prepared including control (no addition of PPCP), T1 (1.0% of PPCP), and T2 (1.5% of PPCP). After batter preparation, the meat batter was stuffed into collagen casings (1.0 m of length and 26 mm of gauge) using an automatic stuffer (VF 610 E8, Handtmann, Biberach an der Riß, Germany) and manually twisted to shape segments of about 10 cm of length.

Table 1. Chicken product formulations with addition of 1.0% (T1) or addition of 1.5% (T2) or without (Control) potentially postbiotic-containing preservative (PPCP).

Ingredients	Semifinished Chicken Products			Raw Chicken Sausages		
	Control	T1	T2	Control	T1	T2
Chicken parts (slit back or thigh)	92–93	91–92	90.5–91.5			
Minced chicken meat				86.34	85.34	84.84
Water	2–5	2–5	2–5	8	8	8
Seasoning [1]	2.1	2.1	2.1	2.87	2.87	2.87
Sodium phosphate	0.5	0.5	0.5	0.25	0.25	0.25
Sodium trypoliphosphate				2.5	2.5	2.5
Sodium erythorbate	0.6	0.6	0.6			
Annatto dye	0.02	0.02	0.02			
Sodium lactate	0–2	0–2	0–2			
Curing Salt [2]				0.12	0.12	0.12
Cochineal carmine dye				0.02	0.02	0.02
PPCP		1.0	1.5		1.0	1.5

[1] Sodium chloride, pepper, sucrose, and monosodium glutamate. [2] Traditional cure with direct addition of curing salt to obtain a final concentration at 150 ppm of sodium nitrite.

Five packages of each sample group were packaged in polyethylene bags and sealed in a heat sealer. Freezing tunnels (Recrusul, Sapucaia do Sul, Brazil) were used to freeze the products at −12 °C. The sample groups were blind coded and shipped to the laboratory in isothermal boxes with ice bricks. Codes were unblinded only after performing the durability study.

2.8. Durability Study

A predictive microbial method, named MicroLab_ShelfLife®, was used to perform a durability study in raw chicken sausages and semifinished chicken products. It was carried out considering a realistic temperature profile in three R, including DC and M.

One package per group was analyzed soon after being received in the laboratory to count of the initial microbial load (time zero). Microbial growth was stimulated to grow by pair incubation at low (3 °C) and high (25 °C) temperatures. BOD incubators were used for precise temperature control. The doors were kept closed, except during sample withdrawals. The method ISO 4833 (2013), with few modifications as reported by Oliveira et al. [30], was used for enumeration of microorganisms in samples, with counts at intervals on days 2 and 4 (low temperature) and on days 1 and 3 (high temperature) of incubation [36]. The MicroLab_ShelfLife® was compiled to calculate results by using at least two successive dilution levels (Equation (9)) and to obtain information about the method parameters and the microbial growth curve at a chosen dynamic temperature profile.

$$N = \frac{\sum C}{V\,[n1 + 0.1n2)\,d} \quad (9)$$

where, $\sum c$—sum of the colonies counted on the two plates retained from two successive dilutions (at least one of which contains a minimum of 10 colonies); V—volume of inoculum placed in each well (mL); $n1$ and $n2$—number of wells selected in the first dilution and number of wells selected in the second dilution, respectively; and d–level of the first dilution retained.

The microbiological limit that separates good quality from marginally acceptable quality (m = 5 log cfu/g) prescribed by the regulatory agencies [8–10] was inserted in the predictive modeling package as borderline to indicate the use-by date of the products.

2.9. Temperature Profile of the Test

An electronic device (QII343, XpressPDF Logger, Emerson, USA) with a temperature range from −40 °C to 85 °C (±0.5 °C accuracy) was used to elucidate the temperatures to

which the products were exposed during transport and sale. The equipment was adjusted as follows: sensor reaction time of 5 min, a sampling frequency of 1 h to 10 days, the data storage capacity of 8000 readings. At the end of the acquisition period, the logger was recovered, and data were downloaded into a computer. Data were grouped for hourly mean over one day to fit the data in the MicroLab_ShelfLife®. Three routes (R1, R2, and R3) were strategically included in the study, encompassing three DC and three M. DC1, which is located in the city of Penha, state of Paraná (Brazil), is a common and mandatory route to other routes. In R1, from DC1 the product is shipped to DC2, located in the city of Bebedouro, state of São Paulo (Brazil), after to DC3 (Bebedouro, São Paulo, Brazil) and finally to the M1 (Bebedouro, São Paulo, Brazil). In R2, from DC1 the product is shipped to M2, located in the city of Cafelândia, state of Paraná (Brazil). In R3, from DC1 the product is shipped to DC2 and to the M3 (Bebedouro, São Paulo, Brazil), as depicted in Figures 1 and 2.

2.10. Statistical Analysis

Results were presented as Mean ± Standard Error (SE) from replicates. The assumption of normal data distribution was assessed with the Shapiro–Wilk test. Grubbs and Tietjen–Moore tests were used for detecting a single or more than one outlier. Data were evaluated by analysis of variance (ANOVA), followed by Fisher's LSD test ($p < 0.05$) using the software Addinsoft (2019)—XLSTAT, Boston, MA, USA.

Confidence interval for the mean and prediction interval for the sample of linear regression were estimated according to Equations (10), (11) and (12), respectively:

$$(\dot{Y} \pm t\alpha/2 * SE * \sqrt{hi}) \quad (10)$$

$$(\dot{Y} \pm t\alpha/2 * SE * \sqrt{1 + hi}) \quad (11)$$

$$(hi = 1/n + (xi - x)^2 / \sum (xi - x)^2) \quad (12)$$

where $\dot{Y}$—value of estimative; $t\alpha/2$—value of Student's t distribution; n—number of observations; xi—value of sample, x—mean.

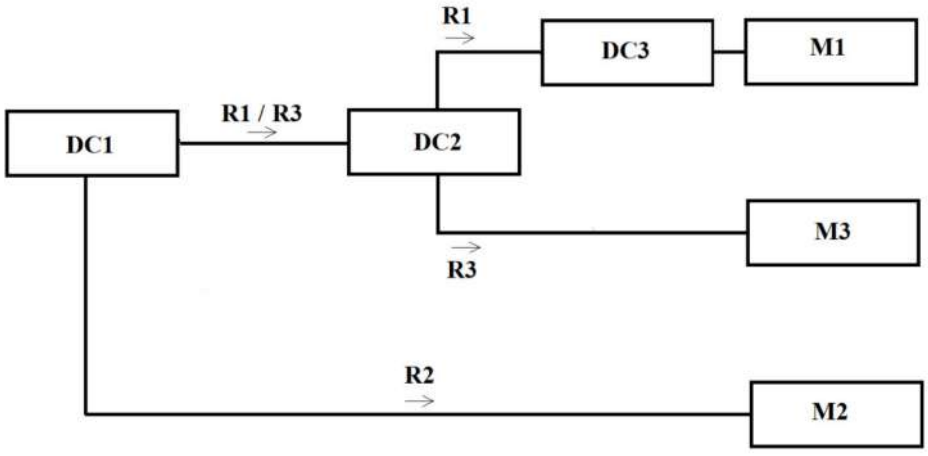

Figure 1. Logistic distribution routes. Route (R1)—from distribution center DC1 (Penha, Paraná, Brazil) to distribution center DC2 (Bebedouro, São Paulo, Brazil), to distribution center DC3 (Bebedouro, São Paulo, Brazil), and to market M1 (Bebedouro, São Paulo, Brazil). Route (R2)—from distribution center DC1 (Penha, Paraná, Brazil) to market M2 (Cafelândia, Paraná, Brazil). Route (R3)—from distribution center DC1 (Penha, Paraná, Brazil) to distribution center DC2 (Bebedouro, São Paulo, Brazil), and to market M3 (Bebedouro, São Paulo, Brazil). Retention time at DC was included in modeling (2 days) according to information from meat industry.

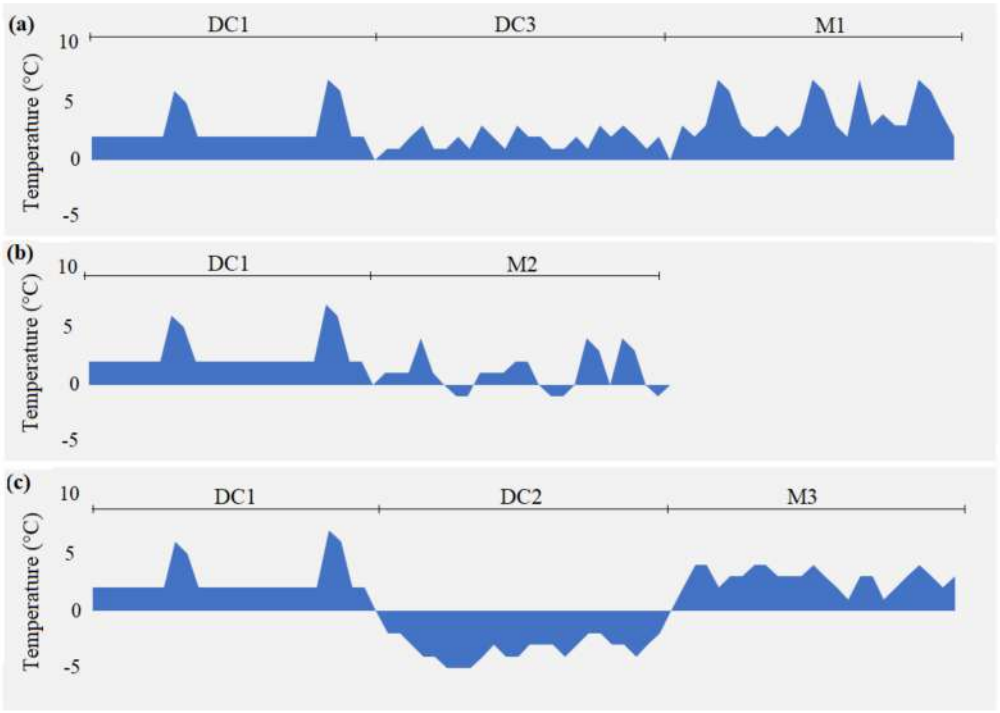

Figure 2. Temperature profile based on hourly variation during a 1-day period to represent distribution centers (DC1, DC2, and DC3) and markets (M1, M2, and M3) in logistic distribution routes R1 (**a**), R2 (**b**), and R3 (**c**). DC2 was eliminated from R1 due to insignificance of microbial growth once products were stored at a temperature profile constantly below zero, keeping frozen storage temperatures.

A computational predictive modeling package named *MicroLab_Shelf-Life*® was used to predict the use-by date of vacuum-packaged cooked sausages (Appendix A). The parameters of the model (*Ngrowth* and *Ndeceleration)* were used to represent daily microbial population growth (log cfu/g) in the microbial growth (log) and deceleration phases. The upper limit for total microbial count of 5 log cfu/g was considered as the method borderline in the durability study.

3. Results

L. paracasei DTA 83 converted glucose into acids and produced PPCP. In addition, *L. paracasei* DTA 83 and *S. boulardii* 17 showed an amensal interaction, without severe prejudice to any strain (Table S2 and Figure 3).

PPCP may be more effective as a preservative than organic acids since semicultured is an adequate fermentation system for the production of lactic and acetic acids by *L. paracasei* DTA 83 and *S. boulardii* 17, respectively. Moreover, other preservatives, such as biocides, may be produced by these strains during fermentation. When the concentrations of produced lactic acid and acetic acid or biocides were not measured in the present study, the stressful effects on chicken-related contaminants were designed and demonstrated in Figure S1.

The in vitro trial showed that microbial susceptibility of chicken-related contaminants was directly proportional to the added concentration of PPCP. When a concentration of PPCP below 0.5% was added to raw chicken sausages or semifinished chicken products, the susceptibility of chicken-related contaminants was not inhibited. Partial inhibition was obtained by adding 1.0 to 2.5% of PPCP and total inhibition was determined by adding above 3.0% ($p > 0.95$).

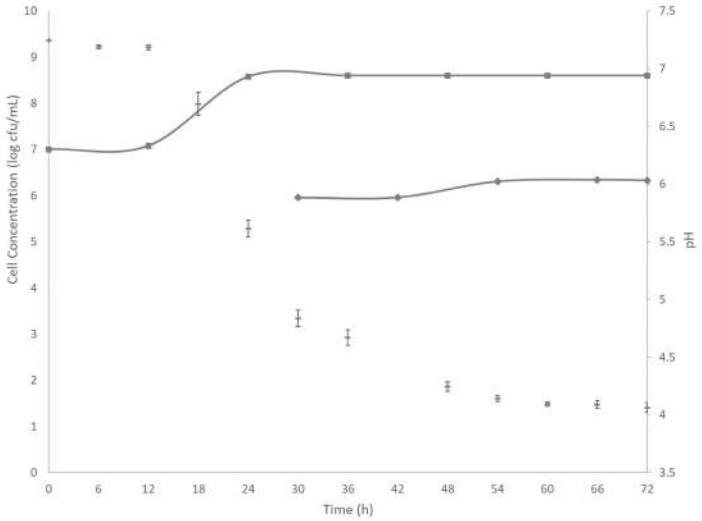

Figure 3. Viability of *L. paracasei* DTA 83 (—■—) and *S. boulardii* 17 (—◆—) and pH measurement (unconnected points) during potentially postbiotic-containing preservative (PPCP) production. (⊥) Standard Error.

Concentrations of 1.0 and 1.5% of PPCP were chosen to be studied in raw chicken sausages and semifinished chicken products to obtain in situ partial inhibitory effects on spoilage microorganisms to extend the use-by date. Although concentrations of PPCP above 3.0% showed a remarkable inhibitory potential, its addition can severely impact the formulation cost.

Linear regression parameters of the microbial growth of chicken-related contaminants at different concentrations of PPCP are shown in Figure 4 and Table S1.

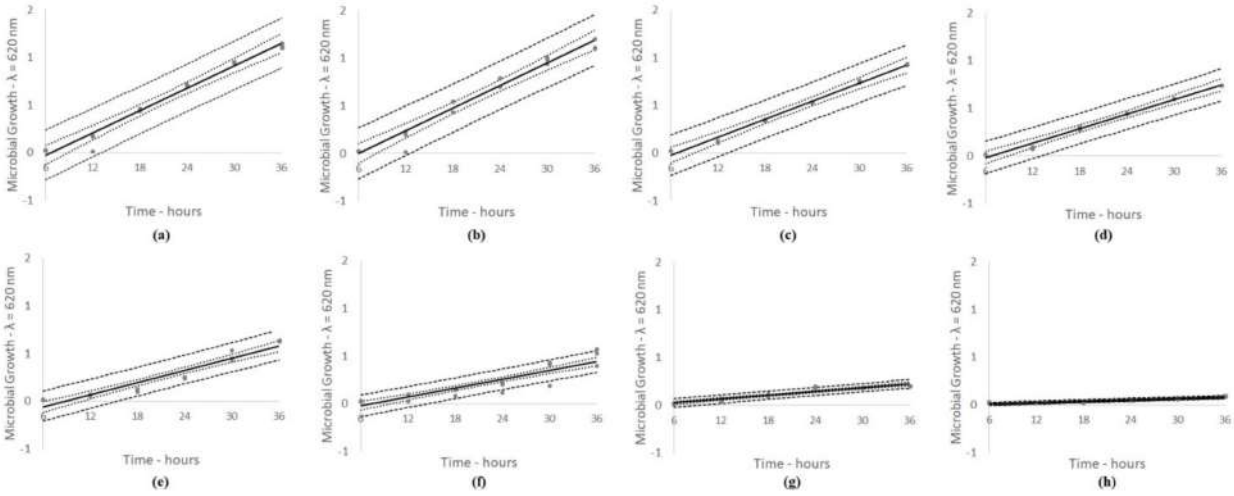

Figure 4. Linear regression (—) parameters of microbial growth of chicken-related contaminants at different concentrations of potentially postbiotic-containing preservative (PPCP). Confidence interval for mean (....) and prediction interval for sample (---) of period with microbial growth (from 6 to 36 h of incubation at 36 °C) at different concentrations of potentially postbiotic-containing preservative (PPCP): (**a**) 0.0%; (**b**) 0.5%; (**c**) 1.0%; (**d**) 1.5%; (**e**) 2.0%; (**f**) 2.5%; (**g**) 3.0%; (**h**) 3.5%. Confidence interval for mean and prediction interval for sample of linear regression were estimated according to Equation (10) ($\dot{Y} \pm t\alpha/2 * SE * \sqrt{hi}$, Equation (11) ($\dot{Y} \pm t\alpha/2 * SE * \sqrt{1 + hi}$), and Equation (12) ($hi = 1/n + (xi - x)^2/\sum(xi - x)^2$), where $\dot{Y}$ —value of estimative; $t\alpha/2$—value of Student's t distribution; n—number of observations; xi—value of sample, x—mean.

All three R included in the study began in DC1, located in Bebedouro, Paraná, Brazil. As expected, *Ngrowth* and *Ndeceleration* parameters of the model were equal in the same sample group (control or T1 or T2) in R1, R2, and R3. However, significant differences were observed when different sample groups were compared (control > T1 > T2), which indicates the potential effects of PPCP to control spoilage growth in raw chicken sausages and semifinished chicken products. These results were in line with those observed during the 2nd and 3rd periods (Tables 2–4).

Table 2. Durability study of raw chicken sausages (control, 1.0% (T1), and 1.5% (T2)) of added potentially postbiotic-containing preservative (PPCP) under a dynamic temperature profile in distribution routes (R).

	Sample Incubation		**Treatments**								
	Temperature (°C)	**Time (Days)**	**Control**			**T1**			**T2**		
Laboratorial data (log cfu/g)		0	3.80			3.75			3.72		
	3	2	3.83			3.77			3.72		
		4	3.84			3.76			3.74		
	25	1	5.53			5.38			5.37		
		3	9.30			6.32			6.11		
Specific maximum growth rate (log cfu/g/day)	3	L phase	0.0125			0.0062			0.0025		
		D phase	0.0110			0.0055			0.0022		
	25	L phase	1.7817			1.2433			1.2233		
		D phase	1.5742			1.0986			1.0809		
			R1	R2	R3	R1	R2	R3	R1	R2	R3
1st period	Ngrowth (log cfu/g/day) [1]		0.0423	0.0423	0.0423	0.0292	0.0292	0.0292	0.0282	0.0282	0.0282
	Ndeceleration (log cfu/g/day) [2]		0.0374	0.0374	0.0374	0.0258	0.0258	0.0258	0.0249	0.0249	0.0249
	Ft(n) [3]		1.1318	1.1318	1.1318	1.1318	1.1318	1.1318	1.1318	1.1318	1.1318
2nd period	Ngrowth (log cfu/g/day) [1]		0.0026	0.0127		0.0013	0.0083		0.0005	0.0075	
	Ndeceleration (log cfu/g/day) [2]		0.0025	0.0126		0.0012	0.0083		0.0005	0.0074	
	Ft(n) [3]		1.0548	1.0034		1.0548	1.0034		1.0548	1.0034	
3rd period	Ngrowth (log cfu/g/day) [1]		0.0998	0.0127	0.0290	0.0679	0.0083	0.0185	0.0643	0.0075	0.0156
	Ndeceleration (log cfu/g/day) [2]		0.0806	0.0141	0.0252	0.0549	0.0093	0.0161	0.0519	0.0083	0.0136
	Ft(n) [3]		1.2381	0.8971	1.1501	1.2381	0.8971	1.1501	1.2381	0.8971	1.1501
Use-by date—days			16	91	43	22	146	69	24	167	83

[1] Ngrowth—daily microbial population growth (log cfu/g) in microbial growth (log) phase determined by microbial growth predictor (MicroLab_ShelfLife®); [2] Ndeceleration—daily microbial population growth (log cfu/g) in microbial deceleration phase determined by microbial growth predictor (MicroLab_ShelfLife®); [3] FT(n)—correlation variable factor that describes specific growth rates between log and deceleration phases at a chosen dynamic temperature profile based on hourly variation according to measurements in loco (Figure 2).

Table 3. Durability study of seasoned chicken slit back (control, 1.0% (T1), and 1.5% (T2)) of added potentially postbiotic-containing preservative (PPCP) under a dynamic temperature profile in distribution routes (R).

	Sample Incubation		**Treatments**		
	Temperature (°C)	**Time (Days)**	**Control**	**T1**	**T2**
Laboratorial data (log cfu/g)		0	3.90	3.92	4.01
	3	2	4.08	4.07	4.01
		4	4.03	4.01	4.05
	25	1	7.11	5.32	5.25
		3	9.40	6.91	7.34

Table 3. *Cont.*

	Sample Incubation		Treatments								
	Temperature (°C)	Time (Days)	Control			T1			T2		
Specific maximum growth rate (log cfu/g/day)	3	L phase	0.0613			0.0488			0.0050		
		D phase	0.0541			0.0431			0.0044		
	25	L phase	2.5217			1.1983			1.1750		
		D phase	2.2281			1.0588			1.0382		
			R1	R2	R3	R1	R2	R3	R1	R2	R3
1st period	Ngrowth (log cfu/g/day) [1]		0.0661	0.0661	0.0661	0.0343	0.0343	0.0343	0.0274	0.0274	0.0274
	Ndeceleration (log cfu/g/day) [2]		0.0584	0.0584	0.0584	0.0303	0.0303	0.0303	0.0242	0.0242	0.0242
	Ft(n)[3]		1.1318	1.1318	1.1318	1.1318	1.1318	1.1318	1.1318	1.1318	1.1318
2nd period	Ngrowth (log cfu/g/day) [1]		0.0128	0.0267		0.0102	0.0167		0.0010	0.0077	
	Ndeceleration (log cfu/g/day) [2]		0.0121	0.0266		0.0096	0.0166		0.0010	0.0077	
	Ft(n) [3]		1.0548	1.0034		1.0548	1.0034		1.0548	1.0034	
3rd period	Ngrowth (log cfu/g/day) [1]		0.1718	0.0267	0.0713	0.0953	0.0167	0.0476	0.0636	0.0077	0.0168
	Ndeceleration (log cfu/g/day) [2]		0.1387	0.0298	0.0620	0.0770	0.0186	0.0414	0.0514	0.0086	0.0146
	Ft(n) [3]		1.2381	0.8971	1.1501	1.2381	0.8971	1.1501	1.2381	0.8971	1.1501
Use-by date—days			10	39	18	15	63	26	19	124	60

[1] Ngrowth—daily microbial population growth (log cfu/g) in microbial growth (log) phase determined by microbial growth predictor (MicroLab_ShelfLife®); [2] Ndeceleration—daily microbial population growth (log cfu/g) in microbial deceleration phase determined by microbial growth predictor (MicroLab_ShelfLife®); [3] FT(n)—correlation variable factor that describes specific growth rates between log and deceleration phases at a chosen dynamic temperature profile based on hourly variation according to measurements in loco (Figure 2).

Table 4. Durability study of seasoned chicken thigh (control, 1.0% (T1), and 1.5% (T2)) of added potentially postbiotic-containing preservative (PPCP) under a dynamic temperature profile in distribution routes (R).

	Sample Incubation		Treatments								
	Temperature (°C)	Time (Days)	Control			T1			T2		
Laboratorial data (log cfu/g)		0	3.91			3.82			3.79		
	3	2	4.00			3.83			3.84		
		4	4.03			3.92			3.93		
	25	1	6.78			6.29			4.41		
		3	9.40			8.01			5.83		
Specific maximum growth rate (log cfu/g/day)	3	L phase	0.0375			0.0150			0.0300		
		D phase	0.0331			0.0133			0.0265		
	25	L phase	2.3500			1.9333			0.6500		
		D phase	2.0764			1.7082			0.5743		
			R1	R2	R3	R1	R2	R3	R1	R2	R3
1st period	Ngrowth (log cfu/g/day) [1]		0.0588	0.0588	0.0588	0.0461	0.0461	0.0461	0.0191	0.0191	0.0191
	Ndeceleration (log cfu/g/day) [2]		0.0520	0.0520	0.0520	0.0407	0.0407	0.0407	0.0169	0.0169	0.0169
	Ft(n)[3]		1.1318	1.1318	1.1318	1.1318	1.1318	1.1318	1.1318	1.1318	1.1318
2nd period	Ngrowth (log cfu/g/day) [1]		0.0062	0.0210		0.0140	0.0140		0.0078	0.0098	
	Ndeceleration (log cfu/g/day) [2]		0.0059	0.0209		0.0140	0.0140		0.0074	0.0097	
	Ft(n) [3]		1.0548	1.0034		1.0548	1.0034		1.0548	1.0034	

Table 4. *Cont.*

	Sample Incubation		Treatments								
	Temperature (°C)	Time (Days)	Control			T1			T2		
3rd period	Ngrowth (log cfu/g/day) [1]		0.1464	0.0210	0.0528	0.1093	0.0140	0.0324	0.0542	0.0098	0.0283
	Ndeceleration (log cfu/g/day) [2]		0.1182	0.0234	0.0459	0.0883	0.0156	0.0282	0.0438	0.0109	0.0246
	Ft(n) [3]		1.2381	0.8971	1.1501	1.2381	0.8971	1.1501	1.2381	0.8971	1.1501
Use-by date—days			11	49	23	14	80	38	26	122	46

[1] Ngrowth —daily microbial population growth (log cfu/g) in microbial growth (log) phase determined by microbial growth predictor (MicroLab_ShelfLife®); [2] Ndeceleration—daily microbial population growth (log cfu/g) in microbial deceleration phase determined by microbial growth predictor (MicroLab_ShelfLife®); [3] FT(n)—correlation variable factor that describes specific growth rates between log and deceleration phases at a chosen dynamic temperature profile based on hourly variation according to measurements in loco (Figure 2).

4. Discussion

Gompertz's modified and Baranyi models indicated greater growth of *L. paracasei* DTA 83 (*ca.* 1.6 log cfu/g) than *S. boulardii* 17 (*ca.* 0.65 log cfu/g) during fermentation for PPCP production (Table S2 and Figure 3). Latency in Lag phase (λ) was longer for *S. boulardii* 17 showing that the metabolites produced by *L. paracasei* DTA 83, especially lactic acid, may be stress factors for the growth of *S. boulardii* 17. Although this point seems to be negative, it was strategically designed once *S. boulardii* 17 can produce acetic acid in stressful conditions as reported by Paula and colleagues [33].

S. boulardii 17 performance to assimilate sugars in acid conditions was previously demonstrated by Silva and colleagues, revealing its suitability to be associated with acid lactic bacteria in a culture system [28]. Moradi and colleagues reported that *S. boulardii* is more tolerant to acidic pH and temperature variation than other *S. cerevisiae* strains and can survive at pH values as low as 2.0 [37]. Otherwise, *L. paracasei* DTA 81, which is a close genetic strain to the *L. paracasei* DTA 83 used in the present study [24], showed sensitivity to the metabolites produced by *S. boulardii*, suggesting that the semiseparated coculture system is an adequate choice to culture the strains in fermentation processes [28].

Stanojević-Nikolić and colleagues related the antimicrobial activity of lactic acid against pathogen and spoilage microorganisms. Lactic acid minimal inhibitory concentration for bacteria was about ten times lesser than to inhibit yeasts. Most of the microorganisms studied by the authors are chicken-related contaminants, indicating that acid lactic can be a potential preservative for chicken products to prevent food-born pathogenic and spoilage microorganisms. The inhibitory effect against spoilage microbial growth may be optimized using PPCP when bacteriocins are produced during lactic acid fermentation [38].

In addition, acetic acid has presented good antimicrobial activity against various microorganisms such as bacteria, yeasts, and molds. Halstead and colleagues demonstrated the effect of acetic acid against *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Escherichia coli*, *Staphylococcus aureus*, and *Klebsiella pneumoniae*. Minimum inhibitory concentrations from 0.16 to 0.31% were found to prevent biofilms formation for all isolates [39].

In R1, the addition of 1.0% or 1.5% of PPCP to raw chicken sausages increased the use-by date from 16 (control) to 22 (T1) and 24 (T2) days, respectively. However, it was not sufficient to guarantee aerobic mesophilic counts below 5 log cfu/g during 60 days of storage, which is the printed shelf-life of the product. For this reason, concentrations of PPCP above 1.5% should be studied, according to the inhibition potential observed in Figure 4 and Figure S1. Additionally, proper management of the cold chain throughout distribution is a suitable strategy to achieve a greater use-by date in this route. As presented in Figure 2, R1 was the route with the highest temperature profile.

In R2, aerobic mesophilic counts below 5 log cfu/g (m) were achieved for more than 60 days only by cold chain management, dispensing the addition of any preservative in the product. However, remarkable increases of 55 (T1) and 76 days (T2) were achieved by adding PPCP in sausages. In R3, it was possible to note the importance of adding

PPCP in sausages. The use-by date increased from 43 days (control) to 69 (T1) and 83 (T2) days, ensuring aerobic mesophilic counts below 5 log cfu/g (m) during 60 days of storage (Table 2).

All sample groups showed use-by date below 60 days in semifinished chicken products, regardless of route. These results indicate the positive effect on shelf life due to cold chain management and PPCP. In this sense, the addition of 1.0% PPCP to seasoned chicken slit back increased the use-by date from 39 to 63 in R2. Only with the addition of PPCP at 1.5% the use-by date was increased to 124 and 60 days in R2 and R3, respectively (Table 3).

In seasoned chicken thigh, aerobic mesophilic counts below 5 log cfu/g were only achieved in R2 with the addition of PPCP at 1.0% (80 days) and 1.5% (122 days). These results reinforce the importance of PPCP to extend the use-by date in semifinished chicken products; however, the temperature profile in logistic distribution routes is a crucial factor for product shelf-life extension (Figure S2, Tables 3 and 4).

In the control group, which is the current industry formulation, only 11% of the chicken products complied the microbiological limit that separates good quality from marginally acceptable quality (m = 5 log cfu/g) prescribed by regulatory agencies during 60 days of printed shelf-life [8–10]. The percentages increased to 44 and 67% by adding 1.0% or 1.5% of PPCP, respectively (Figure S3). This finding demonstrates that besides delivering postbiotic compounds, PPCP may act as a natural preservative in raw chicken sausages and semifinished chicken products to control aerobic mesophilic below 5 log cfu/g during 60 days of cold storage.

Interactions concerning food ingredients and microbes are complex to design in a durability study to estimate use-by dates [40]. Moreover, temperature variations caused by external aspects such as climatic and geographic factors and normal fluctuation over the day may affect microbial growth and impact the food shelf-life period. While some predictive methods allow to carry out a durability study at different temperatures, few methods permit the use of a dynamic temperature profile in the same test to achieve realistic temperature conditions based on the temperatures to which the products are exposed during storage for sale in markets [41]. In the present study, the microbial growth predictor named MicroLab_shelfLife® was designed to perform a durability study of meat products by predicting the microbial growth curve of their natural microbiota under a dynamic temperature profile.

A realistic temperature profile collected in DC and M throughout the R was considered in this study. In addition, a method to perform a durability study of raw chicken sausages and semifinished chicken products by predicting the microbial growth curve of their natural microbiota was used.

5. Conclusions

Potentially postbiotic-containing preservative (PPCP) produced by a semiseparated coculture system with *L. paracasei* DTA 83 and *S. boulardii* 17 may be a functional natural alternative to extend the use-by date of raw chicken sausages and semifinished chicken products. However, cold chain management throughout logistics is the crucial factor to avoid product spoilage. The present study reveals the impact of the logistics on chicken sausage products spoilage and may be useful for guiding the responsible use of preservatives. Food operators should support the use of preservatives regarding the logistic routes to where the product will pass by. Additionally, the beneficial immunomodulatory responses of PPCP in the host must be further studied in an in vivo model. For the preservative effects, a robust study should be designed to draft the temperature profile in distribution routes to verify failures in the cold chain management that may impact the use-by date of products.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su14052646/s1, Figure S1. Susceptibility of spoiling chicken product-related microorganisms to potentially postbiotic-containing preservative (PPCP). Figure S2. Impact of temperature profile by the routes R1 (a), R2 (b), and R3 (c) in the use-by date of chicken

products. Squares represent Mean and bars represent Standard Error. Different letters in the same box indicate a significant difference by Fisher's LSD test at 0.95 of reliability. Figure S3. Percentage of chicken products (raw chicken sausages and semifinished chicken products) in compliance with the minimum limit prescribed by regulatory agencies. (a) no addition of potentially postbiotic-containing preservative (PPCP), (b) 1.0% of PPCP, and (c) 1.5% of PPCP. Table S1. Linear regression parameters of the microbial growth of chicken-related contaminants at different concentrations of potentially postbiotic-containing preservative (PPCP). Table S2. Predicted modeling (Baranyi or modified Gompertz's model) to adjust the *L. paracasei* DTA 83 and *S. boulardii* 17 growth during fermentation. DMFit software version 3.5 (Institute of Food Research, Norwich).

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Appendix A

A computational predictive modeling package named MicroLab_ShelfLife® was developed using the Visual Basic for Application—Excel 2016 (MicroSoft, Washington, DC, USA) to serve as an auxiliary tool for laboratory routine to perform a durability study of meat products.

Packages (n = 5) from the same batch and manufactured under the same conditions are used to estimate 'use-by' or 'best-before' date using the natural microbiota of the meat products. A borderline limit can be entered in the computational package, based on preliminary studies for the matrix, to preview the 'use-by' or 'best-before' date of the test. The method ISO 4833 (2013) may be used for enumeration of microorganisms in samples [42]. A package (n = 1) must be analyzed as soon as the product arrives in the laboratory (time zero). Microbial population in the remained packages (n = 4) are stimulated to grow by pair incubation at a lower and a higher temperature. Laboratories can determine the incubation temperatures; however, lower and higher temperatures with a value comprehended from 4 to 20 °C and 25 to 36 °C must be used, respectively. Except for the time zero, there is no predefined time for microbial counting once the computational predictive modeling package is able to process any time; however, microbial growth (log) phase must be included at least in one of the counts. Results related to the colony counting must be entered in the computational predictive modeling package to obtain information about the parameters of the method and the microbial growth curve at a chosen dynamic temperature profile.

Appendix A.1. Growth Phase Modeling

Specific growth rates per hour (log cfu/g/h) at lower and higher temperatures are obtained by determining the angular coefficient of the microbial growth (log) phase in each growth curve. They are calculated to one unit of degree Celsius (log cfu/g/h/°C) by dividing the mean value of the angular coefficient by the difference between the higher and the lower temperature (Equation (A1)). This parameter is used to calculate the microbial growth per hour at each temperature profile. Hourly microbial growth is obtained by

multiplying the specific growth rate (log cfu/g/h/°C) by the temperature value during 1 h. Daily growth is obtained by the sum of all hourly growth (Equation (A2)):

$$N(Tgrowth)\ (\log\ \text{cfu/g/h/°C}) = \left(\left(\frac{\alpha(HT) - \alpha(LT)}{2}\right)\cdot\left(\frac{1}{HT - LT}\right)\right)/\ 24 \quad \text{(A1)}$$

$$Ngrowth\ (\log\ \text{cfu/g}) = \sum_{k=1}^{24} n\cdot(N(Tgrowth)) \quad \text{(A2)}$$

where, *N(Tgrowth)*—rate of microbial growth per degree Celsius (log cfu/g/day/°C) in the growth (log) phase; *α(HT)* and *α(LT)*—angular coefficients at the higher (HT) and lower (LT) temperatures (°C), respectively; *n*—hourly temperature ranging from 4 to 36 °C; *Ngrowth*—daily microbial growth (log cfu/g) in the log phase; and *k*—time (hour).

Appendix A.2. Deceleration Phase Modeling

Correlation variable factor *FT(n)* (Equation (A3)) was created and inserted in Equations (A1) and (A3) to model the microbial growth in the deceleration phase based on the value of the log phase (Equations (A4) and (A5)). The ratio of the log and deceleration phases period was determined at four different temperatures (4, 12, 24, and 36 °C), and xy-scatter charts were plotted with log/deceleration values (coordinate abscissa) and incubation temperature (axial abscissa). Linear regression was used for mathematical modeling of values. To determine the variable factor *FT(n)* for any temperature profile, the average daily temperature was calculated, and the first-degree equation was considered:

$$FT(n) = L/D \quad \text{(A3)}$$

$$N(Tdeceleration)\ (\log\ \text{cfu/g/h/°C}) = N(Tgrowth)/\ FT(n) \quad \text{(A4)}$$

$$Ndeceleration\ (\log\ \text{cfu/g/dia}) = \sum_{k=1}^{24} n\cdot(N(Tdecedeleration))/\ FT(n) \quad \text{(A5)}$$

where *N(Tdeceleration)*—microbial growth rate per degree Celsius (log cfu/g/day/°C) in the deceleration phase; *N(Tgrowth)*—microbial growth rate per degree Celsius (log cfu/g/day/°C) in the log phase; *FT(n)*—correlation variable factor to describe specific growth rate between log and deceleration phases per degree Celsius; *n*—hourly temperature ranging from 4 to 36 °C; *Ndeceleration*—daily microbial growth (log cfu/g) in the deceleration phase; and *k*—time (hour).

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Introduction: A natural technology for vacuum-packaged cooked sausage preservation with potentially postbiotic-containing preservative

Lactic acid bacteria (LAB) have garnered significant attention in food science due to their potential health benefits and application in food preservation. Among these, *Lacticaseibacillus paracasei* stands out as a promising strain for delivering probiotics in food matrices. However, concerns about the administration of live microorganisms, particularly in vulnerable populations, have prompted exploration into alternative strategies such as postbiotics.

Food spoilage caused by microbial activity remains a critical issue globally, contributing significantly to food waste and loss. The presence and growth of spoilage microorganisms in food products can lead to sensory, nutritional, and safety concerns. In response, biopreservation has emerged as a viable alternative to synthetic preservatives, leveraging natural compounds produced by microorganisms like LAB.

The US Food and Drug Administration (FDA) recognizes certain LAB-derived substances as Generally Recognized as Safe (GRAS), making them suitable for controlling pathogenic and spoilage microorganisms in food. These regulations underscore the importance of using nonpathogenic and nontoxicogenic strains under current good manufacturing practices. Despite these regulatory endorsements, the practical application of LAB-derived bacteriocins in food preservation is hindered by the high cost associated with their isolation and purification, particularly for low-cost products.

In this context, the use of pre-cultured broth mediums containing LAB, without the isolation and purification of specific bacteriocins, presents a promising approach to prevent spoilage in meat products. This study explores the efficacy of a potentially postbiotic-containing preservative (PPCP) produced from *L. paracasei* in an axenic fermentation system. Specifically, the research investigates PPCP's ability to preserve vacuum-packaged cooked sausages by inhibiting spoilage microorganisms both on the sausage surface and within the sausage mass. The findings suggest that PPCP exhibits comparable efficacy to sodium lactate FCC85, a standard preservative, highlighting its potential as a natural technology for developing functional cooked sausages while enhancing their safety and shelf life.

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A natural technology for vacuum-packaged cooked sausage preservation with potentially postbiotic-containing preservative

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Abstract: In this study, a potentially postbiotic-containing preservative (PPCP) was produced in an axenic fermentation system with *Lacticaseibacillus paracasei* DTA 83 as a natural technology alternative for vacuum-packaged cooked sausages preservation. Cooked sausage-related microorganisms were obtained during the induced spoiling process in the packages by pair incubation of sausages at different temperatures. The turbidity method was used to determine the microbiota susceptibility to PPCP. A controlled *in situ* design was performed by adding PPCP on the surface or to the mass of the sausages. Sodium lactate FCC85, which was used according to the manufacturer's recommendation, was included in the design for comparison. The results revealed that PPCP was as efficient as FCC85, which indicates PPCP as a promising alternative to the use of natural technologies to preserve and develop functional cooked sausages. Moreover, a strategy to use preservatives in vacuum-packaged cooked sausages was presented: the concentration needed to achieve the total inhibition of the microbiota determined by an *in vitro* trial should be respected when adding PPCP on the sausages' surface. When adding PPCP to the mass of the sausages, the concentration that showed a partial inhibition *in vitro* can also be applied in *situ*.

Keywords: Biocontrol; Biocin; Heat-Inactivated Microorganism; Food Safety; Sustainability;

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1. Introduction

Lactic acid bacteria (LAB) constitute a heterogeneous group that is extensively reported in the literature due to its potential benefits for consumer health [1,2]. *Lacticaseibacillus paracasei* DTA 83 has been described as a candidate strain to deliver probiotics in food matrices [3-5]. In contrast, since microorganisms may present an invasive potential, studies have pointed out the administration of viable cells by healthy people as a subject of great concern. Thus, the use of postbiotic may be highlighted as a suitable alternative.

The presence of spoilage microorganisms in food represents a critical issue with repercussions on massive food waste and food loss worldwide [6]. The safety and stability of food may be affected by numerous factors such as microbial presence and/or activity, biochemical, physical-chemical, and sensory alterations, nutritional losses, among others. When intrinsic and extrinsic aspects of food allow microbial growth, the microbial spoilage pathway becomes dominant [7,8].

Biopreservation is one of the alternative food preservation technologies applied to replace artificial preservatives. The US Food and Drug Administration (FDA) reports substances from LAB as Generally Recognized as Safe (G.R.A.S) (section 21 CFR184). Thus, they are useful to control the development of pathogens and spoiling microorganisms in food and foodstuff. Moreover, FDA also determines that 'conditions for their use are prescribed in the referent regulations and are predicated on the use of nonpathogenic and nontoxicogenic strains of the respective organisms and on the use of current good manufacturing practice (184.1(b))'. Despite all the advantages, the use of bacteriocins is still limited due to the high cost of their isolation and purification, mainly when considering their application in products of low cost. In this context, the use of pre-cultured broth mediums by LAB, without bacteriocin isolation and purification, may be a promising strategy to prevent spoilage in meat products [9].

Heat-treated meat products, such as vacuum-packaged cooked sausages, are traditionally marketed at room temperature in Brazil, leading to food waste due to spoilage processes that may occur before the shelf-life determined by the manufacturer [10]. The vacuum atmosphere selectively suppresses the growth of specific microbial groups, attributing the initial microbiota to anaerobic and facultative groups [11]. These microorganisms overgrow and produce metabolites that cause the rejection of the products by consumers [12]. As a solution, the food industry often increases the concentration of preservatives in meat products, which may result in abusive use.

Sodium lactate is a widespread commercial preservative commonly used in sausages to control microbial growth and increase shelf-life [13]. However, the higher the concentration of sodium lactate added to a food product, the higher the content of sodium. Therefore, although sodium lactate is a safe preservative for food and foodstuff, its excessive intake may result in the increased blood pressure of consumers [14]. Indeed, natural technologies to preserve food are of great interest to food industries and consumers.

Thus, this study aimed to produce and evaluate the efficacy of potentially postbiotic-containing preservative (PPCP) produced by an axenic fermentation system with *L. paracasei* DTA 83 to extend the use-by date of vacuum-packaged cooked sausages.

2. Materials and Methods

2.1. Microbial Collection

L. paracasei DTA 83 was isolated from newborn's stools at Rio de Janeiro (Brazil) in selective Lawvab agar medium, as reported by Lemos-Junior et al. [15]. The strain was genotypically identified by sequencing of the 16S rDNA region and clustered by genetic similarity with other lacticaseibacilli strains of the collection [16]. Further, the complete genome data was deposited in GenBank, under the accession number QRBH00000000, https://www.ncbi.nlm.nih.gov/nuccore/ QRBH00000000 [17]. The strain has been classified as generally recognized as safe (G.R.A.S) and characterized as a potential probiotic according to Tarrah et al. and Laureano-Melo et al. [3,4]. The technological features of the strain have been assessed in food matrices by Silva et al. [5,18]. Additionally, it has been described as a potential strain to deliver postbiotic compounds as reported by Oliveira et al. [19].

2.2. PPCP Production

A stirred tank bioreactor of 330 L, with automatic control of temperature and pH, was used to produce PPCP in an axenic fermentation system with *L. paracasei* DTA 83. This part of the experiment was carried out at BRC Ingredientes Ltda, located in São Paulo, Brazil. Modified MRS broth was prepared with food-grade ingredients, without the addition of polysorbate 80 (Tween 80). The heat-treatment was performed in the tank (heating up of 1 °C per minute) by the electrical activation of three resistors (3 kw). During heating, the medium was axially agitated at 84 rpm. The binomial 75 °C per 2 hours was used to reduce the contaminants to an acceptable level (*ca.* 3 log cfu/g) and provide a competitive

advantage to *L. paracasei* DTA 83 during the fermentation. After the heat-treatment, the temperature of the medium was reduced to 36 °C (heating down of 0.5 °C per minute). *L. paracasei* DTA 83 biomass was produced in laboratory, scaling up 1/10 (vol/vol) of the culture into sterile modified MRS broth. A biological oxygen demand was used for incubation at 36 °C to obtain 30 L of inoculum. A culture with 18 hours of growth, comprehended into the growth (log) phase, was added (1/10 of inoculum) into the bioreactor containing 270 L of modified MRS medium to obtain a final inoculum concentration of *ca.* 7 log cfu/mL. After 72 hours of fermentation coupled with a pH decay to around 3.5, the medium was heat-treated at 95 °C for 5 minutes (heating up of 1 °C per minute). PPCP was hot bottled in polypropylene containers of 10 L. The presence of remaining cells of *L. paracasei* DTA 83 or contaminants was assessed by plate counting on MRS and plate count agar and potato dextrose agar acidified to pH 3.5 with tartaric acid (all media from HiMedia, Mumbai, India).

2.3. In vitro Efficacy of PPCP

Cooked sausage-related microorganisms were obtained from five packages of sausages, with collection at zero time (n = 1) and after pair incubation of samples at 7 °C (collection on days 3 and 6) and 36 °C (collection on days 2 and 4). A decimal suspension was prepared by weighing the sausages and adding 0.1 % of peptone sterile water to the package. This step was conducted to count the microorganisms in the sausages, as well as the ones accumulated in the liquid inside the package after syneresis. After homogenizing the samples in a stomacher (SP-190, SPLabor, Brazil) for 90 seconds at 230 revolutions per minute (rpm), an aliquot (100 μL) was transferred to tubes with 5 mL of Brain-Heart Infusion, Casoy, deMan, Rogosa, and Sharp, and Yeast-Peptone-Dextrose Extract. The tubes were incubated at 36 °C for 24 - 48 hours. The inoculum was obtained separately from each culture medium by transferring 1 mL of the tube content, with expressive growth (turbidity above 0.5 MacFarland standard) to an empty sterile screw-cap tube. Cells free of toxic compounds were obtained by washing twice the biomass cell pellets with a routine of centrifugation at 6000×*g* for 6 minutes for pellet sedimentation at the bottom of the tube, discarding the liquid fraction, adding 2 mL of phosphate buffer pH 7.2, and homogenizing in vortex. The turbidity of the microbial suspension was adjusted to 0.5 MacFanland standard and 2-fold diluted. PPCP was randomly outlined to final concentrations of 0.0; 0.1; 0.3; 0.5; 1.0; 1.5; 2.0; 2.5; 3.0; and 3.5 % (vol/vol) in the Brain Heart Infusion broth. The dilutions were prepared in the same media used in the test to avoid a shortage of nutrients for microbial growth. Finally, 100 μL of the microbial suspension was added into the tubes to achieve a final microbial concentration of *ca.* 5 log cfu/mL. A digital stirred water bath (SP-156/22, SPLabor, Brazil), with automatic temperature control, was used to incubate the tubes at 36 °C for 72 hours. The absorbance was reading in a photometry device at 600 nm (Spectrum SP-2000UV/2000UVPC, Shanghai, China) for a regular 6-hour period. Before reading, the tubes were vortexed and the absorbance was directly measured in the tubes. A tube without inoculum was used as blank and for the equipment calibration at each reading.

2.3. In situ Efficacy of PPCP

PPCP was tested *in situ* at concentrations of 1.0, 2.0, and 3.0 % by adding the preservatives on the surface or to the mass of the sausages. The sausages were manufactured in an industrial-pilot scale of a meat industry located in Rio de Janeiro, Brazil. The production was performed according to the meat products standard procedures, as follows: input feed of raw materials, defrosting or broken in frozen block crusher, grinding through industrial grinder knife (8 - 12 mm hole diameter (Ø) plate) (PC 106, Canoas, Brazil), mixing and addition of food ingredients (lean meat, pork fat, spices, and food additives) (250L, Cataguases, Brazil), stuffing in 15 x 250 mm inner (diameter x length) natural pork casing (NDX 22 Viscofan, Spain), cooking process to achieve 72 °C (approximately 2 hours)

(MECA2G, Pará de Minas, Brazil) at the coldest point of the sausage, cooling by immersion in a cold water bath, packing using a vacuum-package system with 5 to 7 pieces of sausage per package. PPCP was added to the mass of the sausages with other ingredients during the sausage mass preparation or directly into the packages to hurdle microbial growth after syneresis. The amount of PPCP added to the package was calculated based on the weight of the sausages stored in it. Sodium lactate FCC85 (Corbion, Purak, Brazil), added to the mass or on the sausages' surface, was included in the design to compare the efficacy of the PPCP with a reference widespread commercial preservative. The addition of sodium lactate was performed following the manufacturer's recommendation. Sausages without preservatives or with sterile deionized water, added to the mass or on the sausages' surface, were included as blank and control, respectively (Table 1). After manufacturing, the packages were immediately addressed to the laboratory.

Table 1. Formulation of pork sausage samples.

		Treatments									
		Sausage surface					**Sausage mass**				
Ingredients (%)	**Blank**	**S0**	**S1**	**S2**	**S3**	**S4**	**M0**	**M1**	**M2**	**M3**	**M4**
Lean pork meat	67.33	67.33	67.33	67.33	67.33	67.33	67.33	67.33	67.33	67.33	67.33
Pork fat	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00
Drinking water	10.00	10.00	10.00	10.00	10.00	10.00	8.00	8.00	9.00	8.00	7.00
Salt (sodium chloride)	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80
Seasoning[1]	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Sodium trypoliphosphate	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.32
Sodium erythorbate	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Curing Salt[2]	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Sterile deionized water		2.00					2.00				
Sodium lactate FCC85			2.00					2.00			
PPCP				1.00	2.00	3.00			1.00	2.00	3.00

[1] Garlic powder, onion powder, black pepper, nutmeg, laurel powder, and celery powder. [2] Sodium chloride (90 %), sodium nitrite (6 %), and sodium nitrate (4 %).

2.4. Sample Characterization

2.4.1. Physico-Chemical Analyses

The analyses were carried out following the AOAC procedures [20]. Moisture content (% w/v) was determined by oven drying at 105 °C until constant weight. Ash content (% w/v) was determined by incinerating samples in a muffle furnace at 550 °C for 4 hours. Protein level (% w/v) was obtained by the Kjeldahl method. Soxhlet extraction method with hexane was applied to determine the total fat content (% w/v). The total carbohydrate content was calculated as the difference between 100 and the sum of the percentage of moisture, ash, lipid, and protein. Total energy (kcal/100 g sample) was calculated according to the Atwater specific factor system (4.27 kcal/g for protein or carbohydrate, and 9.02 kcal/g for fat).

2.4.2. Water Activity Measurement

Changes in the electrical conductivity of an electrolyte, in accordance with the method ISO 18787 (2017) [21], were used for water activity measurement in a AcquaLab Lite device (Decagon, Washington, USA), provided with a dielectric humidity sensor and infrared sample surface temperature. Before measuring, the equipment was calibrated with two standard solutions (K2SO4, *aw* 0.973 (CAS 7778–80–5) and KCl, *aw* 0,843 (CAS 7447–40–7)) provided by the manufacturer. A maximum error of ± 0.005 was considered as accuracy. To obtain a uniform sample, a piece of sausage was ground in an electric meat grinder (Centrífuga 1000, Britânia, Brazil). Excessive milling, which could lead to heating

of samples and affect measurements, was avoided. Immediately after grinding, the sample portion was taken as quickly as possible to minimize exposure to humidity in the laboratory. A sample dish with a capacity of 7 mL was 1/3 filled with sample so that there was no empty space at the bottom. During the analytical series, the measurement stability was verified using standard solutions. A waiting time of approximately 15 minutes was established between each measurement, after opening the equipment lid.

2.4.3. pH Values

Non-destructive measurement of pH was performed according to method ISO 2917:1999(E) [22]. A portable meat pHmeter device (pH Classic, Akso, Brazil), equipped with knife probe electrode (IP65, Akso, Brazil) and automatic compensation of temperature, was used. Sausages were randomly withdrawn from the packages and the pH value was determined by direct sticking the electrode in 3 different positions of the sausage, including the two ends and the central section of the pieces. Before measuring, the equipment calibration was performed with buffer solutions, pH 4.00 and pH 6.88 at 20°C. A maximum error of ± 0.01 was considered as accuracy.

2.5. Durability Study

A predictive microbial method, named *MicroLab_ShelfLife*, was used to estimate the use-by date of vacuum-packaged cooked sausages at a chosen dynamic temperature profile (Supplementary Material). The use-by dates for vacuum-packaged cooked sausages were established when spoilage microbial load achieved the maximum limit of *ca.* 9.3 log cfu/g. This is the borderline to determine when changes in sensory attributes related to the appearance of vacuum-packaged cooked sausages occur (Figures S1 and S2, Table S1). The horizontal method for enumeration of microorganisms ISO 4833-1:2013 [23] was performed to determine the total microbial load at the zero time and after stimulating the microbial growth in the packages by pair incubation of samples at 7 and 36 °C, with counts on days 3 and 6 (7 °C) and on days 2 and 4 (36 °C) of incubation (Figure S3). The number of colonies obtained at each dilution level was imputed in the *MicroLab_ShelfLife* computational package to determine the parameters of the microbial growth and to plot the predictive microbial growth curve (Figure S4).

A dynamic temperature profile was entered in the predictive model based on the measurements published by the AccuWeather forecast during 2021. Latitude and longitude coordinates (22° 54' 13'' South; 43° 12' 35'' West; Rio de Janeiro, Brazil) were considered as the climatic location, indicating the place where the sausages are sold. According to the Köppen-Geiger classification, the climate of Rio de Janeiro is a tropical monsoon climate (*Am*) [24]. The temperature data were grouped by season. The daily temperature profile, representing each climate season, was hourly grouped to fit in the *MicroLab_ShelfLife* platform (Figure 1). This profile was used to mimic the temperature during the products storage and disposal for sale in markets.

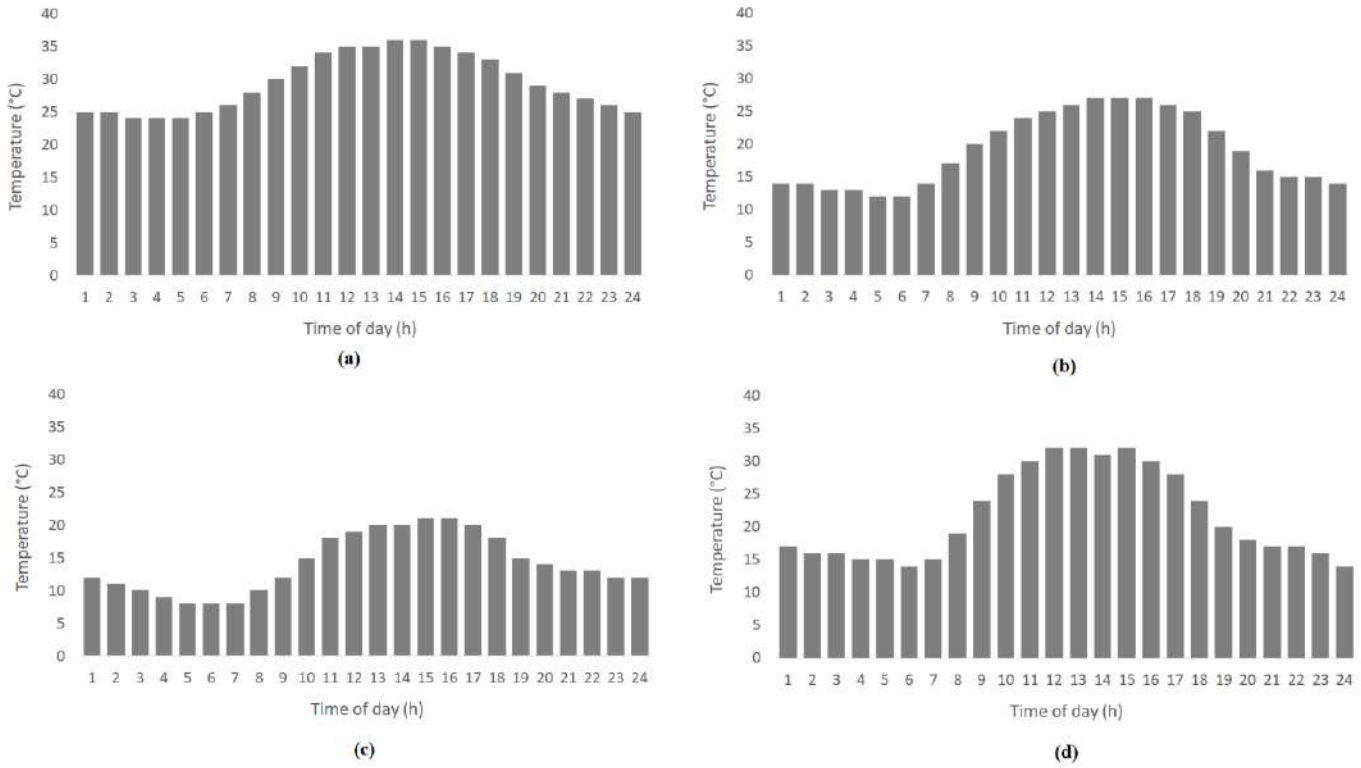

Figure 1. Temperature profile based on hourly variation during a one-day period to represent the seasons: **(a)** summer, **(b)** autumn, **(c)** winter, **(d)** spring. They were determined based on the measurements published by AccuWeather (www.accuweather.com) for 2021. Latitude and longitude coordinates: 22° 54' 13'' South; 43° 12' 35'' West; Rio de Janeiro, Brazil, where a tropical monsoon climate (*Am*) is reported (Köppen-Geiger climatic classification) [24].

2.6. Statistical Analyses

Results were expressed as Mean ± Standard Error (SE) from replicates. Shapiro-Wilk test was used to assume a normal distribution of data and Grubbs. Data were evaluated by analysis of variance (ANOVA), followed by Fisher's (LSD) test ($p < 0.05$).

Linear regression was applied to the incubation time with representative microbial growth in the turbidity method, according to Equations 1 to 3.

$$\dot{Y} \pm \frac{t\alpha}{2} \times SE \times \sqrt{hi} \quad (1)$$

$$\dot{Y} \pm t\alpha/2 \times SE \times \sqrt{1 + hi} \quad (2)$$

$$hi = 1/n + (xi - x)^2 / \sum (xi - x)^2 \quad (3)$$

Where, $\dot{Y}$ - estimated value, tα/2 - value of Student's t distribution, n – number of observations, xi – value of sample, x – mean.

A computational predictive modeling package, *MicroLab_Shelf-Life*, was developed in the present study and used to predict the use-by date of vacuum-packaged cooked sausages (Supplementary Material).

3. Results

In vitro trials revealed that PPCP addition at concentrations up to 0.5 % did not inhibit microbial growth. In samples containing from 1.0 to 3.0 % of PPCP, microbial inhibition was partially achieved. Although the efficacy was directly proportional to the added

concentration of PPCP, similar results were obtained by adding 1.0 or 1.5 % of PPCP ($p > 0.05$). The total inhibition was achieved at concentrations above 3.0 % (Table 2, Figures 2 and 3).

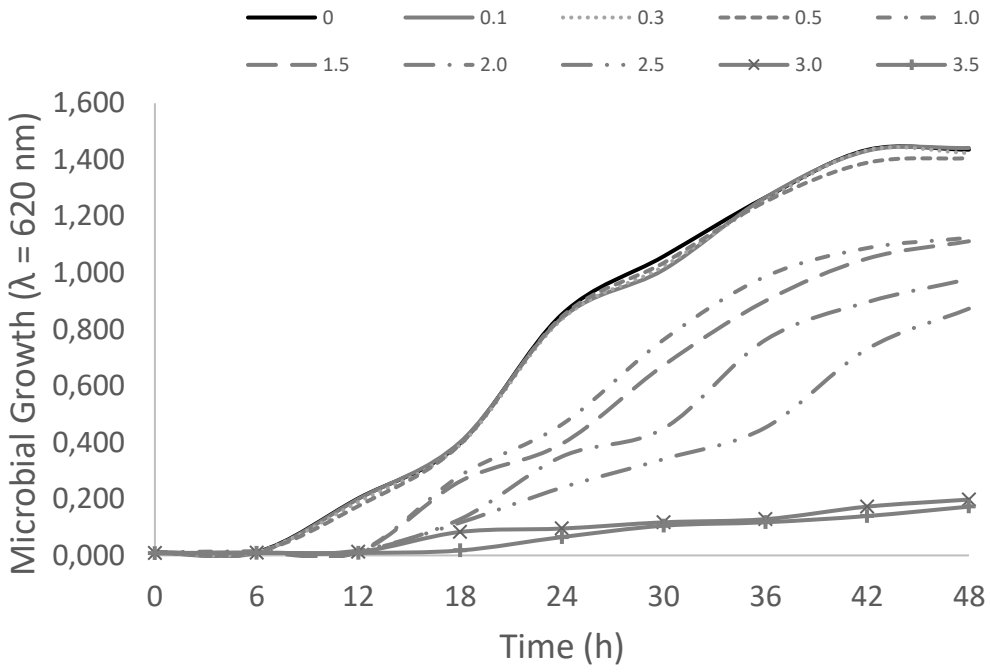

Figure 2. *In vitro* efficacy of PPCP against the growth of natural microbiota of vacuum-packaged cooked sausages. The inoculum was adjusted to *ca.* 5.5 log cfu/g before testing and the turbidity method was used to evaluate the efficacy. .

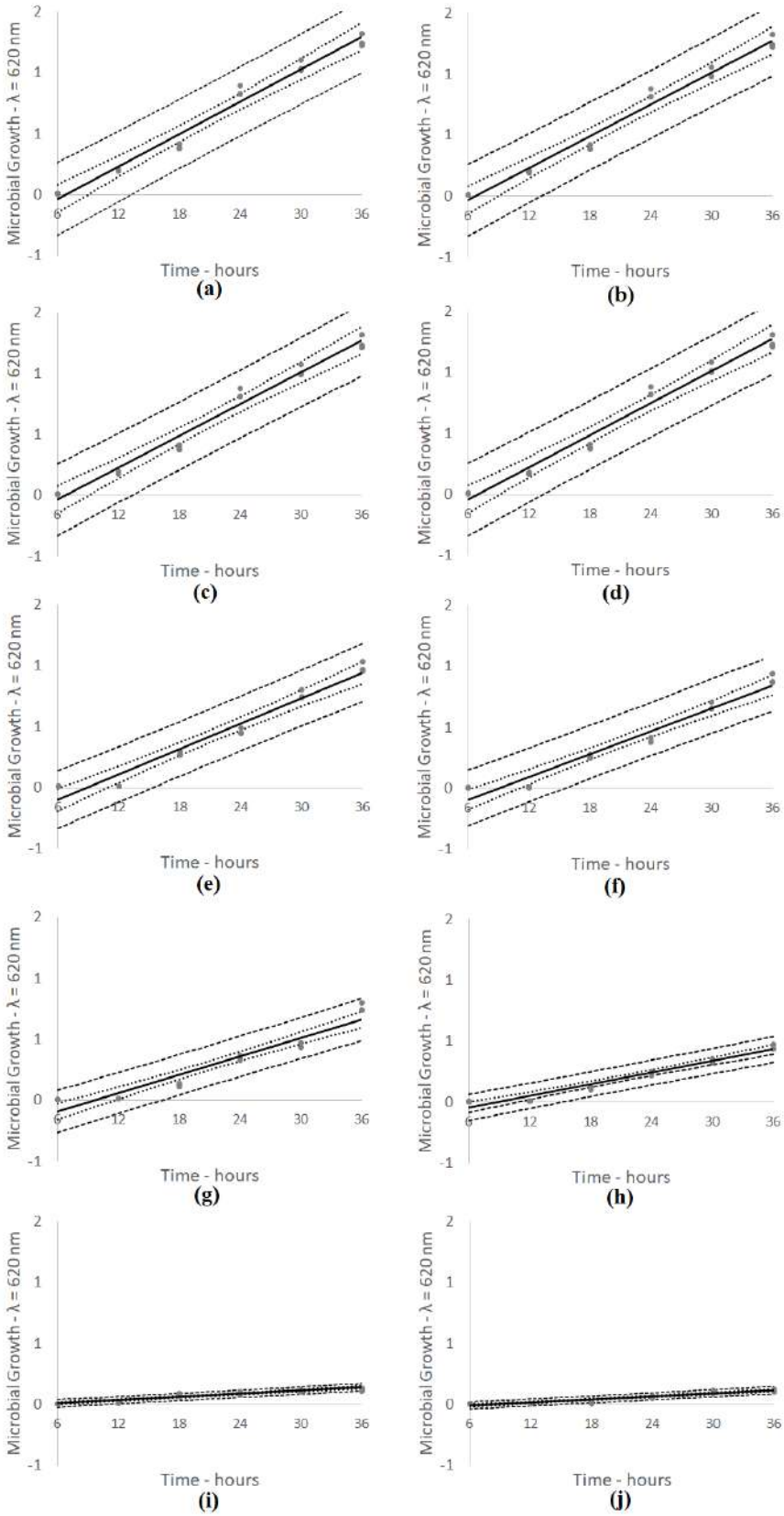

Figure 3. Linear regression (—), confidence interval for the mean (….), and prediction interval for the sample (---) of the period with microbial growth (from 6 to 36 hours of incubation at 36 °C) at different concentrations of potentially postbiotic-containing preservative (PPCP)**: (a)** 0.0%; **(b)** 0.1 %; **(c)** 0.3 %; **(d)** 0.5 %; **(e)** 1.0 %; **(f)** 1.5 %; **(g)** 2.0 %; **(h)** 2.5 %; **(i)** 3.0 %; **(j)** 3.5 %.

Table 2. Linear regression parameters of microbial growth.

	(%) of potentially postbiotic-containing preservative (PPCP)									
Coefficients	**0.0**	**0.1**	**0.3**	**0.5**	**1.0**	**1.5**	**2.0**	**2.5**	**3.0**	3.5
xi	0.044 [a]	0.044 [a]	0.044 [a]	0.044 [a]	0.035 [a]	0.031 [a]	0.025 [b]	0.016 [c]	0.004 [d]	0.004 [d]
yi	-0.299	-0.294	-0.298	-0.305	-0.309	-0.281	-0.242	-0.137	-0.018	-0.034
R2	0.978	0.981	0.979	0.977	0.959	0.955	0.921	0.961	0.881	0.088
SE	0.111	0.109	0.110	0.111	0.088	0.079	0.065	0.040	0.012	0.011
SQ	1890	1890	1890	1890	1890	1890	1890	1890	1890	1890
n	18	18	18	18	18	18	18	18	18	18
DF (n - 2)	16	16	16	16	16	16	16	16	16	16
tα/2	2.4729	2.4729	2.4729	2.4729	2.4729	2.4729	2.4729	2.4729	2.4729	2.4729
Confidence Interval	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95

xi – Angular coefficient; yi – Linear coefficient; R2 – Coefficient of determination; SE – Standard Error; SQ – Sum of Squares; n – Number of observations; DF – Degrees of Freedom; tα/2 - Value of Student's t distribution. Different capital letters indicate significant differences by Fisher's (LSD) test ($P < 0.05$).

Table 3 shows the physico-chemical characterization, water activity measurements, and pH values of sausages.

Table 3. Physico-chemical characterization of vacuum-packaged cooked sausages.

Parameter	**Mean ± Stardard Error**	
Moisture (%)	56.663	± 0.160
Protein (%)	14.434	± 0.288
Fat (%)	23.550	± 0.122
Ash (%)	3.550	± 0.387
Carbohydrates[1] (%)	1.803	± 0.627
Total energy (kcal/100 g sample)	281.749	± 1.714
Potential of hydrogen (pH)	6.878	± 0.004
Water activity (*Aw*)	0.964	± 0.002

[1] Calculated according to the Atwater specific factor system (4.27 kcal/g for protein or carbohydrate, and 9.02 kcal/g for fat).

PPCP and FCC85 reduced the growth of the natural microbiota in vacuum-packaged cooked sausages. However, the strategy of addition, on the surface or into the mass of the sausages, should be carefully designed. When the preservatives were added on the sausages' surface, only the treatment with 3.0 % of PPCP achieved the desired efficacy (S4). A slight efficacy of 2 days of the self-life was observed adding 2.0 % of the PPCP compared to 2.0 % of FCC85 (S1 and S3). The superficial treatments with 1.0% of PPCP and 2.0 % of FCC85 should be discouraged, since these treatments did not present effective results compared to blank and control (S0). The use-by date increased in 8 days (summer), 14 days (autumn), 22 days (winter), and 12 days (spring) in samples added with 2.0 % of PPCP, compared to the same concentration of FCC85. Furthermore, the addition of 1.0 % PPCP was as effective as the addition of 2.0% of FCC85 (Table 4).

The temperature profile entered in the predictive model influenced the growth of the natural microbiota in vacuum-packaged cooked sausages. In summer, the *Ngrowth* and *Ndeceleration* parameters of the predictive model, which represent the kinetics of the microbial growth in the growth (log) and deceleration phases, respectively, were higher than the values obtained during the other seasons. Thus, a shorter shelf-life was observed during summer, with an early achievement of the predictive borderline limit, which can result in changes in sensory attributes related to sausages' appearance. As expected, microbial growth reduced in winter. In addition, the correlation variable factor *FT(n),* which describes specific growth rates between log and deceleration phases, can also be used to

indicate the impact of the temperature profile on microbial growth, highlighting that the critical period for sausages preservation was summer (*FT(n)* = 3.4894), followed by autumn (*FT(n)* = 2.8038), spring (*FT(n)* = 2.5801), and winter (*FT(n)* = 2.1401).

Table 4. Durability study of vacuum-packaged cooked sausage samples.

Sample incubation			Treatments										
					Sausage surface				Sausage mass				
	Temperature (°C)	Time (days)	Blank	S0	S1	S2	S3	S4	M0	M1	M2	M3	M4
Laboratorial data (log cfu/g)	7	0	5.77	5.80	5.71	5.88	5.79	5.80	5.66	5.76	5.81	5.83	5.87
		3	6.01	5.99	5.89	5.99	5.90	5.81	5.95	6.01	6.02	5.92	6.01
		6	6.49	6.48	6.37	6.45	6.23	5.98	6.42	6.14	6.09	6.00	6.32
	36	2	6.69	6.72	6.70	6.80	6.64	6.14	6.59	6.66	6.61	6.33	6.26
		4	8.50	8.48	8.49	8.61	8.45	6.87	8.42	7.12	7.10	6.97	6.94
Specific maximum growth rate (log cfu/g/day)	7	L phase	0.1000	0.0883	0.0850	0.0658	0.0550	0.0167	0.1117	0.0733	0.0583	0.0292	0.0608
		D phase	0.0287	0.0253	0.0244	0.0189	0.0158	0.0048	0.0320	0.0210	0.0167	0.0084	0.0174
	36	L phase	0.5713	0.5650	0.5950	0.5713	0.5450	0.2188	0.5775	0.3950	0.3613	0.2675	0.2313
		D phase	0.1637	0.1619	0.1705	0.1637	0.1562	0.0627	0.1655	0.1132	0.1035	0.0767	0.0663
		Season											
Ngrowth (log cfu/g/day)[1]		Summer	0.4649	0.4575	0.4800	0.4572	0.4345	0.1732	0.4724	0.3224	0.2929	0.2137	0.1928
		Autumn	0.2970	0.2876	0.2982	0.2771	0.2599	0.1012	0.3064	0.2078	0.1850	0.1288	0.1321
		Winter	0.2158	0.2054	0.2103	0.1900	0.1754	0.0663	0.2261	0.1524	0.1328	0.0877	0.0608
		Spring	0.3383	0.3294	0.3429	0.3214	0.3028	0.1189	0.3473	0.2360	0.2115	0.1497	0.1470
Ndeceleration (log cfu/g/day)[2]		Summer	0.1332	0.1311	0.1375	0.1310	0.1245	0.0496	0.1354	0.0924	0.0839	0.0613	0.0553
		Autumn	0.1151	0.1115	0.1156	0.1074	0.1007	0.0392	0.1188	0.0805	0.0717	0.0499	0.0512
		Winter	0.1008	0.0960	0.0983	0.0888	0.0820	0.0310	0.1057	0.0712	0.0620	0.0410	0.0284
		Spring	0.1207	0.1175	0.1223	0.1146	0.1080	0.0424	0.1239	0.0842	0.0754	0.0534	0.0524
Use-by date (days)[3]		Summer	12	12	12	12	14	34	12	19	20	27	30
		Autumn	17	18	17	18	20	50	17	24	27	38	37
		Winter	22	22	22	24	26	70	21	31	35	52	45
		Spring	15	16	15	16	17	43	15	23	25	35	35

[1] *Ngrowth* – daily microbial population growth (log cfu/g) in the microbial growth (log) phase;.[2] *Ndeceleration* - daily microbial population growth (log cfu/g) in the microbial deceleration phase;.[3] The use-by dates for vacuum-packaged cooked sausages were established when spoilage microbial load achieved the maximum limit of *ca.* 9.3 log cfu/g.

3. Discussion

Over the past decade, novel terms have been used to represent the beneficial effects of microorganisms. Postbiotics or paraprobiotics or metabiotics represent structural components of probiotic microorganisms and/or formulation of and/or signaling molecules with a known chemical structure that can optimize host-specific physiological functions and regulate metabolic and/or behavior reactions related to the activity of host natural microbiota[25-27].

In 2001, FAO and WHO proposed a definition of probiotics that is used today as ''live microorganisms which when administered in adequate amounts confer a health benefit on the host'[28]. In October 2013, the International Scientific Association for Probiotics and Prebiotics (ISAPP) met to discuss about probiotics and the FAO/WHO definition of probiotics was reinforced as relevant and adaptable for current and further applications. The panel found that 'probiotic advanced by the FAO/WHO in 2001 is sufficiently broad to enable a wide range of products to be developed, and sufficiently narrow to impose some core requirements The development of metabolic by-products, dead microorganisms, or other microbial-based, nonviable products have potential; however, these do not fall under the probiotic construct' [29]

This FAO/WHO requirement may difficult the development of cooked food matrices containing probiotics, such as cooked sausages. Thus, the delivery of postbiotic compounds can be a suitable alternative for the development of functional cooked foods. Moreover, pre-cultured medium by LAB has been reported in the literature as a promisor natural technology for food preservation [30]

Poor-quality raw material and inadequate handling can anticipate sausages spoilage. In vacuum-packaged cooked sausages, changes in the sensory attributes related to the sausages' appearance, which can be a decisive factor for the consumer's appraisal, occur when the microbial population achieves the stationary phase in the microbial growth curve (*ca.* 9.3 log cfu/g) (Figures S1, S2, Table S1). The use of preservatives may reduce the activity of the natural microbiota, impacting the cell viability.

The meat industry prescribed the use-by date of 90 days for the vacuum packaged cooked sausages studied in this work. None of the treatments maintained the microbial load below the predicted model's borderline during the shelf-life period. Therefore, additional hurdles, such as cold storage, should be used combined with preservatives. When the cold storage temperature profile (7 °C) was entered in the predictive model to estimate the use-by date of the sausages, adding 3.0 % of PPCP on the surface (S4) or adding 2.0 % or more to the mass (M3 and M4), extended the use-by date by more than 90 days (Table 5).

The addition of 2.0 % of FCC85 on the surface (S1) or to the mass of the sausages (M1) little increased the use-by date. However, this is the maximum concentration permitted by the regulatory agency for the use of sodium lactate in heat-treated meat products [31]. This fact casts doubt on the efficacy of sodium lactate to increase the use-by date of vacuum-packaged cooked sausages. Although PPCP showed advantages compared to FCC85 regarding the extension of the use-by date of the sausages, it did not maintain the microbial load below the predictive model's borderline over 90 days either. However, there is no prescribed limit to use natural substances in sausages. Moreover, co-use of preservative and proper management of the cold chain are suitable strategies to achieve a use-by date higher than 90 days.

Cold chain management of meat products, including raw material supply, processing, distribution, and retail, is a crucial factor to prevent spoilage [12]. The specific maximum growth rate obtained at 36 °C was expressively higher than the value determined at 7 °C (Table 2), showing the influence of the temperature in sausage spoilage. Indeed, the temperature profile during the distribution, storage, and disposal in the market plays a role in the durability of meat products.

The addition of 3.0 % of PPCP on the surface (S4) or 2.0 % or more of PPCP to the mass (M3 and M4), combined with a management of the cold chain, resulted in a use-by date higher than 90 days (Table 5).

Table 5. Durability study of vacuum-packaged cooked sausages stored at 7 °C.

Sample incubation			Treatments										
					Sausage surface				Sausage mass				
	Temperature (°C)	Time (days)	Blank	S0	S1	S2	S3	S4	M0	M1	M2	M3	M4
Laboratorial data (log cfu/g)	7	0	5.77	5.80	5.71	5.88	5.79	5.80	5.66	5.76	5.81	5.83	5.87
		3	6.01	5.99	5.89	5.99	5.90	5.81	5.95	6.01	6.02	5.92	6.01
		6	6.49	6.48	6.37	6.45	6.23	5.98	6.42	6.14	6.09	6	6.32
	36	2	6.69	6.72	6.70	6.80	6.64	6.14	6.59	6.66	6.61	6.33	6.26
		4	8.50	8.48	8.49	8.61	8.45	6.87	8.42	7.12	7.10	6.97	6.94
Specific maximum growth rate (log cfu/g/day)	7	L phase	0.1000	0.0883	0.0850	0.0658	0.0550	0.0167	0.1117	0.0733	0.0583	0.0292	0.0608
		D phase	0.0287	0.0253	0.0244	0.0189	0.0158	0.0048	0.0320	0.0210	0.0167	0.0084	0.0174
	36	L phase	0.5713	0.5650	0.5950	0.5713	0.5450	0.2188	0.5775	0.3950	0.3613	0.2675	0.2313
		D phase	0.1637	0.1619	0.1705	0.1637	0.1562	0.0627	0.1655	0.1132	0.1035	0.0767	0.0663
Ngrowth (log cfu/g/day)[1]			0.1000	0.0883	0.0850	0.0658	0.0550	0.0167	0.1117	0.0733	0.0583	0.0292	0.0250
Ndeceleration (log cfu/g/day)[2]			0.0661	0.0584	0.0562	0.0435	0.0363	0.0110	0.0738	0.0485	0.0386	0.0193	0.0165
Use-by date (days)[3]			41	46	49	60	73	240	38	56	68	136	158

[1] *Ngrowth* – daily microbial population growth (log cfu/g) in the microbial growth (log) phase; [2] *Ndeceleration* - daily microbial population growth (log cfu/g) in the microbial deceleration phase; [3] The use-by dates for vacuum-packaged cooked sausages were established when spoilage microbial load achieved the maximum limit of ca. 9.3 log cfu/g.

These results highlighted the potential use of PPCP on the surface of the sausages. However, the concentration to achieve the total inhibition of the microbiota, determined *in vitro*, should be respected. Thus, regarding the addition of PPCP to the mass of the sausages, the concentrations used to achieve a partial inhibition of the microbiota can be used, as observed in M3.

After packaging, syneresis may be induced during the storage and distribution of sausages, resulting in the accumulation of water, nutrients, and microorganisms inside the package. Preservatives are usually added to the mass with other ingredients during meat products preparation. However, after syneresis, there are no barriers to prevent microbial growth in the liquid accumulated inside the package. Even when effective preservatives are added to the mass, this strategy may fail after syneresis due to the partial migration of these additives to the liquid phase. It can be of great concern if the storage temperature allows microbial activities.

The initial microbial load of the sausages may contribute to shorten the use-by date. By fixing the values of predictive model's parameters (*Ngrowth, Ndeceleration*, and *Ft(n)*) for each treatment, the use-by date of 90 days was achieved with the predicted initial microbial loads presented in Table 6.

Table 6. Estimated initial microbial load of vacuum-packaged cooked sausages to achieve the predictive model's borderline of 90 days.

Treatments		Presumed initial microbial load (log cfu/g)				
		Summer	**Autumn**	**Winter**	Spring	Cold Storage
Sausage surface	Blank	-20.00	-13.05	-9.03	-14.90	0.87
	S0	-19.17	-12.22	-8.20	-14.07	1.90
	S1	-19.46	-12.51	-8.49	-14.36	2.21
	S2	-17.59	-10.64	-6.62	-12.49	3.88
	S3	-16.38	-9.43	-5.41	-11.28	4.84
	S4	-6.57	0.38	4.40	-1.47	8.30
Sausage mass	M0	-20.91	-13.96	-9.94	-15.81	-0.24
	M1	-14.21	-7.26	-3.24	-9.11	3.26
	M2	-12.46	-5.51	-1.49	-7.36	4.56
	M3	-7.62	-0.67	3.35	-2.52	7.18
	M4	-6.27	0.68	4.70	-1.17	7.57

Only the treatments with 3.0 % of PPCP on the surface (S4) or 2.0 % or more of PPCP into the mass of the sausages (M3 and M4) during the winter achieved the proposed use-by date. This result highlights the importance of considering additional factors to hurdle microbial growth in the sausages. During the summer and spring, the preservation of the sausages during the proposed use-by date is elusive for any treatment. Considering the co-use of preservatives and the management of the cold chain, the meat industry may reduce the initial microbial load to the levels presented in Table 6.

Satisfactory results regarding the extension of the shelf-life of meat products can be achieved by reducing the initial microbial load, as well as by improving the product formulation to retain syneresis [32]. Indeed, the microbial growth and durability of sausages are greatly influenced by the initial microbial load and the use of effective hurdles [33]. However, sporulated bacteria groups cannot be eliminated by cooking processes and hurdlers. This fact highlights the importance of avoiding the presence of these microorganisms in products by applying microbiological quality control in the meat supply chain [34].

Handlers, utensils, equipment, and microbial load of the raw material are the main microbial vehicles during production [35-37]. The environment is also a factor to meat spoilage [38], and it depends on the region, climate, microclimate, season, anomalous environmental events as forest fires, deforestation, rainwater excess, etc [39].

5. Conclusions

PPCP produced by an axenic fermentation system with *L. paracasei* DTA 83 was as effective as the reference widespread commercial preservative FCC85 in preserving vacuum-packaged cooked sausages. Thus, it can be highlighted as a promisor alternative to the use natural technologies to preserve and produce functional cooked sausages. These results also revealed a logic relation regarding *in vitro* and *in situ* tests to evaluate sausage preservation. Thus, the concentration needed to achieve the total inhibition of the microbiota, determined by an *in vitro* trial, should be respected when adding PPCP on sausages' surface. When adding PPCP to the mass of the sausages, the concentration that showed a partial inhibition *in vitro* can also be applied in *situ*. However, proper chain management during distribution and disposal of products in the market is pivotal to achieve the desired use-by date. Although this study presented a potential postbiotic alternative by adding PPCP to sausages, a robust *in vivo* trial must be further designed to evaluate such effect in the host.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1. The relative abundance (**a** and **b**), Krona plot (**c**), and dendrogram of similarities and discrepancies of high-throughput sequencing of bacterial phyla of vacuum-packaged cooked sausages. Figure S2. Microbial growth curves at 4 °C (**a**), 12 °C (**b**), 24 °C (**c**), and 36 °C (**d**). They were plotted regarding the natural microbiota of vacuum-packaged cooked sausages (sample #1; sample #2; sample #3). Drop-plate technique was used to count total bacteria. Baranyi's mathematical model was applied to model the microbial growth at each temperature. The initial population was ca. 2.8 log cfu/g. The growth (log) phase started suddenly after incubation at 24 and 36 °C, and extended up to 8.2 log cfu/g. Stationary phase started after the population had reached ca. 9.3 log cfu/g. The period between the log and the stationary phases was considered the deceleration phase. Table S1. Instrumental color measurement (on the unopened packaged sausages and after withdrawing the sausages from the packages and cleaning up their surfaces) and slime formation detection. Figure S3. Sample incubation design. Microbial count at time zero must be below 8.2 log cfu/g to validate the test. Besides the time zero, there is no predefined time for microbial counting once the computational predictive modeling can process any time; however, microbial growth (log) phase must be included at least in one of the counts. Laboratories can determine the incubation temperatures; however, lower and higher temperatures between 4 and 20 °C, 25 and 36 °C, respectively, must be used. Figure S4. Illustration of the biological growth curve by predictive modeling. A – adaptation and acceleration growth phase; L – microbial growth (log) phase; D – deceleration phase; S – stationary phase. Correlations between specific growth rate in L and D phases were performed based on the correlation factor *FT(n)* value, according to the chosen temperature profile of the test.

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Introduction: Impact of a Biopreservative Derived from Lactic Fermentation on Quality after Food Processing: A Case Study on Sliced Cooked Ham

The consumption of processed meats, such as sliced cooked ham, remains popular worldwide due to their convenience, taste, and extended shelf life. However, traditional methods of preserving these products often involve synthetic chemicals, raising concerns over their potential health risks and environmental impact. In response to growing consumer awareness and regulatory scrutiny, there is increasing pressure on the food industry to adopt safer and more sustainable preservation practices.

Lactic fermentation, a process where lactic acid bacteria ferment sugars like glucose to produce lactic acid, offers a natural alternative for food preservation. This approach not only lowers the pH of food products, creating unfavorable conditions for pathogenic microorganisms, but also enhances their nutritional and sensory properties. Historically, ham production involved injecting pork with brine, followed by controlled cooking and cooling steps. However, recent innovations have integrated biopreservatives derived from lactic fermentation into this process, aiming to improve shelf life and maintain crucial quality attributes such as color and texture.

This study focuses on the application of a biopreservative produced through axenic lactic fermentation of glucose to sliced cooked ham. The research explores optimal concentrations of the biopreservative to effectively extend shelf life under varying storage conditions. Physicochemical and microbiological analyses ensure that the biopreservative meets rigorous safety and quality standards, particularly concerning color stability, which is pivotal for consumer acceptance and product safety.

The use of fermentation products as natural biopreservatives offers several advantages over synthetic counterparts. Lactic acid bacteria used in fermentation are generally regarded as safe (GRAS) by regulatory authorities, mitigating health concerns associated with synthetic preservatives. Furthermore, natural fermentation processes can potentially enhance food products with health benefits, such as probiotics, while minimizing environmental impact, contributing to sustainable food production practices.

This research contributes to broader efforts in sustainable food processing by demonstrating the feasibility and efficacy of a natural biopreservative derived from lactic acid fermentation in preserving sliced cooked ham. By highlighting significant improvements in shelf life and quality maintenance, particularly in color stability, this study supports the adoption of environmentally friendly alternatives in food preservation. Ultimately, the findings underscore the potential of leveraging bioactive compounds from lactic fermentations to develop natural preservation solutions that align with evolving consumer preferences and global sustainability goals in food production.

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Impact of a Biopreservative Derived from Lactic Fermentation on Quality after Food Processing: A Case Study on Sliced Cooked Ham

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Abstract: This study presents an innovative approach to enhancing the shelf life and maintaining the quality of sliced cooked ham through the application of a natural biopreservative derived from lactic fermentation. The biopreservative, at concentrations ranging from 1% to 3.5%, demonstrated substantial efficacy in microbial inhibition, keeping the microbial density low and relatively constant over time ($p < 0.05$). Remarkably, even at the lower concentration of 1%, the microbial growth rates were significantly reduced, with the treated samples showing notable stability over 24 days at both 7 °C and 25 °C. The microbial count in the treated with biopreservative group was significantly lower (3.19 log cfu/g) compared to the blank (4.59 log cfu/g) and control (5.01 log cfu/g) over 4 days at 7 °C. The shelf life of the ham was 24 days for the blank, 20 days for the control, and 101 days for the treated group at 7 °C. Moreover, colorimetric analysis revealed that the treated samples maintained better color stability, experiencing less variation in the hue angle and chroma, suggesting a protective effect against quality degradation over time. The successful application of the biopreservative aligns with the growing consumer demand for natural food additives and underscores the movement toward sustainable, health-conscious food preservation practices. The findings of this study indicate a promising avenue for the food industry to adopt environmentally friendly alternatives to synthetic additives, which could significantly influence future standards in food processing and preservation.

Keywords: biopreservative; lactic fermentation; sliced cooked ham; shelf life; microbial inhibition; color stability; sustainable food practices

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1. Introduction

The consumption of processed meats like cooked ham is widespread due to their convenience, taste, and long shelf life. However, the preservation of these meats typically involves synthetic chemicals that can have negative health implications and contribute to environmental degradation [1]. As consumer awareness increases and regulatory bodies tighten the rules, the food industry faces significant pressure to find greener and safer preservation methods [2]. Lactic fermentation involves lactic acid bacteria fermenting sugars like glucose to produce lactic acid, which acts as a natural preservative [3]. This approach not only enhances food quality by lowering the pH and creating conditions unfavorable for pathogenic microorganisms but also improves the nutritional and sensory properties of the food products [3].

Traditionally, ham was manufactured by injecting pork raw materials with a brine solution, followed by a series of controlled cooking and cooling steps [4]. The innovative part of the process involved the incorporation of a biopreservative produced through an axenic lactic fermentation of glucose. This process was optimized to achieve a biopreservative that is effective at extending the shelf life and maintaining the color and texture of the ham, which are critical quality attributes that influence consumer acceptance [5].

In-depth physicochemical and microbiological analyses were conducted to ensure that the biopreservative meets safety and quality standards [6]. Color degradation not only affects the product's appearance but can also underlie chemical changes that may affect safety and palatability [7].

The use of fermentation products as natural biopreservatives offers several advantages over synthetic alternatives. Firstly, the involved bacteria are generally regarded as safe (GRAS) by regulatory authorities, reducing the health risks associated with synthetic preservatives. Secondly, natural fermentation processes can enhance the potential addition of health benefits, such as probiotics, to the food. The environmental impact of producing natural preservatives is typically lower, contributing to a more sustainable food production system [8], given the pressing need for sustainable food production and preservation methods [9].

This study explores an innovative approach to extend the shelf life of sliced cooked ham using a natural biopreservative (fermented solution) produced from the lactic fermentation of glucose. Therefore, the aim of this study is to move beyond traditional food microbiology practices and storage settings, demonstrating the feasibility and benefits of using a natural biopreservative in meat products. Specifically, this study contributes to the broader goals of sustainable food processing. Initially, this study focuses on optimizing the best concentration of biopreservative to identify the most effective conditions. Subsequently, assays are conducted on hams, considering various storage temperatures and their fluctuations. Additionally, the quality parameter of color is evaluated to understand the impact of the biopreservative on ham preservation.

2. Materials and Methods

2.1. Biopreservative Assays

2.1.1. Biopreservative Manufacturing

The lactic fermentation was conducted in the presence of 0.1% of glucose, 0.5% of yeast extract, 0.1% of magnesium sulphate, 0.05% of ammonium citrate tribasic, and 0.05% of dipotassium phosphate by adding *Lacticaseibacillus paracasei* DTA-83. The fermentation was carried out at BRC Ingredientes Ltda., located in the city of Rio Claro, in the state of São Paulo, Brazil. In the production, the medium was prepared with food-grade ingredients in a stirred-tank bioreactor with a nominal load to 2000 L. The powdered ingredients were dissolved in drinking water in the vessel of the tank with gentle agitation at about 80 rpm. After complete dissolution, the temperature of the medium was heated up (1 °C per minute) to 75 °C for 2 h by an indirect injection of steam provided by a steam-boiler at 115 °C in the tank jacket. During heating, the medium was axially agitated at 84 rpm using a mechanical stirrer with a four-blade propeller (50 × 15 mm, length × width) and a pitch coupled to the bioreactor. After the heat treatment, the temperature of the medium was reduced to 36 °C (heating down by 1.0 °C per minute) by an indirect circulation of cold water in the tank jacket. In the starter solution, *L. paracasei* DTA-83 was growth in sterile modified MRS broth. An 18 h culture, at the log-phase stage, was added (1/100 of inoculum) into the bioreactor containing 2000 L of medium to obtain a final inoculum concentration of ca. 7 log cfu/mL. After 15 h of fermentation coupled with a pH decay to around 3.5, the medium was heat-treated at 95 °C for 5 min. The biopreservative was hot-bottled in polypropylene containers of 50 L.

2.1.2. The Minimum Concentration Assays

The in vitro antimicrobial activity of the biopreservative to inhibit the growth of the microbiota isolated from the cooked ham was assessed by the minimum inhibitory concentration test [10].

Serial dilutions of the biopreservative were prepared to achieve final concentrations ranging from 0.05% to 3.5%. The broth microdilution method in 96-well microtiter plates was performed according to the Clinical and Laboratory Standards Institute consensus process [11]. A positive control was included, consisting of microbial cultures grown in the absence of the inhibitory substance.

Microbial growth was monitored by measuring the optical density at 600 nm (OD600) using a spectrophotometer. Measurements were taken at predetermined intervals over a period of 30 days. All the tests were performed in triplicate. The mean and standard deviation were calculated for each time point and concentration.

The Minimum Inhibitory Concentration (MIC) was determined as the lowest concentration of the inhibitory substance that resulted in no visible growth of the microbe compared to the positive control. Growth was assessed by measuring the OD600 after 24 days of incubation.

2.2. Sliced Cooked Production

Sliced Cooked Ham Manufacturing

The cooked ham was produced at an agroindustry producer located in the city of Patos de Minas, in the state of Minas Gerais, Brazil, by injecting the pork raw materials with 50% brine injection at −1 to −5 °C.

The raw materials were placed in a vacuum meat tumbler for 6 h at 12 rpm (40 min clockwise and for 20 min counterclockwise). The mass obtained was packed in stainless steel boxes and taken to the curing chamber at 0 °C from 12 to 24 h. After the curing process, the product was placed in cook-in packaging and placed in molds, which were closed under pressure and taken to cooking tanks with water with the following binomials of time and temperature: 60 °C for 40 min, 65 °C for 40 min, 70 °C for 40 min, 75 °C for 40 min and 80 °C until the coldest point of the piece achieve 72 °C. Then, the product was immersed in another tank containing ice water to cool it and stored in a refrigerated chamber at 0 °C. Upon reaching 15 °C, the ham was unmolded, visually inspected and stored in chambers for 24 h.

2.3. Sliced Cooked Characterization

2.3.1. Physicochemical Analyses

General Physicochemical Analyses

The protocol reported in [12] was performed to determine the moisture (%w/v) in an oven-drying process at 105 °C until constant weight, ash (%w/v) by incinerating in a muffle furnace at 550 °C for 4 h, protein (%w/v) by the Kjeldahl method, total fat content (%w/v) by the Soxhlet extraction method with hexane (%w/v), and carbohydrate as the difference between a 100% and the sum of the percentages of moisture, ash, lipid, and protein [2].

Water Activity Measurement

The protocol reported in ISO 18787:2017 [13] was performed for the water activity measurements. An AcquaLab Lite device (Decagon, Pullman, WA, USA) equipped with a dielectric humidity sensor and infrared sample surface temperature sensor, which determine water activity by the electrical conductivity of an electrolyte, was used for the water activity measurements. The equipment was calibrated using standard solutions of K_2SO_4 (*aw* 0.973, CAS 7778–80–5) and KCl (*aw* 0.843, CAS 7447–40–7)). The sample was cut into small pieces approximately 0.3 mm in size with the help of a knife. The sample portion was positioned in a dish so that there was no empty space at the bottom. The equipment's reading stability was checked during the analytical series.

pH Values

The protocol reported in ISO 2917:1999(E) was performed for the pH determination [14]. A potentiometer device equipped with a calibrated surface pH probe (2015PC, Akso, Brazil) was used for the measurements. The pH value was calculated as the mean value of the measurements at 15 positions in a slice, which included the central and peripheral sections.

2.3.2. Texture Profile Analyses

The protocol reported in ISO 11036:2020 was performed for the texture profile analysis [15]. A texture analyzer (TA.XT, Stable Micro Systems, USA) and TA-XT+ software (TA.XT, Stable Micro Systems, USA) were used for the measurements. The sample was sectioned axially (2.0 cm) using a sampler. Measurements were carried out at six positions in the sample, covering the central and peripheral sections. All the measurements were performed at room temperature (about 23 °C). A 9.1 cm diameter circular plate was clamped onto a 500 N load cell and the sample was compressed to 60% of its original height at a crosshead speed of 200 mm/min in two cycles. The TPA parameters of hardness-1, hardness-2, cohesiveness, elasticity, and chewability were determined. Briefly, hardness-1 is the force required for the first compression and hardness-2 is the peak force for the second compression. Elasticity is the distance that the sample recovers in height after the first compression. Cohesiveness is the relationship between the two total areas under the compression curves (A1/A2). Chewing is the product of hardness-1, elasticity and cohesion [15].These parameters, hardness-1 and -2, were expressed in N/cm^2; elasticity was expressed in m/cm^2; and chewiness in J/cm.

2.4. Slice Cooked Ham Preservation

2.4.1. Microbial Load

The protocol reported in the method of MicroLab_ShelfLife [16] was performed to estimate the durability of sliced cooked ham at three temperature profiles (Figure 1). The end of the shelf life was determined when the total microbial count achieved the stationary growth phase (*ca.* 9.3 log cfu/g). The horizontal method for enumeration of microorganisms [17] was performed to determine the total microbial load, using plate count agar medium (HiMedia, Mumbai, India), at the zero time and after stimulating the microbial growth by incubation of the samples at 7 and 25 °C. The microbial load was counted at time zero and on days 2 and 4 of the incubation. The MicroLab_ShelfLife software [16] was used to estimate the microbial growth parameters and to model the microbial growth (log) phase, deceleration phase and the entrance of the microbiota in the stationary phase. Three simulations were performed for each sample group to mimic various conditions that the product may experience when for sale in markets.

2.4.2. Minimal Inhibitory Concentration

Concomitantly with the sample incubation in the durability study, spoilage microorganisms were obtained from five packages. Aliquots (0.1 mL) from the initial decimal suspension was transferred to tubes containing 5 mL of brain–heart infusion (BHI) broth or deMan–Rogosa–Sharpe (MRS) broth or yeast extract–peptone–dextrose (YPD) broth. The tubes were incubated at 30 °C for 24 h. The inoculum was obtained separately from each culture medium by transferring 1 mL of the tube content, with expressive growth (turbidity above 0.5 MacFarland standard), to an empty sterile screw-cap tube. Cells free of toxic compounds were obtained by twice washing the biomass cell pellets with a routine of centrifugation at 6000× *g* for 6 min for pellet sedimentation at the bottom of the tube, discarding the liquid fraction, adding 2 mL of phosphate buffer pH 7.2, and homogenizing in a vortex. The turbidity of the microbial suspension was adjusted to 0.5 McFarland standard and 2-fold diluted. The biopreservative was outlined to the final concentrations from 0.0 to 3.5% (*v*/*v*) in the brain–heart infusion broth. The dilutions were prepared in the same media used in the test to avoid a shortage of nutrients for microbial growth. Finally,

10 µL of the microbial suspension was added into the tubes to achieve a final microbial concentration of ca. 5 log cfu/mL.

The microbial density was measured in an automated microplate reader (Epoch2, Biotek, Agilent, Santa Clara, CA, USA) and Gen5 software (Biotek, Agilent, Santa Clara, CA, USA) for 24 h. The reader was equipped with an automated temperature control system and automated for homogenization, as activated before each reading record. It was adjusted to an incubation temperature of 30 °C, with a monochromatic wavelength of 620 nm, without a temperature gradient, with a recording of the optical density values, in absorbance, at regular intervals of 30 min, for 24 h. To homogenize the cell biomass before each reading, the microplate was agitated automatically by orbital movements, with a frequency of 282 cpm (3 mm) for 5 s. The absorbance values were plotted on a scatter plot with the variable time on the abscissa axis (x) and the absorbance values on the coordinate axis (y).

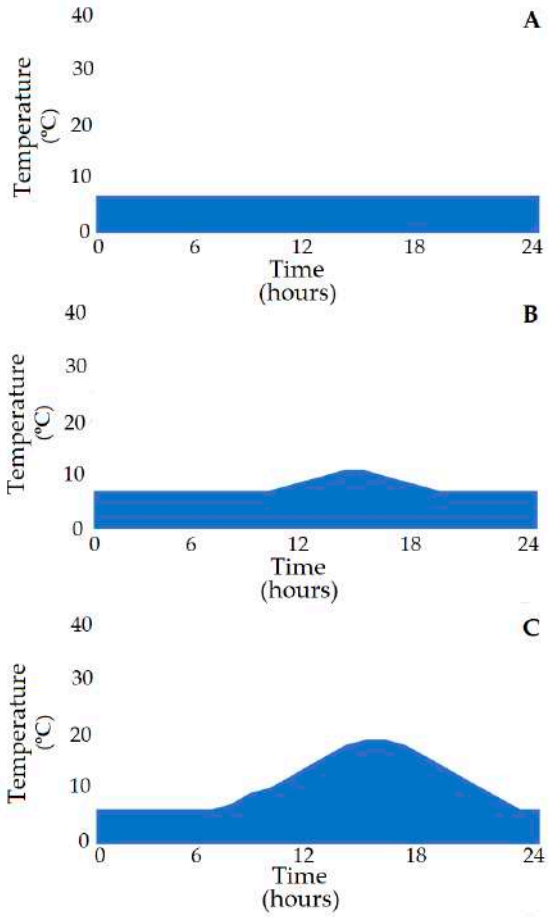

Figure 1. Dynamic temperature profiles based on the hourly variation over a period of one day to simulate realistic temperature conditions during the distribution of the products for sale in markets: (**A**)—cold storage at 7 °C; (**B**)—cold storage with a small abuse; and (**C**)—cold storage with a large abuse.

2.5. Quality Evaluation

Color Assays

A piece of ham was sliced in an electric slicer machine (BM 06 NR, Bermar, Brazil) with a thickness of 0.4 mm and placed in expanded polypropylene trays (N = 18), with one slice per tray. The packages were covered with polyvinyl chloride (PVC) film and divided into three groups: treatment (with 2.0% biopreservative added), control (with 2.0% sterile deionized water added) and white (no addition of biopreservative or water). All the groups were stored at 7 °C and 25 °C as follows:

B1—Blank cold storage at 7 °C;
B2—Blank storage at 25 °C;
C1—Control cold storage at 7 °C;
C2—Control storage at 25 °C;
T1—Treatment cold storage at 7 °C;
T2—Control storage at 25 °C;

The color was analyzed with a colorimeter (450 G, Delta Color, Brazil) provided with an 8 mm diameter measuring area and a 50 mm diameter illumination area. Illuminant A (average incandescent, tungsten-filament lighting, 2857 K) and a 10° standard observer were used to place more emphasis on the proportion of red wavelengths and captures# a larger portion of the scanned sample [18,19]. The readings were performed randomly for each treatment (n = 15), before and after cooking. Raw and cooked sausages with no addition of nitrite (T1) were used as the control. The color values were expressed as the L* (100 = white, 0 = black), A* (positive values = redness, negative values = greenness), and B* (positive values = yellowness, negative values = blueness).

2.6. Statistical Analysis

The samples were described using the microbial growth predictor MicroLab_ShelfLife, with a natural microbial load. All the data were evaluated by parametric and non-parametric tests using XLSTAT statistical and R 3.6.

3. Results and Discussion

3.1. Screening Biopreservative Concentration

Initially, we evaluate the MIC values to consider the best concentration of biopreservative at 10 different points from 0.03 up to 3.5%.

The growth curves (Figure 2) indicate that the microbial density increases over time in all the samples, including the positive control. The positive control shows a robust growth pattern, reaching a high optical density, suggesting that under normal conditions, the microbial population proliferates effectively, as accords with [20].

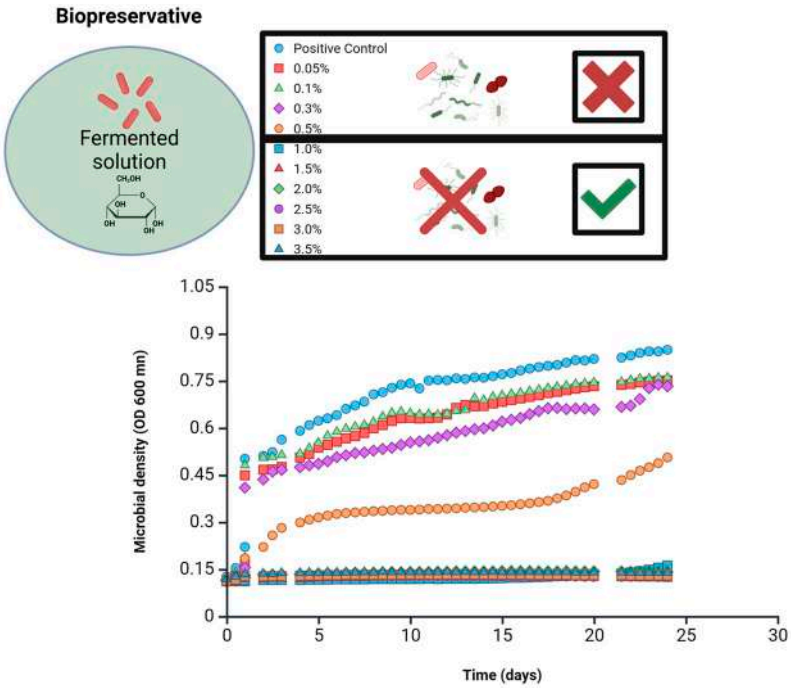

Figure 2. Microbial density at OD 600 nm over a 24-day period for 10 different concentrations of biopreservative (0.03% up to 3.5%).

At lower concentrations of the inhibitory substance (0.05% to 0.5%), there is a noticeable increase in the microbial density, although it is less than the positive control. At a 1% concentration, microbial growth is significantly suppressed, suggesting that this concentration is the minimum required to achieve good inhibition. It is at this concentration that the microbial density remains relatively flat, indicating effective inhibition of growth over 24 days.

At higher concentrations (1% to 3.5%), the microbial density remains low and relatively constant over time ($p < 0.05$), suggesting that these concentrations maintain strong inhibition and potentially bactericidal activity, preventing any significant increase in the microbial population.

Therefore, we selected for the next steps the minimum percentage of biopreservative (1%) to be applied as treatment in the ham samples.

3.2. Physicochemical Characterization of the Ham

The analysis of the physicochemical properties (Table 1) of the ham sample reveals a high moisture content (77.46 ± 0.021%), which is indicative of a water-rich food matrix. The protein content is substantial (16.87 ± 0.247%), suggesting the sample is a significant source of protein. The fat content is relatively low (3.61 ± 0.091%), aligning with leaner food products, and the carbohydrate content is minimal (0.84%), which could be beneficial for low-carbohydrate dietary needs. The ash content, representing the total mineral content, is modest (1.22 ± 0.080%). The pH is slightly acidic (6.49 ± 0.021), and the water activity (aw) is high (0.976 ± 0.002), potentially affecting the microbial stability and shelf life.

Table 1. Physico-chemical parameters of the cooked ham (mean ± standard deviation).

Parameter	Test	Quantity
Physico-chemical	Moisture (%)	77.46 ± 0.021
	Protein (%)	16.87 ± 0.247
	Fat (%)	3.61 ± 0.091
	Ash (%)	1.22 ± 0.080
	Carbohydrate (%)	0.84
	Potential of hydrogen (pH)	6.49 ± 0.021
	Water activity (*aw*)	0.976 ± 0.002
Texture profile	Fracturability (N/cm^2)	19.453 ± 0.983
	Hardness-1	21.366 ± 1.001
	Hardness-2	19.753 ± 0.894
	Cohesiveness	1.205 ± 0.222
	Springiness (mm)	13.600 ± 0.432
	Chewiness (J/cm^2)	35.015 ± 0.567

The texture profile analysis shows considerable fracturability (19.453 ± 0.983 N/cm^2), indicating that the sample requires a significant force to fracture, suggesting a firm structure. The hardness is also significant (hardness-1: 21.366 ± 1.001 N; hardness-2: 19.753 ± 0.894 N), which correlates with the fracturability results. The cohesiveness (1.205 ± 0.222) and springiness (13.600 ± 0.432 mm) values suggest that the sample has a notable internal binding and elastic nature. The chewiness (35.015 ± 0.567 J/cm^2), which combines the hardness, cohesiveness, and springiness, indicates that the sample has a substantial chew, which could contribute to consumer satiety and mouthfeel during consumption.

In this study, we developed the experimental design by considering a ham with a high moisture content coupled with high water activity, which suggests that while the sample may offer a desirable mouthfeel, due to its composition, it might be prone to microbial proliferation, potentially reducing the shelf life unless properly stored. The low fat and carbohydrate contents are favorable for those seeking healthier food options with reduced caloric intake [21].

From a textural perspective, the high fracturability and hardness reflect a firm and dense food product, possibly requiring sturdy packaging to prevent damage during transportation. The texture properties measured, including the cohesiveness and chewiness, provide insight into the potential sensory experience of the product, which is an important consideration for consumer acceptance [22].

After this characterization the potential for microbial growth was evaluated, considering the samples with water as a preservative (blank), the traditional method (control), and with the biopreservative (1%), exploring the use of natural preservatives or modified atmosphere packaging on enhancing the shelf life.

3.3. Impact of Storage Temperature on Microbial Growth in Sliced Cooked Ham

A concentration of 1.0% biopreservative was applied to sliced cooked hams and the impact of the storage temperature was evaluated in comparison with hams treated with 1.0% sterile deionized water (control) and hams without any application (blank).

The investigation assessed the efficacy of a treatment on the microbial inhibition at 7 °C and 25 °C over 4 days. Initially, the treatment started with a lower microbial count (3 log cfu/g) compared to the blank (3.82 log cfu/g) and control (3.65 log cfu/g).

After 2 and 4 days at 7 °C, the microbial count in the treated group was significantly lower (3.02 and 3.19 log cfu/g, respectively) compared to the blank (4.02 and 4.59 log cfu/g) and control (4.13 and 5.01 log cfu/g), suggesting effective microbial inhibition by the treatment.

At the higher temperature of 25 °C, the microbial counts increased across all the conditions. However, the treated group maintained lower counts (3.81 log cfu/g after 1 day and 4.13 log cfu/g after 3 days) compared to the blank (6.24 log cfu/g after 1 day and 7 log cfu/g after 3 days) and the control (6.99 log cfu/g after 1 day and 8.01 log cfu/g after 3 days), indicating that the treatment remained effective even at elevated temperatures.

In Figure 3A,C, we can observe the overall microbial growth across the different treatments at both temperatures. At 7 °C, all the treatment groups show a lower microbial count compared to the control and blank, with the treated group t exhibiting the most significant reduction ($p < 0.001$). This suggests that treatment is particularly effective at this refrigeration temperature over 4 days.

In Figure 3B,D, the box plots provide a distributional view of the microbial counts for the blank, control, and treated groups at both temperatures. At 7 °C, the treated group shows a broad distribution but a lower median log UFC/g.

The rapid growth in the control at 25 °C highlights the role of temperature in accelerating microbial proliferation. There is a hypothesis to explain this effect and these differences regarding the microbial densities at 7 °C. For instance, CO_2 is dissolved in the samples in a temperature-dependent manner, while increasing the temperature might lead to CO_2 release, thereby increasing the pH, as reported by Lamichhane et al. [23]. These authors described a linear decrease in the CO_2 concentration with the increase of both the temperature and the salt-in-moisture content, whereas the solubility of CO_2 increased, increasing the pH. In addition, *L. paracasei* DTA-83 showed high performance as heterofermentative bacteria, as described in previous studies [19,24,25]; therefore, its capability to produce organic acids as well as lactic acid can interfere with the ionic strength of the preservative liquid. When this biopreservative is exposed to high temperatures, the molecular ionization may decrease, resulting in a higher pH [26].

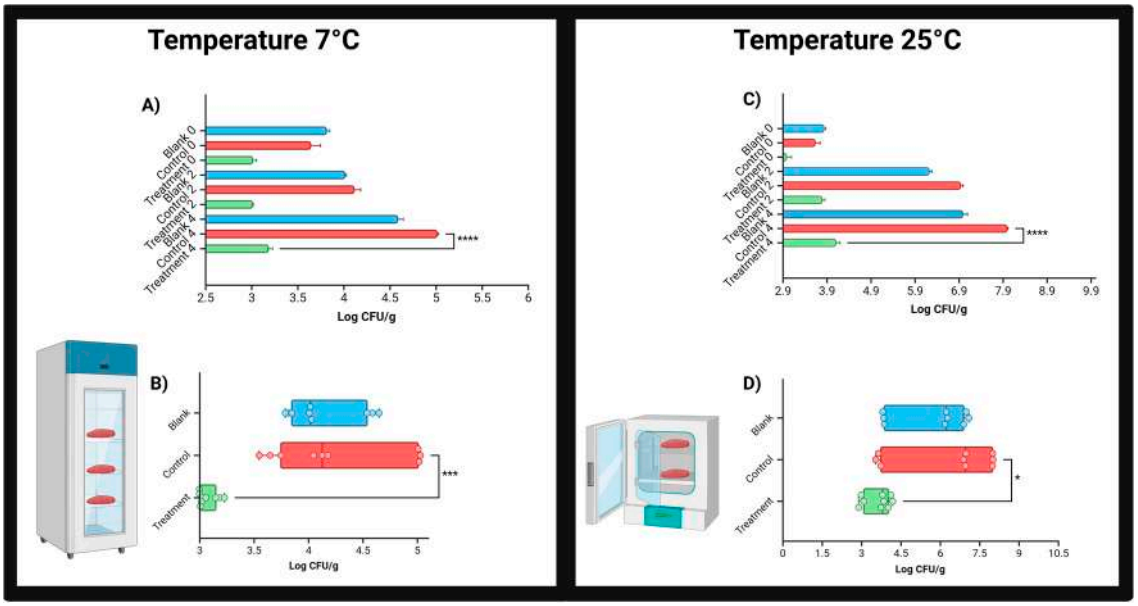

Figure 3. Impact of storage temperature on microbial growth in sliced cooked ham. (**A**) Bar graph depicting the log CFU/g of microbial populations at 7 °C for different times 0, 2 and 4 days (****) means $p < 0.001$ generated by the Mann–Whitney test). (**B**) Box plot representation of the microbial count variability at 7 °C considering 0, 2 and 4 days (***) means $p < 0.05$ generated by the *t* test). (**C**) Bar graph depicting the log CFU/g of microbial populations at 25 °C for different times 0, 2 and 4 days (****) means $p < 0.001$ generated by the Mann–Whitney test). (**D**) Box plot representation of the microbial count variability at 7 °C considering 0, 2 and 4 days (* means $p < 0.05$ generated by the *t* test).

3.4. Shelf-Life Evaluation

The durability of sliced cooked hams was assessed by applying a 1.0% biopreservative treatment and comparing with hams treated with 1.0% sterile deionized water (control) and hams without any application (blank). The specific microbial growth rates (log cfu/g/day) were measured under various temperature conditions (Table 2).

Table 2. MicroLab test.

Temperature Profile		Blank (Log cfu/g/Day)	Control (Log cfu/g/Day)	Treatment (Log cfu/g/Day)
Cold storage at 7 °C	*N (Tgrowth)*	0.2562	0.3124	0.0677
	N (Tdeceleration)	0.1693	0.2235	0.0447
Cold storage with soft abuse	*N (Tgrowth)*	0.302	0.3454	0.0839
	N (Tdeceleration)	0.1903	0.2145	0.0555
Cold storage with large abuse	*N (Tgrowth)*	1.018	1.111	0.4947
	N (Tdeceleration)	0.3726	0.4532	0.181

At 7 °C, the growth rate during the growth phase (N(T_growth)) was lowest in the treated group (0.0677) compared to the control (0.3124) and the blank (0.2562). During the deceleration phase (N(T_deceleration)), the treated group also showed a lower rate (0.0447) compared to the control (0.2235) and the blank (0.1693).

Under soft-abuse conditions, the treated group maintained a lower growth rates during both the growth (0.0839) and deceleration (0.0555) phases than the control (0.3454 and 0.2145, respectively) and the blank (0.302 and 0.1903, respectively).

When subjected to large abuse, the treated group's growth rates were significantly lower during the growth (0.4947) and deceleration (0.181) phases compared to the control (1.111 and 0.4532, respectively) and the blank (1.018 and 0.3726, respectively).

The application of a 1.0% biopreservative significantly inhibited microbial growth on the sliced cooked hams under all the storage conditions. The efficacy of the biopreserva-

tive was particularly notable under abusive temperature conditions, which are known to accelerate microbial proliferation.

The results at 7 °C suggest that the biopreservative is effective and the data also indicate that even under mild temperature fluctuations (soft abuse), the biopreservative continues to inhibit microbial growth effectively, although the rate of growth was higher compared to the ideal storage temperature.

However, the substantial increase in the microbial growth rate under large-abuse conditions, despite the presence of the biopreservative, highlights the challenges in maintaining food quality when storage temperatures are not strictly controlled.

The shelf-life assays were evaluated on sliced cooked hams stored under three different conditions (Figure 4).

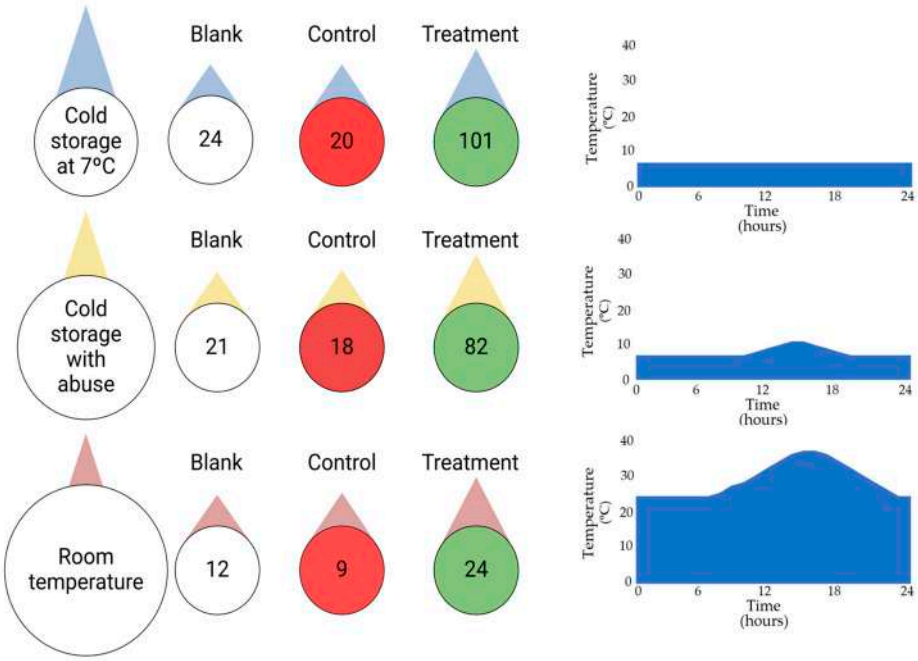

Figure 4. Shelf life of samples evaluated by MicroLab_ShelfLife.

Under cold storage at 7 °C, the durability of the hams was extended to 24 days for the blank, 20 days for the control, and markedly higher at 101 days for the treated group (Figure 4).

When exposed to cold storage with abuse, which likely represents temperature fluctuations, the durability decreased to 21 days for the blank, 18 days for the control, and 82 days for the treated group.

At room temperature, the durability was significantly reduced, with the blank lasting only 12 days, the control 9 days, and the treated group showing a durability of 24 days.

The application of 1.0% biopreservative (treated group) substantially increased the durability of the sliced cooked hams across all the storage conditions. Notably, at 7 °C, the treatment prolonged more than four times the durability comparted to the blank and five times compared to the control, highlighting the effectiveness of the biopreservative under optimal cold storage conditions. It is important to note that the promising effects of the biopreservative treatment observed in this study are contingent upon the initial microbial load being relatively low. In our case, the initial microbial load was around 3 log CFU/g, which significantly influenced the outcome of the treatment.

The reduced durability assessed in the abuse conditions suggests that although the biopreservative enhances the shelf life, the treatment is not a substitute for proper storage practices. However, the treatment still provided substantial durability benefits under these less-than-ideal conditions.

The results at room temperature are particularly compelling, as they indicate that the biopreservative still had a protective effect even when the hams were stored outside of the recommended temperatures, where spoilage typically accelerates.

The increased shelf life of the treated samples emphasizes the potential of biopreservatives in storing meat products [27].

3.5. Quality Impact Assays

After storage, the evaluation of some important quality parameters was considered. Color was selected to understand the changes in quality aspects, and at the same time, the test was carried out following the same experimental design. As shown in Table 3, the treated group and controls recorded no significant differences as storage at both temperatures T1 (7 °C) and T2 (25 °C) recorded similar lightness values of 68.650 ± 0.240 and 68.261 ± 0.245, respectively. This similarity suggests that the range of temperature conditions studied had a negligible impact on the lightness parameter of the samples.

Table 3. Colorimetric properties determined over a period of seven days. Two temperature-controlled groups, T1 (7 °C) and T2 (25 °C), were assessed alongside their counterparts, B1, C1, and B2, C2. The parameters examined included *L** (lightness), *a** (red–green coordinate), *b** (yellow–blue coordinate), C* (chroma), hab (hue angle), and ΔE (total color difference). The results are expressed as the mean ± standard deviation, with statistical significance denoted by superscript letters.

Groups	*L**	*a**	*b**	C*	hab	ΔE
B1	65.198 ± 4.358 ^{bcd}	6.626 ± 0.067 ^{a}	10.890 ± 0.050 ^{ab}	12.748 ± 0.007 ^{bc}	58.680 ± 0.375 ^{ab}	7.092 ± 0.100 ^{b}
C1	68.652 ± 0.401 ^{a}	6.637 ± 0.058 ^{a}	11.647 ± 0.132 ^{a}	13.405 ± 0.143 ^{a}	60.322 ± 0.065 ^{a}	8.402 ± 0.575 ^{a}
T1	68.650 ± 0.240 ^{a}	8.115 ± 0.106 ^{c}	9.585 ± 0.474 ^{c}	12.562 ± 0.293 ^{bc}	49.727 ± 1.766 ^{c}	6.549 ± 1.509 ^{b}
B2	68.579 ± 0.550 ^{a}	6.887 ± 0.219 ^{b}	11.020 ± 0.669 ^{d}	12.995 ± 0.684 ^{bc}	57.978 ± 0.747 ^{ab}	8.417 ± 0.934 ^{a}
C2	68.261 ± 0.245 ^{ab}	7.057 ± 0.460 ^{b}	10.660 ± 0.160 ^{b}	12.786 ± 0.388 ^{bc}	56.514 ± 1.323 ^{b}	6.589 ± 0.317 ^{b}
T2	68.261 ± 0.245 ^{ab}	8.480 ± 0.028 ^{c}	9.130 ± 0.948 ^{c}	12.468 ± 0.713 ^{cd}	47.032 ± 2.873 ^{c}	6.877 ± 0.061 ^{b}

A marked distinction was observed in the *a** parameter, with a T1 value of 8.115 ± 0.106, which was not significantly different from T2 (8.480 ± 0.028).

The *b** values for T1 and T2 (9.585 ± 0.474 and 9.130 ± 0.948, respectively) were lower than the controls. The lower values at both T1 and T2 may be due to the fact that temperature variations might cause a decrease in the perceived yellowness of the samples.

T1 had a chroma value of 12.562 ± 0.293, whereas T2 displayed a lower value (12.468 ± 0.713). This finding suggests that the low intensity of color at a higher temperature is potentially due to pigment or structural changes within the samples.

The hab presented notable differences between the two temperatures, with T1 having a higher hue angle of 49.727 ± 1.766 compared to T2 (47.032 ± 2.873).

The color difference for T1 was 6.549 ± 1.509, in contrast to T2, which had a ΔE of 6.877 ± 0.061. Although they exhibited relatively lower ΔE values compared to the control groups, the small increase in the ΔE at the higher temperature points to a more discernible change in color from the standard or initial state.

Based on these results, we can conclude that the treatment do not influence the color. Additionally, *L. paracasei* DTA-83 can influence biochemical processes, such as lactic acid production, that can be modulated by temperature [19,24].

4. Conclusions

Our research validated the application of a natural biopreservative derived from lactic acid bacteria as an effective means of extending the shelf life and preserving the quality of sliced cooked ham. Our study revealed that even at a low concentration of 1%, the biopreservative significantly curtailed microbial growth, showcasing its potency in both optimal and fluctuating temperature conditions over an extended period of 24 days. The

treated samples demonstrated superior color stability, indicating the biopreservative's role in mitigating quality degradation.

Our outcomes resonate with the increasing consumer preference for natural food additives and can support a shift of the food industry toward more sustainable and health-conscious preservation methods. The results underline the potential of leveraging bioactive compounds such as lactic acid produced by lactic fermentations to develop natural preservation solutions that not only meet quality standards but also align with the global drive for environmentally responsible food processing following sustainable practices.

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Introduction: Probiotic potential and biofilm inhibitory activity of Lactobacillus casei group strains isolated from infant feces

The term "probiotic," derived from the Greek word meaning "for life," refers to microorganisms that provide beneficial effects to humans and animals by interacting with their gut microbiota (De Vrese & Schrezenmeir, 2008). *Lactic acid bacteria* (LAB), particularly within the genus *Lactobacillus*, are extensively studied for their probiotic potential. With over 170 species, *Lactobacilli* are widely distributed in nature, found in fermented foods, plant materials, soil, and the human gut (Pogačić et al., 2010; Campanaro et al., 2014; Kim et al., 2018; Guerra et al., 2018; Zhang et al., 2018).

The increasing understanding of probiotics has led to the development of functional foods that offer health benefits beyond basic nutrition. Probiotic foods constitute a significant segment of the global functional food market, projected to reach a value of US $46.55 billion by 2020 (Singh, Singh, & Singh, 2018). According to the WHO/FAO definition, probiotics are live microorganisms that confer a health benefit to the host when consumed in adequate amounts. Health benefits attributed to probiotics are strain-specific, requiring thorough strain identification and safety evaluations (Motevaseli, Dianatpour, & Ghafouri-Fard, 2017).

The *Lactobacillus* genus includes the highest number of Generally Recognized As Safe (GRAS) species among LAB (Klaenhammer & de Vos, 2011). Criteria for defining a strain as probiotic include survival through gastrointestinal conditions, adherence to intestinal surfaces (indicating potential antimicrobial activity), and advantageous technological traits (Mattila-Sandholm et al., 2002; Ouwehand, Salminen, & Isolauri, 2002).

Probiotics are also investigated for health benefits like colon cancer prevention. Early studies correlated gut microbiota with colon cancer incidence, prompting research into probiotics' roles in cancer cell development regulation (Kubota, 1990; Bruno-Bárcena et al., 2004; Lee et al., 2004; Takagi et al., 2008; Shahid et al., 2018).

This study focuses on *Lactobacillus* strains isolated from infant feces, evaluating their probiotic potential through tests for gastrointestinal survival, bile salt hydrolysis, prebiotic utilization, absence of hemolytic activity, antibiotic resistance, and ability to inhibit biofilm formation and modulate HT-29 colorectal cancer cells. Strains *L. paracasei* DTA81 and DTA93, isolated from infants aged 7-21 days in Rio de Janeiro hospitals, exhibited promising probiotic and anti-cancer activities comparable to or surpassing commercial strains like *L. rhamnosus* GG.

In summary, this research highlights *Lactobacillus* as a valuable source for discovering health-promoting microbes. Further investigation, including in vivo studies, is necessary to fully assess these strains' potential health benefits in their intended hosts. This study contributes to ongoing efforts to leverage beneficial microbes from infant feces for improving human health and wellness.

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Probiotic potential and biofilm inhibitory activity of *Lactobacillus casei* group strains isolated from infant feces

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ABSTRACT

Probiotic bacteria are receiving growing interest, particularly for the preparation of functional foods. In the present study, eight *Lactobacillus* strains, newly isolated from infant feces, were investigated for the presence of probiotic properties such as antimicrobial susceptibility, hemolytic activity, resistance to simulated gastro-intestinal conditions, bile salts hydrolytic activity, inhibitory ability against biofilm formation by other bacteria, attachment to HT-29 human cancer cells and anti-cancer activity. All the strains tested highlighted interesting properties, but *L. paracasei* DTA93 and *L. paracasei* DTA81 appeared of particular interest. Some properties of these two strains resulted similar, and in some cases superior, to the reference widespread probiotic commercial strain *L. rhamnosus* GG. Strain *L. paracasei* DTA81 possesses amazingly high adherence ability to HT-29 cells, about ten times higher than that of *L. rhamnosus* GG. Moreover, *L. paracasei* DTA93 and *L. paracasei* DTA81 were able to effectively inhibit biofilm formation of *Escherichia coli* and *Listeria innocua*.

1. Introduction

The word "probiotic" is derived from the Greek word meaning "for life". It is usually referred to microorganisms that confer beneficial effects to human and animals by interacting with their gut microbiota (De Vrese & Schrezenmeir, 2008). Lactic Acid Bacteria (LAB) are presently the most studied and characterized bacterial group containing probiotic strains, particularly in the genus *Lactobacillus*. This genus contains more than 170 species and it is considered a taxonomically complex group (Foschi et al., 2017; Goldstein, Tyrrell, & Citron, 2015). Lactobacilli are very widespread in nature and are commonly isolated from several different matrices, such as fermented foods (Pogačić et al., 2010), plant material (Campanaro et al., 2014), soil (Kim et al., 2018) and human gut (Guerra et al., 2018; Zhang et al., 2018). The increase in knowledge on probiotics has led to the development of food products that can provide health benefits beyond basic nutrition. Probiotic foods represent a huge segment of functional food available on the market worldwide, projected to reach a value of US $46.55 billion by 2020 (Singh, Singh, & Singh, 2018). According to the WHO/FAO definition, probiotics can be considered live microbes which confer a health benefit to the host when ingested in adequate amounts. It has been demonstrated that probiotic effects are strains-specific (Motevaseli, Dianatpour, & Ghafouri-Fard, 2017), thus strain identification and evaluation of its safety aspects must be performed prior to connect a specific strain to its own health benefit. Among LAB, the *Lactobacillus* genus includes the highest number of GRAS (Generally Recognized As Safe) species (Klaenhammer & de Vos, 2011). Besides the safety aspects such as resistance to antibiotics and blood hemolytic activity, other important criteria to define a strain as probiotic include survival to the human gastro-intestinal conditions, ability to adhere to the intestinal epithelial surface, which indicates possible antimicrobial activity against pathogens, prevention of colon cancer. In addition, the presence of potentially interesting technological traits would be desirable, such as good sensory properties, phage resistance, viability during processing and stability in production and during storage (Mattila-Sandholm et al., 2002; Ouwehand, Salminen, & Isolauri, 2002). Among all benefits from probiotics, anti-cancer effect has been one of the most interesting characteristic studied during last decade (Kumar & Dhanda, 2017). In

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1990, Kubota reported a correlation between gut microbiota and colon cancer incidence (Kubota, 1990), since illness incidence was lower when the presence of probiotic strains was higher. Lactobacilli can inhibit cancer cells development by inactivate reactive oxygen species (ROS), increase TNF-α, interferon-γ (IFN-γ), downregulating nuclear factor-kappaB (NF-κB) and increase the level of natural killer cell (NK) in the human body (Bruno-Bárcena, Andrus, Libby, Klaenhammer, & Hassan, 2004; Lee, Kim, Yim, & Joo, 2004; Takagi et al., 2008). More recently, Shahid et al. (2018) demonstrated that cell-free culture supernatant (CFCS) of *L. casei* and *L. paracasei* isolated from human breast milk can upregulate the expression of apoptosis-related genes on cervix cancer (HeLa) cells. In this study, we identified and selected some potential *Lactobacillus* probiotic strains isolated from infant feces. We studied the capability to withstand the transit through the gastro-intestinal tract and to hydrolyze bile salts and utilize prebiotic molecules. The absence of hemolytic activity and of transmissible antibiotic resistance were also studied. Finally, we looked for health-related traits, namely ability to attach and contrast the development of HT-29 colorectal cancer cells.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The strains used in the present work were isolated from feces of infants aged between 7 and 21 days from different hospitals in Rio de Janeiro (RJ, Brazil). Samples were collected in duplicate in sterile 10-mL plastic tubes and immediately placed on ice inside separate plastic bags. A hand pump was used to remove the air from the bags. Decimal dilutions were performed in Anaerobic Wilkins-Chalgren broth (Oxoid, Basingstoke, United Kingdom) and 100-µl aliquots of each dilutions were plated on Lamvab agar medium (Hartemink, Domenech, & Rombouts, 1997) specific for the isolation of lactobacilli and incubated at 37 °C for 48 h under microaerophilic conditions. Colonies showing typical morphologies were further characterized by Gram staining and catalase test.

2.2. Molecular identification of strains belonging to the Lactobacillus casei group

For DNA extraction one colony was picked from MRS agar plate (Bottari et al., 2017) and transferred into a 0.5 mL Eppendorf tube containing 50 µl of lysis solution (0.25% SDS and 50 mM NaOH). Cell lysis was obtained by incubating the tube at 95 °C for 15 min in a thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). The supernatant was collected by centrifugation at 10,000*g* for 10 min and diluted 1:100 in ultrapure sterile water. DNA yield and purity were assessed by NanoDrop 2000c (Thermo Fischer Scientific, Wilmington, DE, USA).

The multiplex PCR assay was performed using the primer pairs and PCR conditions described previously (Bottari et al., 2017). Results were visualized by gel electrophoresis on SYBR Safe stained 1.5% agarose gel. The type strains *L. paracasei* subsp. *paracasei* DSM 5622, *L. casei* DSM 20011 and *L. rhamnosus* DSM 20021 were used as reference for the species and *L. plantarum* subsp. *plantarum* DSM 20174 was used as negative control.

2.3. Determination of minimum inhibitory concentration (MIC)

The Minimum Inhibitory Concentration test was performed by the broth microdilution method, in 96-well microtiter plates (Wiegand, Hilpert, & Hancock, 2008). The following antibiotics: ampicillin, ciprofloxacin, erythromycin, gentamycin, kanamycin, penicillin G, tetracycline, and vancomycin (Sigma-Aldrich, Saint Louis, MO, USA) which are recommended by the European Food Safety Authority (EFSA, 2008), were dissolved in MRS plus ISO-Sensitest broth (Sigma-Aldrich) (ratio 10:90) and distributed as 2-fold serial dilutions in the microtiter plate wells. Each microtiter well was then inoculated with 50 µl of bacterial culture to reach 5×10^5 cfu/mL (final concentration). After incubation for 24 h, the MIC was defined as the drug concentration in the first well with no visible growth. The test was performed in triplicate.

2.4. Hemolytic activity test

The hemolytic activity test was performed by streaking fresh cultures of strains to be tested on MRS agar containing 5% (w/v) defibrinated sheep blood (cat. SR0051D, Thermo Fisher Scientific, Waltham, MA, USA) and incubated at 37 °C. Hemolytic activity was checked after 48 h of incubation. *Staphylococcus aureus* ATCC 6538 and *L. rhamnosus* GG (ATCC 53103) were used as positive and negative control, respectively. The experiment was performed with three technical and three biological replicates (Pieniz, Andreazza, Anghinoni, Camargo, & Brandelli, 2014).

2.5. Resistance to simulated gastrointestinal conditions

The resistance of strains to simulated gastro-intestinal conditions was carried out as previously described (Tarrah, Castilhos, et al., 2018). The basic juice for the gastrointestinal test contained (per liter) calcium chloride, 0.11 g; potassium chloride, 1.12 g; sodium chloride, 2.0 g; potassium dihydrogen phosphate, 0.4 g. The artificial gastric juice contained (per liter) 3.5 g of swine mucin (Sigma-Aldrich) and 0.26 g of swine pepsin (Sigma-Aldrich). The gastric juice was adjusted to pH 2.5 with 1 N HCl, filter sterilized and then added to the basic gastro-intestinal juice. The simulated intestinal juice contained (per liter) 3 g Ox-bile extract (Sigma-Aldrich), 1.95 g pancreatin (Sigma-Aldrich) and 0.1 g lysozyme (Sigma-Aldrich). The pH was adjusted to 8.0 with 1 N sodium bicarbonate and the medium was then filter sterilized. Regarding resistance to gastric conditions, aliquots of 0.1 mL of cell suspension obtained after three subcultures for 24 h in MRS broth was transferred to 0.9 mL of artificial gastric juice (pH 2.5) and incubated for 1 h at 200 rpm agitation at 37 °C. Regarding the intestinal conditions, 1 mL of artificial intestinal solution (pH 8) was added after incubation to gastric juice and incubated at 37 °C at 200 rpm stirring for 180 and 300 min. Microbial viability was evaluated by the micro drop technique. The experiment was repeated three times with three technical replicates each.

2.6. Bile salts hydrolytic activity

Fresh cultures were streaked on MRS agar containing 0.5% taurodeoxycholic acid (Sigma-Aldrich Saint Louis, MO, USA). The hydrolytic activity was checked after 48 h of anaerobic incubation at 37 °C for the presence of a precipitation halo. MRS plates without taurodeoxycholic acid were used as negative controls, whereas *Leuconostoc mesenteroides* SJRP 55 was used as a positive control (Jeronymo-Ceneviva et al., 2014).

2.7. Adhesion to HT-29 cells

The adhesion potential of newly isolated *Lactobacillus* strains to HT-29 cell lines was assessed as previously described (Jacobsen et al., 1999). The adherent strains were counted in 20 random microscopic fields. According to (Jacobsen et al., 1999), strains were scored as non-adhesive if they were fewer than 40, adhesive if the number was between 41 and 100, and strongly adhesive if there were more than 100 bacteria in 20 fields. The test was repeated three times with three technical replicates each.

2.8. Anti-proliferative activity against HT-29 cells

The anti-proliferative activity of the *Lactobacillus* strains on HT-29 cells was assessed by the microculture tetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) assay (Mosmann, 1983). Aliquots of 100 µl of HT-29 cells culture (1.2×10^5 cells/mL) in McCoy's 5A (Sigma-Aldrich) growth medium were inoculated into each well of 96-wells microplates. The strains to be tested were grown for 24 h, then pellets were removed by centrifugation at 5000 rpm for 10 min and pH normalized to 7.0 with 1 N NaOH. The supernatant was then freeze-dried and diluted in McCoy's 5A broth to obtain the following concentrations: 0.125, 0.25, 0.5, 0.75, 1, 2, 4 and 8 mg/mL. When 50% confluence of HT-29 cell was reached, the McCoy's 5A was replaced with 0.1 mL filtered supernatant of strain cultures at different concentrations and cells were incubated in 5% CO_2 atmosphere for 48 h at 37 °C. Then 20 µl of MTT (5 mg/mL diluted in PBS) was added to each well and incubated for further 4 h at 37 °C to allow cells interaction with MTT. Following incubation, the formazan blue crystals formed were dissolved in 100 µl DMSO (Sigma-Aldrich). After 20 min stirring at 200 rpm, the reduction of MTT was measured by reading the absorbance at 570 nm using a microplate reader (Biotek ELx 800, Thermo Fisher Scientific) and viability percentage of HT-29 cells was calculated by using the following formula:

$$\text{Viability } \% = (OD_{570} \text{ of treated cells}/OD_{570} \text{ of cells without treatment}) \times 100$$

Cells incubated with MRS (bacterial culture medium) only and with DMSO 3% (anticancer molecule used as reference) were used as negative and positive controls, respectively. The experiment was repeated two times (biological replicates) in three technical replicates each.

2.9. Biofilm inhibitory activity

The capability of the *Lactobacillus* strains to inhibit biofilm formation by *Escherichia coli* DSM 30083^T and *Listeria innocua* DSM 20649^T was evaluated as previously described (Woo & Ahn, 2013), with some modifications. Biofilm-producing strains were grown on the hydrophobic surface of a 24-wells polystyrene plate with flat bottom. The biofilm inhibitory activity was evaluated in two different conditions, namely competition and exclusion. In the first test the *Lactobacillus* strains were co-inoculated with *E. coli* or *L. innocua*, at a concentration of 10^7 cfu/mL in a 24-wells plate and incubated at 37 °C for 18 h. In the exclusion experiment *Lactobacillus* cell suspensions containing 10^7 cfu/mL were inoculated inside 24 wells plate and incubated for 18 h at 37 °C. The wells were then washed three times with PBS, the wells inoculated with *E. coli* or *L. innocua* cell suspensions at the same concentration (10^7 cfu/mL) and incubated for further 18 h at 37 °C. Wells inoculated with *E. coli* or *L. innocua* alone were used as controls. Following incubation, each well was washed three times to remove non-adherent cells. Biofilms were collected using a sterilized swab and cells were serially diluted using sterile PBS. All dilutions were plated on VRBA (DIFCO, Maryland, USA) for *E. coli* and BHI (DIFCO, Maryland, USA) containing 1.5% LiCl for *L. innocua*. Plates were incubated at 37 °C for 48 h and then colony were counted. The level of inhibition was determined by comparing the values of the co-inoculated cultures with those containing only *E. coli* or *L. innocua*. The experiment was repeated three times.

2.10. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using GraphPad Prism software (version 7, GraphPad Software, Inc., San Diego, CA).

3. Result and discussion

3.1. Molecular identification of Lactobacillus isolates

Thirty-five isolates belonging to this genus, previously isolated from infant stools, were identified at species level by 16 rDNA sequencing and RAPD analysis (Guerra et al., 2018) and grouped into 9 cluster according to a RAPD similarity profile percentage of more than 80%. A molecular method to discriminate species belonging to the *L. casei* group, namely *L. casei, L. paracasei* and *L. rhamnosus*, was recently proposed based on a multiplex PCR assay targeting the *mutL* gene (Bottari et al., 2017). Its application on one strains chosen from each branch of the above mentioned cluster allowed to identify six *L. paracasei* (DTA72, DTA76, DTA81, DTA83, DTA93 and DTA96) and two *L. rhamnosus* (DTA79 and DTA105) (Fig. S1). According to this approach, three strains, namely DTA72 DTA76 and DTA105 were reclassified with respect to their initial attribution. Strain DTA106, that did not give amplification bands, was excluded from this study since it does not belong to the *Lactobacillus* "*casei*" group.

3.2. Hemolytic activity test

According to the European Food Safety Authority (EFSA), the assessment of hemolytic activity is strongly recommended for bacteria intended for food use, even if they are known to be safe or possessing the GRAS or QPS status (WHO/FAO, 2002). In this study, all strains were ɣ-hemolytic, i.e. negative, and none of them showed β-hemolytic activity when grown on MRS agar containing 5% (w/v) sheep blood. *Staphylococcus aureus* ATCC 6538, inserted as positive control, showed β-hemolytic activity.

3.3. Determination of minimum inhibitory concentration (MIC)

To be considered for food use, bacteria should not contain antibiotic resistance genes that could be horizontally transferred to other bacteria, particularly to human pathogens (Ashraf & Shah, 2011). For this reason, the antimicrobial susceptibility tests have become one of the most important assessments needed to evaluate potential probiotic bacteria (Tarrah, Treu, et al., 2018). Although acquired antibiotic resistance is an undesirable characteristic, intrinsic (i.e. non transmissible) resistance could contrary be considered favorable, due to the capability of the probiotic bacteria of withstanding antibiotic treatments on their human host (Charteris, Kelly, Morelli, & Collins, 1998). The results of the Minimum Inhibitory Concentration test on the *Lactobacillus* strains are reported in Table 1. All strains were resistant to kanamycin (K) and vancomycin (VA), while all showed susceptibility to penicillin G ampicillin (AMP), erythromycin (E), tetracycline (TE), ciprofloxacin (CIP) and gentamycin (CN), according to the cut-off values reported by EFSA (EFSA, 2008). Several lactobacilli often display a range of antibiotic resistance but in most cases, this is not transmissible and therefore does not usually represent a safety concern (Saarela, Lähteenmäki, Crittenden, Salminen, & Mattila-Sandholm, 2002). Several species of *Lactobacillus* including *L. paracasei* and *L. rhamnosus* are intrinsically resistant to vancomycin and kanamycin (Blandino, Milazzo, & Fazio, 2008). Resistance towards kanamycin and streptomycin in *L. delbrueckii* is conferred by the occurrence of the *aph*(3′)-IIIa and *ant*(6) genes (Devirgiliis, Zinno, & Perozzi, 2013). In addition, resistance against inhibitors of nucleic acid synthesis, such as trimethoprim was reported to be intrinsic (Ammor, Belén Flórez, & Mayo, 2007). On the other hand, lactobacilli are usually sensitive to inhibitors of protein synthesis such as erythromycin, and tetracycline (Ammor et al., 2007; Coppola et al., 2005; Danielsen & Wind, 2003; Halami, Chandrashekar, & Nand, 2000). According to our Minimum Inhibitory Concentration test results, the resistances found could therefore be considered as natural (intrinsic resistance) and hence do not pose a safety issue on the use of these strains.

Table1
Minimum Inhibitory Concentration (MIC) of 8 antibiotics on *Lactobacillus* strains (μg/mL).

Antibiotic	Strain								
	DTA72	DTA76	DTA79	DTA81	DTA83	DTA93	DTA96	DTA105	GG
Ampicillin	4 (4)	2 (4)	4 (4)	4 (4)	4 (4)	2 (4)	2 (4)	4 (4)	4 (4)
Ciprofloxacin	4 (4)[a]	2 (4)[a]	2 (4)[a]	4 (4)[a]	2 (4)[a]	4 (4)[a]	4 (4)[a]	2 (4)[a]	2 (4)[a]
Erythromycin	0.125 (1)	0.0625 (1)	0.125 (1)	0.125 (1)	0.0625 (1)	0.0625 (1)	0.125 (1)	0.125 (1)	0.125 (1)
Gentamycin	8 (32)	8 (32)	8 (16)	8 (32)	8 (32)	8 (32)	8 (32)	8 (16)	8 (16)
Kanamycin	*> 64 (64)*	*> 64 (64)*	*> 64 (64)*	*> 64 (64)*	*> 64 (64)*	*> 64 (64)*	*> 64 (64)*	*> 64 (64)*	*> 64 (64)*
Penicillin G	1 (4)[a]	0.5 (4)[a]	1 (4)[a]	0.5 (4)[a]	1 (4)[a]	1 (4)[a]	1 (4)[a]	0.5 (4)[a]	0.5 (4)[a]
Tetracycline	1 (4)	1 (4)	1 (8)	1 (4)	1 (4)	1 (4)	1 (4)	1 (8)	1 (8)
Vancomycin	*> 64 (2)*	*> 64 (2)*	*> 64 (2)*	*> 64 (2)*	*> 64 (2)*	*> 64 (2)*	*> 64 (2)*	*> 64 (2)*	*> 64 (2)*

EFSA suggested breakpoints (μg/mL) for each LAB strain are reported in parentheses. Strains with MICs higher than breakpoints are considered resistant and indicated in italics.

[a] According to (Danielsen & Wind, 2003).

3.4. Resistance to simulated gastrointestinal conditions

Resistance to the gastrointestinal juices during passage through stomach and intestine is the key factor for the probiotic strains to reach the gut and benefit the host (Bezkorovainy, 2001). Several studies have assessed the resistance of different species of *Lactobacillus* to the gastrointestinal conditions (Charteris et al., 1998; Fernández, Boris, & Barbes, 2003). The pH in the human stomach can vary from 1.3 to 2.5 during fasting and can increase up to 4.5 soon after a meal (Kong & Singh, 2008). Since the survival of lactobacilli at pH 4.2–4.4, which represents the common value of many fermented dairy products, is well known, pH 2 was selected to evaluate their ability to withstand gastric juice. Several studies reported survival of different *Lactobacillus* strains at that pH (Argyri et al., 2013). In our study, this evaluation was performed in two successive steps. First, the strains were incubated in artificial gastric juice for 60 min and then immediately transferred into the intestinal juice for 180 min (short incubation) followed by further 120 min (prolonged incubation, total 300 min). Cell concentration of bacterial cultures ranged from 8.7 to 9.8 log CFU/mL. All strains exhibited a very good resistance to gastric juice (Fig. 1), as viability decrement was always below 1 log and strains DTA72 and DTA83 showed the lowest viability loss together with the commercial strain GG. Our results on viability of *Lactobacillus* strains in the presence of mucin and pepsin at pH 2 are comparable with data available in the literature (Charteris et al., 1998; Fernández et al., 2003). Besides, considering that most probiotics are used in milk-based products, it is worth mentioning that some studies evidenced how milk proteins can play a significant role in protecting these bacteria (Charteris et al., 1998; Conway, Gorbach, & Goldin, 1987; Fernández et al., 2003).

Regarding incubation in gastro-intestinal juice, after 180 min all strains evidenced a statistically significant reduction, with the sole exception of DTA79, which was interestingly did not show any significant decrease after 1 h of gastric incubation followed by 3 h in gastrointestinal juice.

After prolonged incubation (300 min) in intestinal juice, strains showed a further significant decrease in viability, with the exception of strains DTA96 and DTA 105 that maintained the same level of viability shown after the short incubation.

Although tolerance to intestinal juice is considered to be strain dependent, it has been reported that lactobacilli can progressively adapt to the presence of bile salts and other components of the intestinal juice (Burns et al., 2010; Noriega, Gueimonde, Sánchez, Margolles, & de los Reyes-Gavilán, 2004) due to global cellular responses such as preservation of internal pH, cell membrane integrity/functionality and activation of bile salt efflux pumps (Bustos, Raya, de Valdez, & Taranto, 2011; Wu, He, & Zhang, 2014; Wu, Zhang, Wang, Du, & Chen, 2012). Interestingly, our results showed that all strains had a stronger overall resistance with respect to the commercial strains. Indeed, considering that all cultures started from a cell concentration roughly around 10^9 cfu/mL, all newly isolated strains evidenced a decrease within 2 logs while total *L. rhamnosus* GG decrease was more than 3 logs. Although this strain has great resistance to the gastric juice, it appears to be much more sensitive to intestinal incubation with respect to the lactobacilli tested.

3.5. Bile salts hydrolytic activity

None of the tested *Lactobacillus* strains showed capability to hydrolyze bile salts when grown on MRS agar medium containing 0.5% taurodeoxycholic acid. The meaning of BSH activity in probiotic bacteria has been questioned during last years. Although BSH is somehow connected to the intestinal survival of bacteria and reduction of cholesterol in humans, there are several undesirable effects from de-conjugated bile salts that can induce serious problems to the human body, such as DNA damage, promote colon cancer, diarrhea or inflammation (Berr, Kullak-Ublick, Paumgartner, Munzing, & Hylemon, 1996; Mamianetti, Garrido, Carducci, & Cristina Vescina, 1999). Therefore, we do not consider this capability as a desirable feature for probiotic strains.

3.6. Adhesion to HT-29 cells

The ability to adhere to intestinal cells is another important property for probiotic bacteria to stably colonize the host gut. Results of adhesion test are reported in Table 2 and Fig. 2 shows some images of *Lactobacillus* strains adhesion to HT-29 colorectal cancer cells. Strains DTA93, DTA81 and DTA79 strongly adhered to HT-29 cells whereas strains DTA96 and DTA76 showed normal adhesive characteristic and strains DTA72, DTA83 and DTA105 were non-adhesive. The *in-vitro* adherence ability of probiotic bacteria to HT-29 cells line has been extensively used during the last years (Ahmad, Yap, Kofli, & Ghazali, 2018; Bernet, Brassart, Neeser, & Servin, 1994; Wang et al., 2008). Indeed, there are two different ways by which bacteria can interact with cell surfaces, i.e. specific or non-specific. The latter is a consequence of the physicochemical properties of the cell wall, especially its outer constituents (Schaer-Zammaretti & Ubbink, 2003) and depends on the hydrophobic properties of the surfaces and on the balance of electrostatic interactions (Boonaert & Rouxhet, 2000). Differently, specific adhesion is related to the recognition of a specific site or ligand by a receptor on the bacterial surface (Schaer-Zammaretti & Ubbink, 2003). Many lactobacilli exhibit specific interactions, and the colonization ability of epithelial cells of Lactobacillus strains has been reported (Bouzaine, Dauphin, Thonart, Urdaci, & Hamdi, 2005; Jin, Ho, Abdullah, Ali, & Jalaludin, 1996). In our study, three out of eight strains tested, namely DTA93, DTA81 and DTA79, exhibited strong adherence to HT-29 cells line and particularly *L. paracasei* DTA81 revealed a dramatically strong adherence ability, about ten times higher than that of the commercial strain *L. rhamnosus* GG, thus indicating it as a very

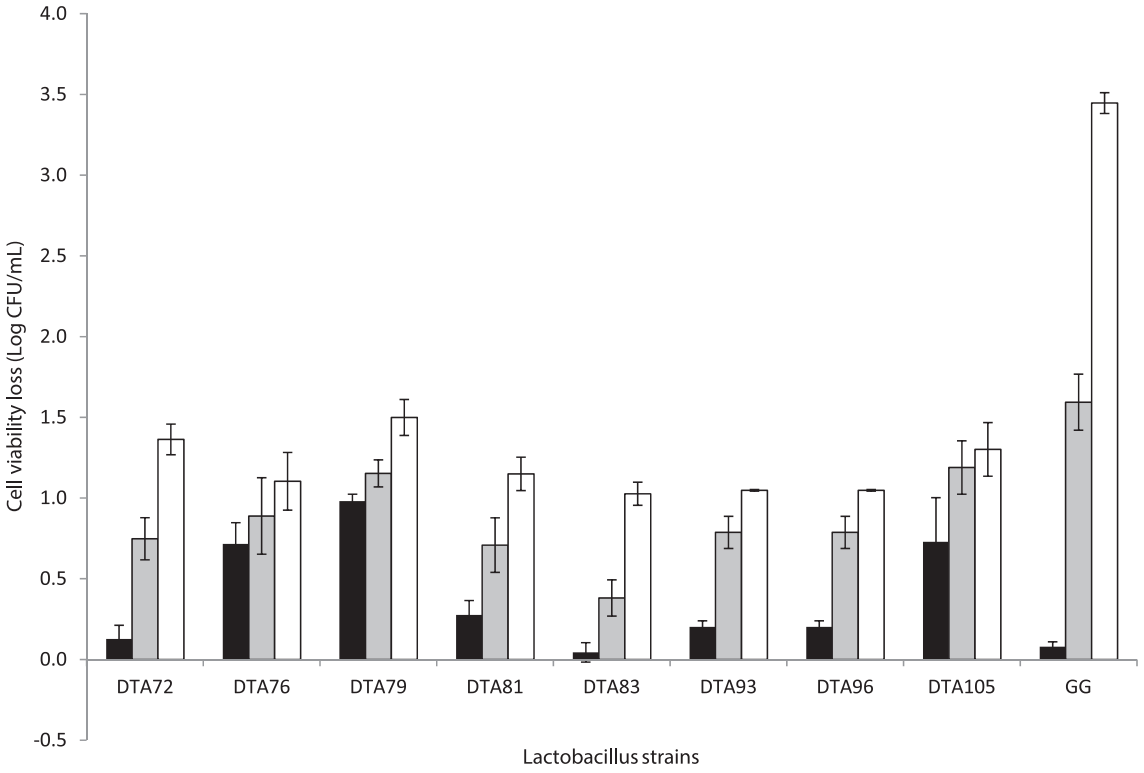

Fig. 1. Cell viability loss of *Lactobacillus* cultures upon exposure to simulated gastrointestinal conditions. Black bars: after 1 h incubation (gastric test); grey bars: after 3 h incubation (short gastrointestinal test); white bars: after 5 h incubation (prolonged gastrointestinal test). Results are expressed as means ± SD (n = 3) of viable cells.

Table 2
Adhesion potential of bacterial strains. Scores are the average number of adhering cells in 20 microscopic fields ± SD (n = 3).

Strain	Adhesion score	Category
L. paracasei DTA72	25.5 ± 2.3	Non-adhesive
L. paracasei DTA76	46.4 ± 4.02	Adhesive
L. rhamnosus DTA79	359.1 ± 7.2	Strongly adhesive
L. paracasei DTA81	4044.0 ± 10.2	Extremely adhesive
L. paracasei DTA83	20.6 ± 1.9	Non-adhesive
L. paracasei DTA93	294.5 ± 5.2	Strongly adhesive
L. paracasei DTA96	41.1 ± 2.7	Adhesive
L. rhamnosus DTA105	28.6 ± 3.5	Non-adhesive
L. rhamnosus GG	420.8 ± 8.1	Strongly adhesive

interesting probiotic candidate.

3.7. Anti-proliferative activity against HT-29 cells

Colorectal cancer is a disease mostly determined by Caco-2 and HT-29 cells that can cause death widely in the world. Strains *L. paracasei* DTA93, DTA96, DTA81 and *L. rhamnosus* DTA79 which had showed good adhesive activity to HT-29 cells and indicated good probiotic potential were tested for anti-proliferative activity. Results (Table 3) indicate that HT-29 cancer cells were significantly inhibited by some lactobacilli supernatants. No significant difference ($P < 0.05$) was found between *L. paracasei* DTA93 and the commercial strain *L. rhamnosus* GG when examined with multiple comparison tests (Tukey's test). The remaining strains, apart from *L. paracasei* DTA96, showed anti-

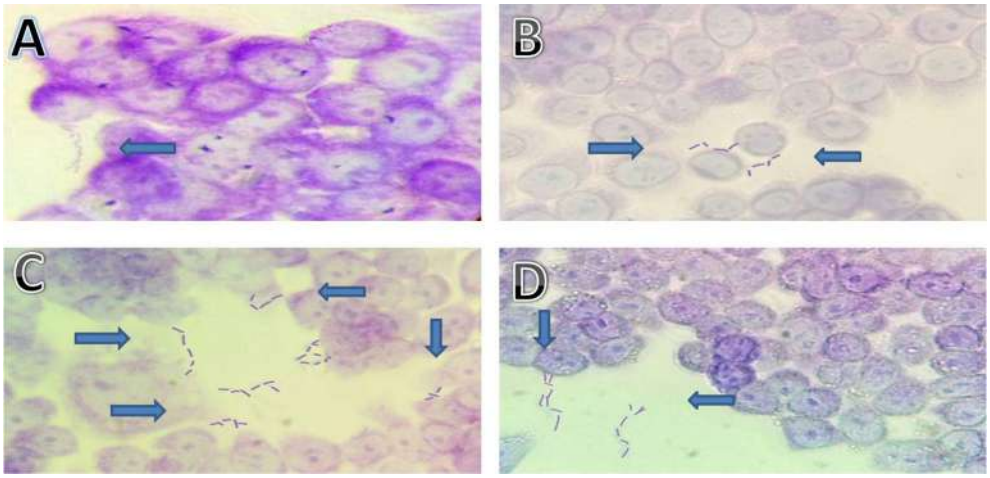

Fig. 2. Adhesion of *Lactobacillus* strains to HT-29 cells observed under optical microscope (1000X). Strains: (A) GG, (B) DTA79, (C) DTA81, (D) DTA93.

Table 3
Antiproliferative effect of lyophilized supernatants collected from lactobacilli cultures on HT-29 cancer cells after 48 h.

Supernatant concentration (μg/mL)	HT-29 cells viability (%)					
	DTA79	DTA81	DTA93	DTA96	GG	MRS medium
125	97.48 ± 0.28	97.81 ± 0.03	70.20 ± 0.01	98.2 ± 0.05	65.8 ± 0.01	90.27 ± 0.10
250	74.94 ± 0.12	97.51 ± 0.05	63.00 ± 0.03	86.9 ± 0.06	63.9 ± 0.09	91.67 ± 0.03
500	65.56 ± 0.07	93.10 ± 0.11	59.98 ± 0.07	88.3 ± 0.11	58.9 ± 0.02	90.03 ± 0.05
1000	57.33 ± 0.05	53.27 ± 0.07	47.42 ± 0.01	84.7 ± 0.06	54.1 ± 0.02	89.47 ± 0.28
2000	46.64 ± 0.03	39.42 ± 0.05	44.79 ± 0.10	89.2 ± 0.05	48.2 ± 0.02	88.61 ± 0.19
4000	37.78 ± 0.00	34.09 ± 0.03	40.48 ± 0.01	90.5 ± 0.05	41.1 ± 0.03	82.63 ± 0.20
8000	31.27 ± 0.07	21.48 ± 0.02	28.56 ± 0.02	88.8 ± 0.24	29.1 ± 0.02	89.44 ± 0.06

Table 4
IC_{50} of probiotic strains against HT-29 cell line (All values are mean ± SD of 2 experiments).

Strains	IC_{50} (mg/mL)[a]
L. rhamnosus DTA79	1.96 ± 0.20
L. paracasei DTA81	1.40 ± 0.25
L. paracasei DTA93	1.30 ± 0.13
L. rhamnosus GG	1.42 ± 0.12

[a] IC_{50}: half minimal inhibitory concentration.

proliferative activity. However, the lowest cell viability was observed in HT-29 cells treated with *L. paracasei* DTA81 supernatant, i.e. 39.4% ± 0.05, 34.1% ± 0.03, 21.5% ± 0.02 at concentrations of 2000, 4000, 8000 μg/mL respectively (Table 3). By considering the half minimal inhibitory concentration (IC_{50}) value related to the strain supernatants (Table 4) it appears that there is no significant difference between *L. paracasei* DTA93, *L. paracasei* DTA81 and the commercial strain *L. rhamnosus* GG. Many lactobacilli, such as *L. acidophilus* and *L. casei* have been reported to possess anti-cancer effects by suppressing the duplication of tumor cells (Lee et al., 2004). Regarding the possible mechanisms of action, in a recent study (Haghshenas et al., 2014) demonstrated that metabolites such as bioactive peptides in the supernatant of several species of lactobacilli could behave as anti-cancer agents, as they can play a crucial role in cytotoxicity by linking to pre-carcinogenic molecules, carcinogenic enzymes or mutagenic compounds or by exerting some immunomodulatory effects (Bermudez-Brito et al., 2012). Moreover, some *L. paracasei* and *L. casei* strains showed effective anticancer activity against cervix cancer (HeLa) cells by upregulating the expression of apoptotic genes *BAX, BAD, caspase3, caspase8*, and *caspase9* and by downregulating the expression of the *BCl-2* gene (Shahid et al., 2018), while other *L. casei* induced up-regulation of TRAIL protein expression (Tiptiri-Kourpeti et al., 2016), known to selectively induce apoptosis in many tumor cell lines without affecting normal cells and tissues, thus appearing as a promising therapeutic drug (Galligan et al., 2005). In another study (Ewaschuk, Walker, Diaz, & Madsen, 2006), the production of Conjugated Linoleic Acid (CLA) as anti-carcinogenic component by different species lactobacilli on HT-29 cell line was demonstrated. Anti-carcinogenic effects of CLA have been also proved by *in-vivo* studies on animals (Liew, Schut, Chin, Pariza, & Dashwood, 1995; Petrik, McEntee, Johnson, Obukowicz, & Whelan, 2000). According to our result, it can be concluded that *L. paracasei* DTA93 and DTA81 which had shown exceptionally good adhesion ability, can be considered as effective strains in anti-proliferative activity against HT-29 cell. Further studies will be needed to understand the mechanics through which the strains exert their activity.

3.8. Inhibition of biofilm formation

The inhibitory activity of the newly isolated *Lactobacillus* strains against biofilm formation by *L. innocua* and *E. coli* was tested by two different approaches. The first strain was chosen because it is physiologically very close to the pathogen *L. monocytogenes*, whose strong ability to form biofilm is well documented and represents a serious problem for surfaces and industrial settings (Klančnik, Toplak, Kovač, Marquis, & Jeršek, 2015). *E. coli* is the most present bacterium in the small intestine and therefore the possibility to displace it represents for a strain a good potentiality to establish in the gut. Fig. 3 shows the results of the biofilm inhibitory activity obtained by inoculating simultaneously one *Lactobacillus* strain together with either *E. coli* (Fig. 3A) or *L. innocua* (Fig. 3B) (competition test) and by inoculating a *Lactobacillus* strain first and subsequently either *E. coli* (Fig. 3A) or *L. innocua* (Fig. 3B) (exclusion test). In the competition assay, all lactobacilli were able to reduce the number of attached *E. coli* and *L. innocua* cells to different extent, but the co-culture with *L. paracasei* DTA81 and DTA93 showed the highest inhibitory effects on both *E. coli* (cells reduction of 0.78 and 0.65 log, respectively) and *L. innocua* (cells reduction of 0.29 and 0.42 log, respectively), comparable or better to the commercial strain *L. rhamnosus* GG (0.59 log on *E. coli* and 0.040 log in *L. innocua*). Similar outcomes were evidenced by the exclusion test where strains DTA81 and DTA93 still evidenced the best inhibition ability (cells reduction of 1.05 and 0.80 log, respectively on *E. coli* and 0.58 and 0.60 log, respectively on *L. innocua*) that resulted higher than that of strain GG on *E. coli* (0.73 log) and slightly lower on *L. innocua* (0.85). Probably, the inhibitory effect of DTA81 and DTA93 can be linked to their strong attachment capability shown in the HT-29 adhesion test. For all lactobacilli, the exclusion effect was always equal or stronger to the respective competition one, with the sole exception of *L. paracasei* DTA83 that clearly inhibited *E. coli* during the competition test (0.52 log decrease) but produced a negligible exclusion effect (0.03 log). A similar behavior was evidenced by DTA83 on another *E. coli* strain, namely ATCC25922 (Guerra et al., 2018), thus allowing to hypothesize that this inhibitory activity could be related to some strain specific antimicrobial effect, such as production of a bacteriocin, rather than to biofilm activity. This idea is also reinforced by the fact that the same effect was not obtained on *L. innocua* and that *L. paracasei* DTA83 showed the worst performance to the adhesion to HT-29 cells test.

4. Conclusions

The results of the present study indicate that the two newly isolated strains *L. paracasei* DTA81 and DTA93 were found to possess *in-vitro* probiotic properties and anti-cancer activity. Some traits resulted very close to and in some cases superior to those of the widespread commercial probiotic strains *L. rhamnosus* GG that we used as reference. Strain *L. paracasei* DTA81 showed an amazingly high adherence ability to HT-29 cells line which resulted about ten times stronger than that of the commercial strain *L. rhamnosus* GG and, to our knowledge, represents the highest level reported to date for this type of cells. Besides, both *L. paracasei* DTA81 and *L. paracasei* DTA93 were able to effectively inhibit biofilm formation by other bacteria. Therefore, further investigation, including *in-vivo* studies, is strongly advisable to evaluate the potential health benefits in the real host. Overall, our study

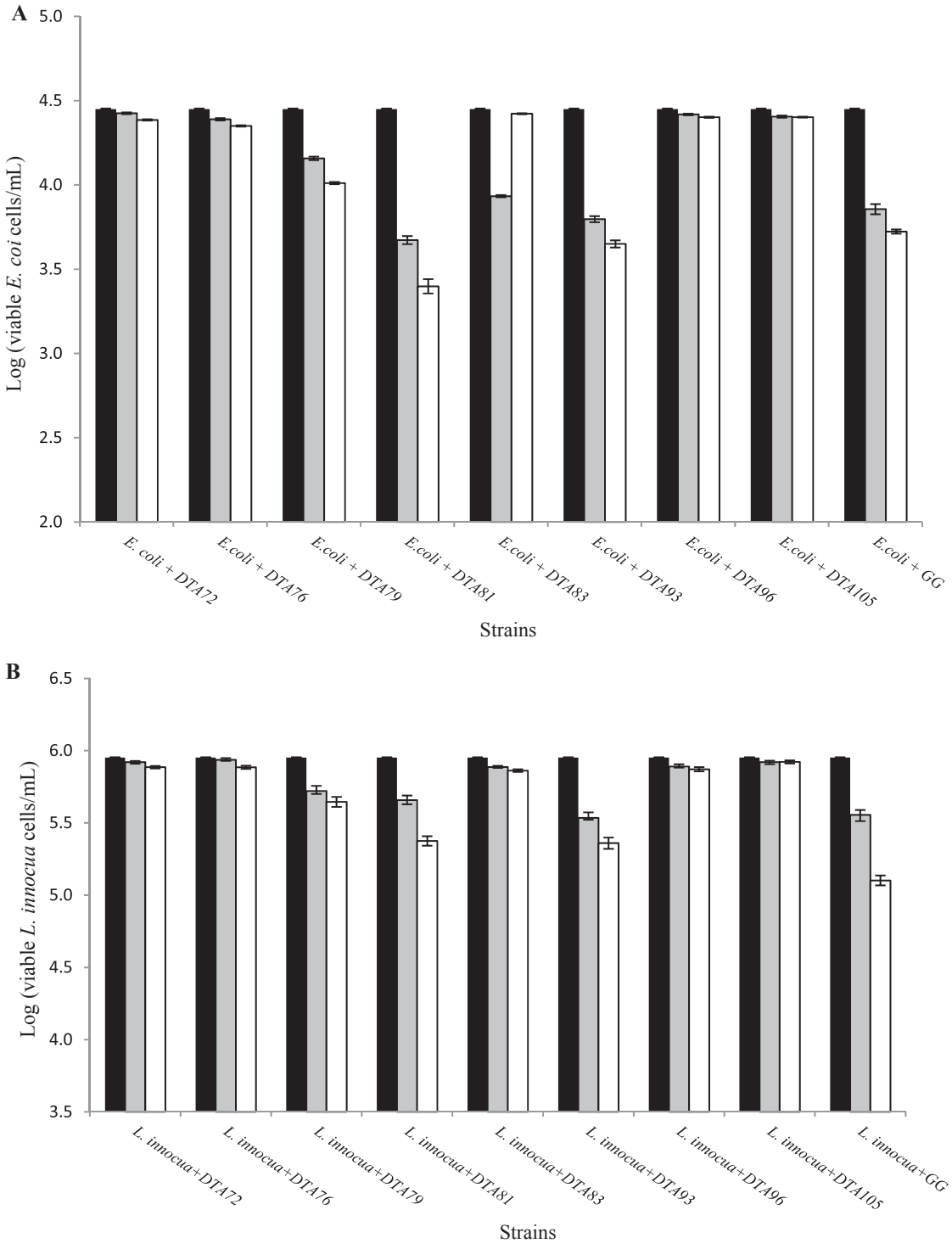

Fig. 3. Biofilm inhibitory activity of *Lactobacillus* strains against *E. coli* (A) and *L. innocua* (B) in competition and exclusion tests. Results are express as mean ± SD (n = 3) of *E. coli* and *L. innocua* viable cells. Black bars: *E. coli* and *L. innocua* population alone; grey bars: *E. coli* and *L. innocua* after competition test; white bars: *E. coli* and *L. innocua* after exclusion test.

confirms what is demonstrated in many other studies regarding the potential of the genus *Lactobacillus* as a very interesting source for the discovery of new health beneficial microbes.

Ethics statement

Our research did not include any human subjects and animal experiments.

Conflict of interest statement

All authors declare there are no conflicts of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2019.02.004.

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Introduction: Maternal supplementation with *Lactobacillus paracasei* DTA 83 alters emotional behavior in Swiss mice Offspring

The concept of fetal programming, introduced by epidemiologist David Barker, underscores how disruptions in homeostasis during fetal development can lead to long-term health implications in adulthood. Among the critical environmental factors influencing fetal health, maternal microbiota has emerged as pivotal.

Historically, it was thought that the fetus develops in a sterile environment, but recent evidence challenges this belief, indicating microbial colonization starts prenatally. Microbes have been isolated from placental, umbilical cord, and amniotic fluid samples, highlighting birth as the initial exposure opportunity for microbial colonization. The mode of delivery significantly influences the infant microbiota: vaginal births expose newborns to microbes resembling maternal vaginal flora, whereas cesarean sections result in skin flora dominance. Such early microbial exposures have enduring impacts on newborn health outcomes.

Probiotics, live microorganisms beneficial when consumed in adequate quantities, have gained attention for their preventive effects against conditions like pre-eclampsia, gestational diabetes, and infections during pregnancy. Maternal probiotic supplementation has also shown promise in preventing childhood eczema, food allergies, asthma, and gastrointestinal infections. Beyond physical health, emerging research suggests a link between microbiota and psychiatric disorders such as anxiety and depression. While certain probiotics, particularly from *Lactobacillus* and *Bifidobacterium* genera, mitigate stress responses and reduce anxiety and depressive symptoms, their impact on behavioral development due to maternal supplementation remains understudied.

This study aims to evaluate whether maternal supplementation with *Lactobacillus paracasei* DTA 83 during pregnancy and lactation alters neurobehavioral parameters in mouse offspring. Animals were housed under controlled conditions with standard diets and lighting cycles. Offspring were assessed for neurobehavioral changes at postnatal day 70, and hippocampal tissues were analyzed for gene expression profiles related to the GABAergic system and other neurochemical targets.

Lactobacillus paracasei DTA 83, isolated from a fecal sample of a two-week-old infant, has been characterized for its safety and probiotic properties. Maternal supplementation involved administering DTA 83 during pregnancy and lactation, with subsequent analysis focusing on its effects on offspring behavior and neurochemistry.

To our knowledge, this study represents the first investigation into the long-term neurobehavioral effects of maternal lactobacilli supplementation in healthy mouse offspring. Our findings suggest that this treatment modulates the transcriptional profile of the GABAergic system, resulting in behavioral alterations characterized by reduced anxiety in adult offspring. While microbial analysis of fetal tissues and milk was not conducted in this study, we hypothesize that probiotic cells could translocate to mammary glands and placenta during maternal supplementation, influenced by both vaginal delivery and nursing practices.

This research contributes to understanding the potential of maternal probiotic supplementation to influence offspring neurodevelopment, highlighting pathways for future investigation into microbiota-based interventions in maternal and child health.

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Maternal supplementation with *Lactobacillus paracasei* DTA 83 alters emotional behavior in Swiss mice offspring

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ABSTRACT

The objective of our study was to evaluate whether maternal supplementation with lactic acid bacteria is able to alter neurobehavioral parameters of Swiss mice offspring. For this purpose, female mice were treated during pregnancy and lactation with milk or milk inoculated with *L. paracasei* DTA 83 at a concentration of 10^7 cells/ml (*p.o*, daily). After the end of the treatment, 6 offspring per group were submitted to euthanasia and the hippocampus was dissected for RNA analysis. After completing 70 days of life, 12 male offspring per group were submitted to a battery of behavioral tests. According to our results, treated offspring had a reduction in the expression of GAD 65, GAD 67 and $GABA_A$ receptor α3 subunit in the hippocampus. In the adult phase, the offspring of the treated group had higher time in the central area in the open field test, lower latency to the light side in the light-dark box test and lower number of decision-making behaviors in the elevated plus maze test. These results indicate that maternal supplementation with *L. paracasei* DTA 83 modulates offspring hippocampal GABAergic system. Such changes could possibly be associated with the reduction of anxiety-like behavior upon reaching adulthood.

1. Introduction

According to studies published by epidemiologist David Barker, the prevalence of some diseases in adulthood presents a considerable correlation with homeostatic disruption still during the fetal phase. In fact, some insults could promote structural and functional changes in the fetus, which could subsequently lead to long-term disease. This process is known as fetal programming [1,2]. In this context, one of the main environmental factors that affect fetal health is the maternal microbiome [3].

Until recently, it was believed that the fetus was completely sterile of bacteria. However, new evidences have suggested that microbial colonization of the fetus begins even before birth [4]. Surprisingly, microorganisms were already isolated from the placenta, umbilical cord and amniotic fluids [5]. Birth is the first opportunity for microbial exposure outside the uterus. During this process, infant microbiota is strongly dependent on the delivery type. In vaginal deliveries, newborns are colonized by microorganisms that are very similar to maternal vaginal populations. On the other hand, skin bacteria colonization is more common in infants born by cesarean section [6]. This initial exposure to such distinct microbial populations has long-term consequences to newborn health [7].

Based on this assumption, probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host [8]. In this context, maternal probiotic supplementation during and after pregnancy prevents pre-eclampsia, gestational diabetes and vaginal infections [9]. Perinatal administration of probiotics also prevents eczema, food allergy, asthma, respiratory and intestinal tract

Abbreviations: GABA, gamma-aminobutyric acid; GAD, glutamic acid decarboxylase; 5-HT, serotonin; TPH2, tryptophan hydroxylase; TH, tyrosine hydroxylase; BDNF, brain-derived neurotrophic factor

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Table 1
List of primers used for qRT-PCR.

Gen Bank	Coded Protein	Function	Primers
GAD1 (NM_008077.5)	Glutamate decarboxylase 1 (GAD67)	GABA synthesis	F: 5'-CTCAGGCTGTATGTCAGATGTTC-3' R: 5'-AAGCGAGTCACAGAGATTGGTC-3'
GAD2 (NM_008078.2)	Glutamate Decarboxylase 2 (GAD65)	GABA synthesis	F: 5'-TCAACTAAGTCCCACCCTAAG-3' R: 5'-CCCTGTAGAGTCAATACCTGC-3'
Gabra 2 (NM_008066.3)	GABA A receptor α2 subunit	Binding site of the benzodiazepines	F: 5'-TTACAGTCCAAGCCG AATGTCC-3' R: 5'-ACTGGCCCAGCAAATCATACTG-3'
Gabra 3 (NM_008067.4)	GABA A receptor α3 subunit	Binding site of the benzodiazepines	F: 5'-CCGCACAGTCTTTGGTGTCA-3' R: 5'-GAAGAAGCACTGGGA GCAGC-3'
Gabra 5 (NM_176942.4)	GABA A receptor α5 subunit	Binding site of the benzodiazepines	F: CAATGGATGCCCATGCCTGT R: TATTCACCTGTGCTGGTGCTG
TPH2 (NM_173391.3)	Tryptophan hydroxylase (TPH2)	Serotonin synthesis	F: 5'-AGTCTACATCCATCCCAACTGCTG-3' R: 5'-CATTCCTCGCACAATTCCAGTCG-3'
Th (NM_009377.1)	Tyrosine hydroxylase (TH)	Dopamine synthesis	F: 5'-GTCAG GGAGCCCGAGGTC-3' R: 5'-CGCTGGATACGAGAG GCATAG-3'
Bdnf (NM_001048139.1)	Brain-derived neurotrophic factor (BDNF)	Neuroplasticiy	F: 5'-AGCAGAGTCCATTCAGCACC-3' R: 5'-TGGCTTGACAGCGAGGAAAA-3'
Ppia (NM_008907.1)	Cyclophilin A	Housekeeping (Internal Control)	F: 5'- GCCGATGACGAGCCCTTG-3' R: 5'- TGCCGCCAGTGCCATTATG-3'

infections [10–12].

In addition, some studies also support that the microbiome can participate to the physiopathology of various psychiatric disorders, such as: anxiety and depression [13]. Although probiotic bacteria, especially those from *Lactobacillus* and *Bifidobacterium*, have been demonstrated to prevent stress-related responses and to reduce anxiety and depressive symptoms [14], there are no reports of the impacts caused by maternal supplementation with lactobacilli on the behavioral development. Thus, the objective of our study was to evaluate whether maternal *Lactobacillus* treatment during pregnancy and lactation is able to alter neurobehavioral parameters of mice offspring.

2. Material and methods

2.1. Preparation of bacterial strain and administration to mice

L. paracasei DTA 83 was isolated on Lamvab agar from fecal sample of an infant of two weeks old. This strain was identified by amplifying and sequencing of the 16S rDNA region as described by Andrighetto et al. [15] and is part of the culture collection of the Laboratory of Microbiology, Department of Food Technology, Federal Rural University of Rio de Janeiro. The *L. paracasei* DTA 83 safety and probiotic properties were reported in an article recently published by our group [16].

The working culture was prepared by activation of the frozen stock culture through three successive daily growth in MRS broth (Himedia, Mumbai, India) at 36 °C. At the last sub culture, the tubes were centrifuged at 6000*g* for 5 min. Then, the supernatant was discarded, and the cell pellet was washed twice before re-suspending in phosphate buffer pH 7.2 and inoculating in 12% reconstituted skimmed milk (sterilized at 110 °C for 10 min) to contain 10^7 cfu/ml.

2.2. Experimental design

Swiss Webster mice with 60 days of age (˜35 g) derived from Federal Rural University of Rio de Janeiro colony were used in this protocol. After acclimatization period of 15 days, the mice were housed in plastic cages (35 cm × 50 cm × 20 cm) and were mated with a ratio of two females to one male. Day 1 of pregnancy was determined by the presence of spermatozoa in the vaginal smear. Following confirmation that mating had occurred, females were randomly assigned into two groups (n = 6 per group): control and *Lactobacillus*-treated groups. Each group was administered daily 100 µl of either milk (control) or 10^7 CFU/ml of *L. paracasei* DTA 83 by oral gavage during all pregnancy and lactation. Therefore, the delivered dose was 10^6 CFU/day/mouse.

All animals were housed at controlled temperature (20 ± 2 °C) with daily exposure to a 12 h light-dark cycle and free access to water and standard rodent chow. After birth, the male mice were removed and the obtained offspring were standardized in maximum of 8 animals (4 males and 4 females) per litter. The offspring was weaned at postnatal day (PND) 21. At PND 23, 6 animals per group (3 males and 3 females) were euthanized and the hippocampus were dissected from the hole brain under cold plate and kept at −70 °C to RNA analysis. Finally, 12 male offspring per group were submitted to the behavioral analysis at PND 70.

2.3. Ethics committee

This investigation was carried out according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996) and was approved by the institutional animal welfare committee in consonance with pertinent Brazilian legislation, under protocol number: 23083.014791/2018-15.

2.4. RNA analysis

Total RNA was extracted using a standard method (TRIzol reagent; Invitrogen, Carlsbad, CA, USA). The RT-PCR analyses were carried out from 1 µg of total RNA extracted from hippocampus of 23 PND male puppies using the Superscript III kit (Invitrogen).

Real-time RT-PCR analyses were performed in a fluorescent temperature cycler (Applied Biosystems 7500; Life Technologies Co., Carlsbad, CA, USA) according to the recommendations of the manufacturer. Briefly, after initial incubation at 50∘C for 2 min and 95∘C for 10 min, reactions were cycled 40 times using the following parameters for all genes studied: 95∘C for 15 s, 60∘C for 30 s and 72∘C for 45 s. SYBR Green (Applied BioSystems, Foster City, CA, USA) fluorescence was detected at the end of each cycle to monitor the amount of PCR product formed during that cycle. We used genes that coded proteins related to GABAergic system (Gad 65, Gad 67, GABA α3 and γ2 subunits), serotonergic system (Tph2), dopaminergic system (Th) and neuroplasticity (Bdnf). Primers used for the amplification of cDNAs of interest were synthesized by Extend Biotecnologia Ltda. The forward and reverse primers sequences were listed in Table 1.

We determined relative mRNA levels ($2^{-\Delta\Delta Ct}$) by comparing the PCR cycle threshold (Ct) between groups, after correcting for the internal control cyclophilin A2 [17]. Assays were repeated two or three times and the data were merged after normalization.

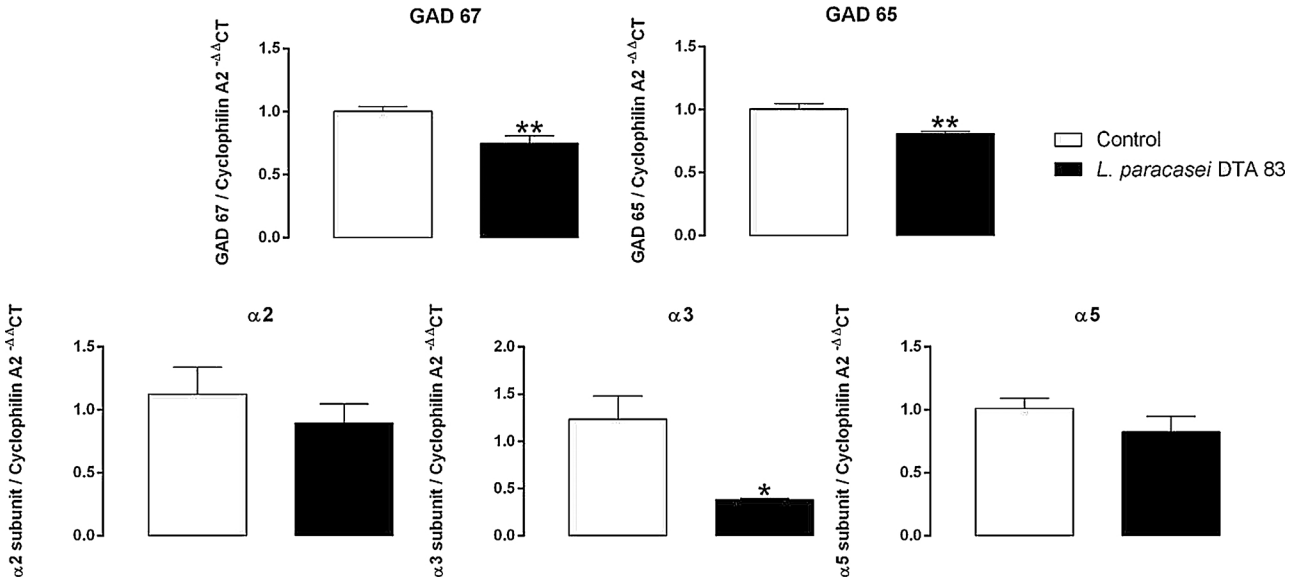

Fig. 1. Hippocampal gene expression of GAD 65, GAD 67, α2, α3 and α5 subunits of $GABA_A$ receptor in mice with PND 23, whose mothers were treated with milk (control) or milk inoculated with *L. paracasei* DTA 83. In this evaluation, significant differences were observed in the expression of GAD 65, GAD 67 and α 3 subunit of GABA A receptor. * represents $p < 0.05$; ** and $p < 0.01$. N = 6.

2.5. Behavioral tests

Between 70–80 days of age, the puppies underwent to a battery tests consisted of the following tests: open field, light-dark box, elevated plus maze and tail suspension test. The tests were performed with an interval of one day and the tests' order within the battery was determined according to the progressive invasiveness degree. All behavioral testswas performed between 7 and 10 a.m. During each test, the experimenter remained outside the testing room except between trials. Each test was recorded and behavior parameters were analyzed by at least two observers.

2.5.1. Open field test

Each mouse was placed individually in the center of a white acrylic cage (30 cm x 30 cm x 15 cm) and it was allowed to explore the cage for five minutes. During this time, total distance travelled, number of rearings, (standing on hind legs with paws pressed against the wall of the arena) episodes and time of grooming, time in center zone, center distance (the distance traveled in the center of the arena), time in corner zone, inactivity time and center ratio (center distance : total distance ratio) were assessed. At the end of testing, the number of fecal boli was also measured and the arena was cleaned with a 10% ethanol solution.

2.5.2. Light-dark box test

The animals were placed individually in an acrylic cage (45 cm x 27 cm x 27 cm) unequally divided into two chambers by a black partition containing a small opening. Two thirds of this chamber is illuminated (400 lx) and the remainder section is closed and dark. Mice were placed into the dark side and they were allowed to move freely between the two chambers for 5 min. During this time, the time spent in light side, number of transitions, attempts, rearings and latency to first entry in light side were verified.

2.5.3. Elevated plus maze test

The apparatus consisting of four arms (30 cm × 5 cm) were placed 50 cm above the floor. Two opposite arms were delimited by acrylic vertical walls, whereas the other two, opposite arms had unprotected edges (open arms). Mice were placed in the center of maze and allowed to move freely for 5 min. During this period, the cumulative time and frequency of entries into open and closed arms were registered. An arm entry was defined as the entry of four paws into an arm. Then, the percentage of entries in opens arms, percentage of time in open arms and time in central platform were calculated.

2.5.4. Tail suspension test

In this protocol, the mice were suspended 100 cm above the stand by adhesive tape placed approximately 1 cm from the tip of the tail. The test was videotaped for five minutes. During this period, time of immobility and latency to the first immobility episode were evaluated.

2.6. Statistical analysis

All results are presented as means ± SEM. The assumption of normal data distribution was assessed with the Shapiro-Wilk test. Parametric comparisons were performed when the data passed the normality test. In this case, comparisons between groups were analyzed with Student's unpaired-test. In the other hand, the Mann-Whitney test was used to compare data without normal distribution. Grubbs test was used for detecting outliers. Cohen's d analysis was used to evaluate the effect sizes between the groups, which is the difference between means divided by standard deviation. In this measure, effect sizes were interpreted as small ($0.2 < d < 0.5$), moderate ($0.5 < d < 0.8$) and large ($d > 0.8$). Differences were considered statistically significant when values $P < 0.05$. GraphPad Prism 5 statistical software (La Jolla, CA) was used for all statistical analysis.

3. Results

3.1. RNA analysis

Regarding GABAergic system (Fig. 1), it was verified that maternal supplementation with L. *paracasei* DTA 83 during pregnancy and lactation downregulated hippocampal genes which encode the GAD 65 (0.81 ± 0.1 vs. 1.00 ± 0.04, p = 0.006) and GAD 67 (0.75 ± 0.05 vs. 1.00 ± 0.03, p = 0.001) enzymes, as well as of $GABA_A$ receptor α3 subunit (0.38 ± 0.01 vs. 1.23 ± 0.24, p = 0.009) in 23 PND offspring. There were no significant transcriptional differences in α2 (p = 0.42) and α5 (p = 0.22) $GABA_A$ receptor subunits. In relation to the other neurochemical targets (Fig. 2), it was also not observed

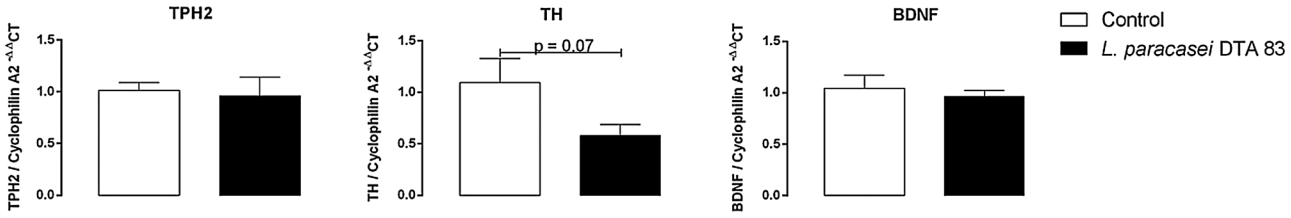

Fig. 2. Hippocampal gene expression of TPH2, TH, BDNF in 23 DPN mice, whose mothers were treated with milk (control) or milk inoculated with *L. paracasei* DTA 83. No significant differences were observed in the expression of such genes. N = 6.

Table 2
Calculation of Cohen's *d* effect size between groups for RNA analysis.

Coded Protein	Cohen's *d*
GAD 65	**2.61**
GAD 67	**2.30**
$GABA_A$ α2 subunit	*0.61*
$GABA_A$ α3 subunit	**2.01**
$GABA_A$ α5 subunit	*0.75*
TPH2	0.15
TH	**1.14**
BDNF	0.33

Numbers in italic represent medium magnitude of the effect, whereas bold numbers represent large magnitude of the effect.

differences in TPH2 (p = 0.79), BDNF (p = 0.61) expression, although it has been demonstrated a trend to TH downregulation (p = 0.07).

In Table 2, according to Cohen's d analysis, maternal treatment with *L. paracasei* DTA 83 promotes a huge effect in the GAD 65 (d = 2.61), GAD 67 (d = 2.30), α3 $GABA_A$ receptor subunit (d = 2.01) and TH (d = 1.14). Medium magnitude of the effect was verified in expression of α2 (d = 0.61) and α5 (d = 0.75) $GABA_A$ receptor subunits. A weak effect of the treatment was observed in TPH2 (d = 0.15) and BDNF (d = 0.33) expression.

3.2. Behavioral assessment

In the open field test (Fig. 3), it was observed that the animals whose dams were submitted to *Lactobacillus* supplementation had an increase in the number of central quadrants crossed (35.0 ± 2.65 vs. 24.9 ± 3.93, p = 0.04), higher central ratio (0.34 ± 0.03 vs. 0.24 ± 0.02, p = 0.02) and increased time in the central area (56.5 ± 7.00 s vs. 35.2 ± 6.71 s, p = 0.04) than control group subjects. The increased of these parameters is associated with anxiolysis. In addition, we observed an increase in the number of fecal pellets in the animals of the treated group, which may indicate a gastrointestinal motility change (5.27 ± 0.33 vs. 3.90 ± 0.51, p = 0.03). There was also a tendency to reduce grooming time (p = 0.06).

In the light-dark box (Fig. 4), maternal supplementation with *Lactobacillus*-inoculated milk promoted significant differences in latency to light side (19.9 ± 2.89 s vs. 67.8 ± 17.4 s, p = 0.01). In the elevated plus maze test (Fig. 5), treated animals had a lower SAP number (24.8 ± 1.40 s vs. 34.5 ± 3.36, p = 0.02) when compared to the control group. We also observed a tendency to increase time (p = 0.08) and percentage of entries in the opened arms (p = 0.07). Again, such behavior profile indicates that treatment promotes a reduction in anxiety-like behavior. No difference was observed in tail suspension test (Fig. 6).

In Table 3, according to Cohen's d analysis, maternal supplementation with *Lactobacillus*-inoculated milk induced a strong effect on some behavioral parameters, such as: the number of central quadrants crossed (d = 0.90) center ratio (d = 1.07), time in the center area (d = 0.93), time of grooming (d = 0.89) and fecal pellets (d = 0.95) in open field test; latency to light in light-dark box test (d = 1.21); entries in closed arms (d = 1.2), time in opened arms (d = 0.81) and in the percentage of entries in opened arms (d = 0.84). In the tail suspension, medium magnitude of the effect was observed in the latency to immobility (d = 0.68). Similarly, in the light-dark box test, a medium effect was also verified in attempts.

4. Discussion

As far as we know, this is the first work which investigated the neurobehavioral impacts of maternal lactobacilli supplementation during pregnancy and lactation in healthy mice offspring. According our results, this treatment modified GABAergic system transcriptional profile, promoting long-term behavioral alterations in adult offspring, characterized by low anxiety. Although we have not performed the microbiological analysis of fetal tissues and milk, we strongly believe

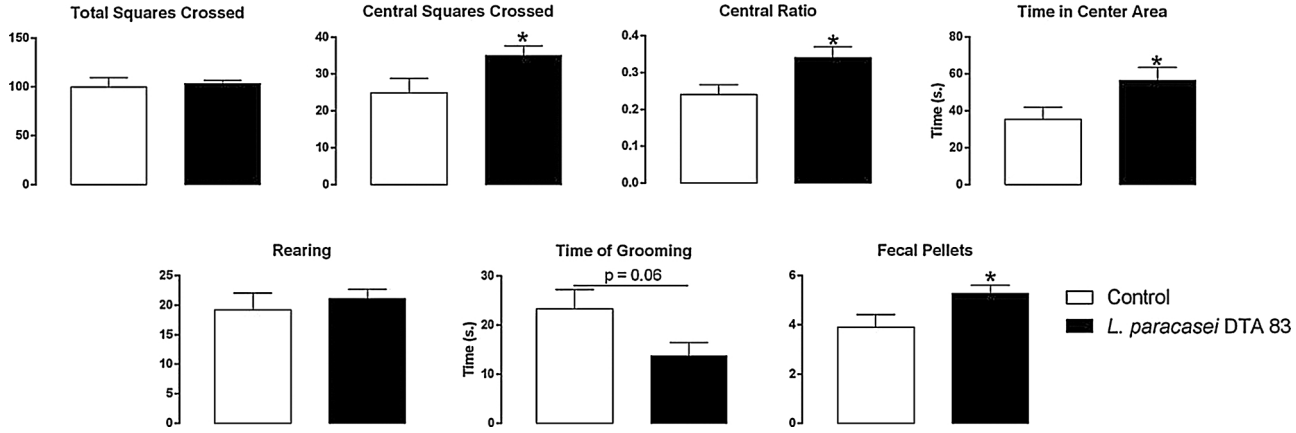

Fig. 3. Evaluation of exploratory activity and anxiety-like behavior in mice with PND 70 whose mothers were treated with milk (control) or milk inoculated with *L. paracasei* DTA 83 through the open field test. In this protocol, it was demonstrated that the treated group had reduction of anxiety-like behavior. * represents p < 005. N = 12 per group.

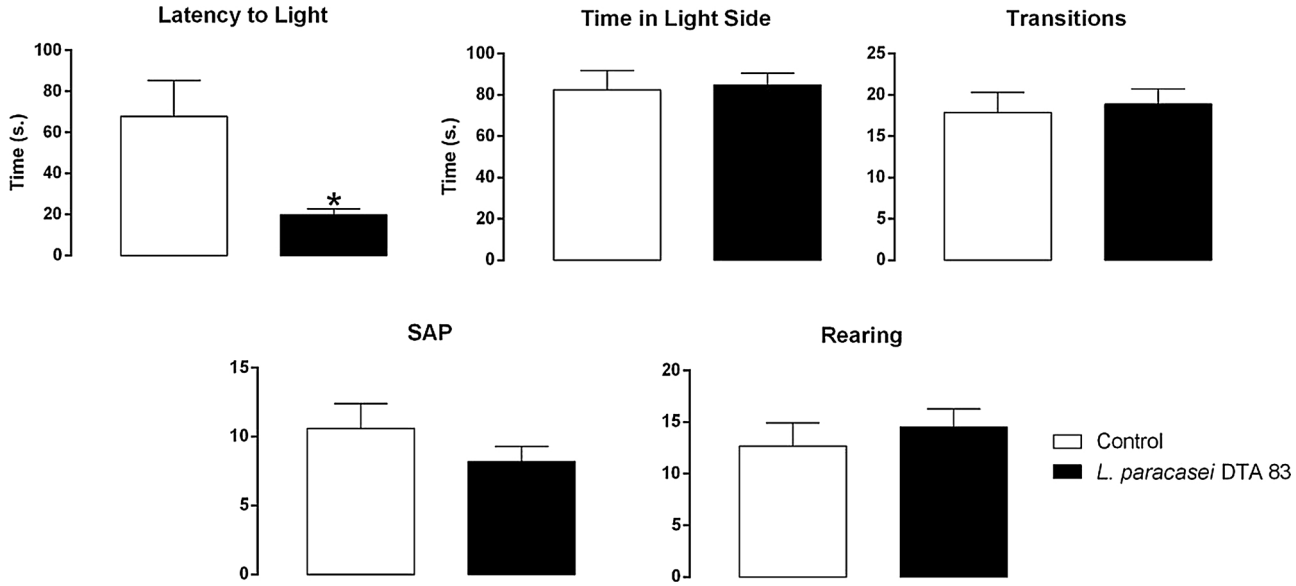

Fig. 4. Evaluation of anxiety-like behavior in mice with PND 71 whose mothers were treated with milk (control) or milk inoculated with *L. paracasei* DTA 83 through the light-dark box test. In this protocol, it was demonstrated that the treated group had reduction of anxiety-like behavior. * represents p < 005. N = 12 per group.

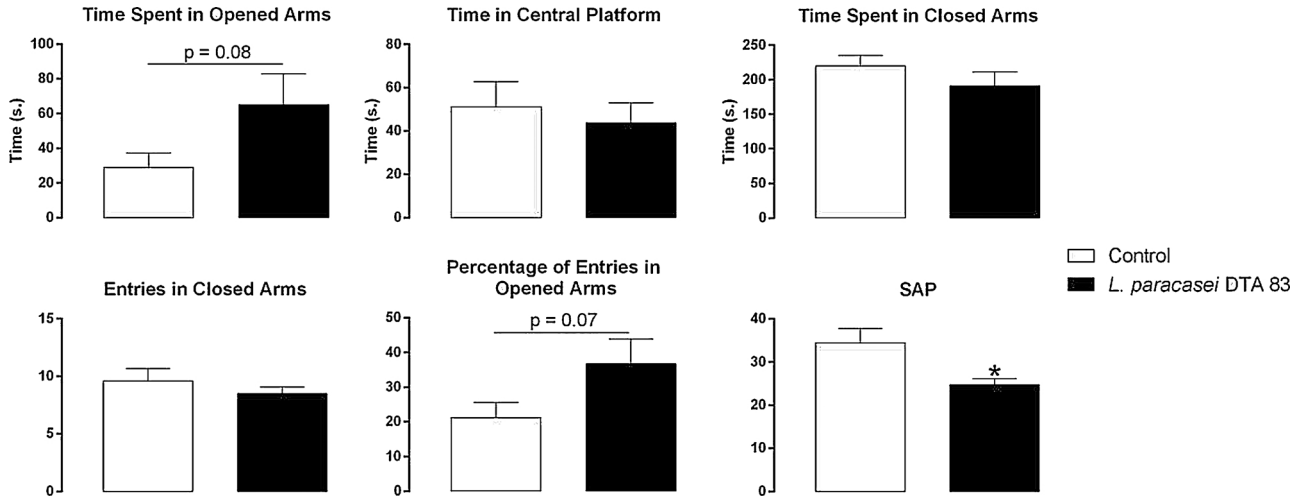

Fig. 5. Evaluation of anxiety-like behavior in mice with PND 72 whose mothers were treated with milk (control) or milk inoculated with *L. paracasei* DTA 83 through the elevated plus maze test. In this protocol, it was demonstrated that the treated group had reduction of anxiety-like behavior. * represents p < 005. N = 12 per group.

that probiotic cells could suffer translocation to mammary glands and placenta. Moreover, the vaginal delivery itself and nursing are important sources of maternal lactobacilli to newborns.

An elegant work that supports our results was published by Bravo et al. Although this study has used non-programmed adult animals, this group verified that *Lactobacillus rhamnosus* supplementation promoted region-dependent alterations in metabotropic and ionotropic GABA receptors [18]. On the other hand, Liang and colleagues showed that

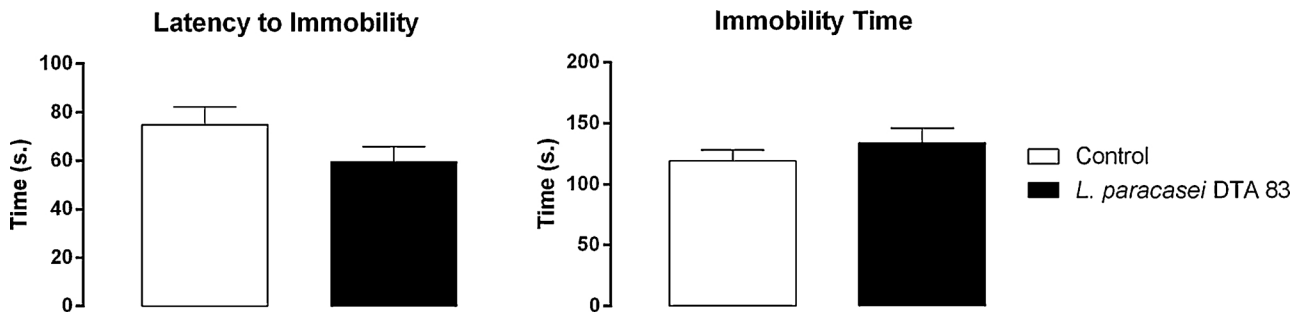

Fig. 6. Evaluation of anxiety-like behavior in mice with PND 73 whose mothers were treated with milk (control) or milk inoculated with *L. paracasei* DTA 83 through the Tail Suspension Test. In this protocol, no significant differences were observed. N = 12 per group.

Table 3
Calculation of Cohen's *d* effect size between groups for all behavioral parameters.

Behavioral Parameters		Cohen's *d*
Open Field Test	Total squares crossed	0.15
	Rearing	0.24
	Central squares crossed	**0.90**
	Center ratio	**1.07**
	Time in center area	**0.93**
	Time of grooming	**0.89**
	Fecal pellets	**0.95**
Light-Dark Box Test	Latency	**1.21**
	Transitions	0.14
	Time in light side	0.09
	Rearing	0.28
	Attempts	*0.51*
Elevated Plus Maze Test	Time in opened arms	**0.81**
	Time in closed arms	0.49
	Time in central platform	0.22
	Percentage of entries in opened arms	**0.84**
	Entries in closed arms	**1.20**
	SAP	
Tail Suspension Test	Latency to immobility	*0.68*
	Immobility time	0.41

Numbers in italic represent medium magnitude of the effect, whereas bold numbers represent large magnitude of the effect.

gut microbiota disturbance during early life also induced reduced expression of GABA-A receptor α5 and *δ* subunits in the hippocampus of adult rats. However, these changes were better reversed by probiotic administration than with the short-term benzodiazepine treatment [19]. In our work, perinatal probiotic exposure disrupts the expression of mRNAs related to the GABA-shift. To explain these transcriptional characteristics, we need to highlight an important property of lactic acid bacteria: these microorganisms, including *L. paracasei*, express GABA-synthesizing enzymes [20]. In such bacteria, the intracellular GABA production confers resistance to acidic pH [21]. It is possible that gut microbiota products, including GABA, exerts an epigenetic modulation role in central nervous system, promoting changes in GABAergic-related genes transcription and eventually alters host behavior not only during early life, but also in adulthood.

According to literature, microbiome-gut-brain axis seems to play an important role in hippocampal BDNF and 5-HT levels. In germ-free mice, transcript levels of BDNF were decreased in the hippocampal CA1 region compared to controls [22] and decreased similarly after antibiotic treatment of specific-pathogen-free) mice [23]. Germ-free mice also exhibited substantial increases in hippocampal 5-HT [24]. Indeed, there are few works that correlates probiotic supplementation and the aforementioned genes regulation. However, chronic *Lactobacillus rhamnosus* administration alters *Bdnf* and 5-HT-related genes expression in zebrafish brain [25]. Contrary to what was observed in the GABAergic system, we did not verified transcriptional changes in *Bdnf*, *Th* and *TPH2* genes. Although there were no differences, we need to remember that gene expression is not necessarily related to protein levels and activity. Thus, further studies will be necessary to assess the impact of maternal *Lactobacillus* supplementation on offspring's neurochemistry.

From the neurobehavioral standpoint, we showed that treatment with *L. paracasei* DTA 83 during pregnancy and lactation promotes anxiolytic effects in open field test (increased central area time and center ratio), in light-dark box (decreased latency to light) and in elevated plus maze test (reduced number of SAP). In elevated plus maze, there was a trend to increase time and percentage of entries in opened arms. However, as demonstrated previously, SAP is a natural behavior observed in rodents under risk-assessment and fundamentally can be more sensitive to estimate anxiety levels than spatiotemporal measures in this protocol [26]. To reinforce the our obtained results, experimental evidences have demonstrated that treatment with Lactobacillus or Bifidobacterium is able to reduce stress-related behavior in adult offspring underwent to model of early-life stress induced by maternal separation [27,28,29,30]. In most of these studies, the probiotic treatment normalizes corticosterone release. Moreover, according to McVey-Neufeld and coworkers, probiotic treatment also restores hippocampal glucocorticoid receptors expression. Taken together, these findings suggest that probiotic bacteria can reestablish hypothalamus-pituitary-adrenal axis set point altered by early life stress [30].

Another work that helps us to understand the potential therapeutic use of maternal probiotic treatment was published by Hsiao and co-authors. This group observed that maternal immune activation model promotes autism-related behavior in adult offspring [31]. According these authors, enteral treatment of MIA offspring with a single commensal organism *Bacteroides fragilis* improved autism related behavioral abnormalities, improved intestinal barrier function, restored microbiota changes and normalized cytokine profiles. The interaction between probiotics-induced changes during pregnancy is poorly understood. However, such effects seem to be mediated by changes in Toll-like receptors expression and regulatory T cells populations [32,33].

Maternal immune activation offspring also display an altered serum metabolomic profile. Some of these metabolites, such as 4-ethylphenylsulfate, promote autism-related behavior in non-programmed animals. Curiously, probiotic treatment reversed these metabolomic and behavioral abnormalities in both protocols [31]. This fact raises the possibility that metabolites derived from maternal gut microbiome modulate the neurotransmitter, synaptic, and neurotrophic signaling systems, thus influencing fetal brain development. According Arentsen and coworkers, bacterial peptidoglycan derived from the commensal gut microbiota can be translocated into the brain and sensed by specific pattern-recognition receptors of the innate immune system. The central activation of these receptors by microbial products could be one of the signaling pathways mediating the communication between the gut microbiota and the developing brain [34]. It is important highlight that anxiolytic behavior observed in our study could be also related to early-life GABAergic system modulation induced by maternal GABA-producing *Lactobacillus* supplementation, being this transcriptional profile important to program neurobehavioral phenotypes in adulthood. Further studies will be necessary to investigate this hypothesis in programmed offspring.

In summary, we demonstrated that maternal supplementation with *L. paracasei* DTA 83 can promote beneficial effects in offspring behavior. However, further studies will be necessary to understand the epigenetic mechanisms by which the maternal probiotic supplementation programs offspring neurochemical profile. The hodological and the pharmacodynamic consequences in GABAergic system should be investigated. Moreover, we need to study not only the relationship between the perinatal brain-gut-microbiome axis and affective disorders, but also its implication with other psychiatric diseases, such as autism, schizophrenia and attention deficit hyperactivity disorder. Undoubtedly, the microbiome can affect gene-environment interaction, braking the mechanisms involved with the fetal origin of diseases.

Conflict of interest

The authors declare that there are no conflicts of interest associated with this and there was no financial support for this work that could have influenced its outcome.

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Introduction: Chemoprevention of DMH-Induce Early Colon Carcinogenesis in Male BALB/c Mice by Administration of *Lactobacillus paracasei* DTA81

Non-communicable diseases (NCDs) are responsible for a substantial portion of global mortality, with colorectal cancer (CRC) ranking as the third most commonly diagnosed cancer and the second leading cause of cancer-related deaths worldwide. The increasing incidence of CRC in Western societies correlates with dietary shifts towards high consumption of ultra-processed foods and poor lifestyle choices.

CRC development involves complex interactions of metabolic dysregulation, chronic oxidative stress, and persistent inflammation. Imbalances between reactive oxygen and nitrogen species (ROS/RNS) production and antioxidant defenses can lead to DNA damage and genetic mutations, initiating carcinogenesis. Furthermore, inflammatory processes, exemplified in conditions like inflammatory bowel disease (IBD), contribute significantly to CRC progression through heightened production of pro-inflammatory cytokines such as IL-6, IL-17, IFN-γ, and TNF-α aimed at tissue repair.

The intestinal microbiota, pivotal in regulating immune responses and various metabolic functions, has emerged as a critical player in CRC pathogenesis. Dysbiosis, characterized by microbial community disruptions, along with identification of CRC-associated microbes, has opened avenues for diagnostic biomarker discovery and therapeutic interventions targeting specific bacterial genera.

In this context, considerable efforts have focused on identifying novel chemopreventive agents and functional foods with potential benefits in CRC prevention. Probiotics and synbiotics, by modulating intestinal microbiota, immune responses, inflammation, and biosynthesis of anticarcinogenic compounds, have shown promise in CRC prevention, as highlighted in recent reviews.

Dairy products represent a readily available source of lactic acid bacteria (LAB) with recognized probiotic potential. Among LAB species used in dairy fermentation, *Lactobacillus* strains like L. rhamnosus, L. casei, *L. paracasei*, L. plantarum, and L. acidophilus are widely acknowledged for their safety and health benefits [18].

Lactobacillus paracasei DTA81, isolated from the stool of a Brazilian infant, has demonstrated notable anti-proliferative activity and adherence capabilities against human colorectal adenocarcinoma cells in vitro. However, its prophylactic efficacy against precancerous lesions in animal models remains unexplored. Therefore, this study aimed to assess the impact of *L. paracasei* DTA81 on oxidative stress reduction, modulation of colonic cytokine profiles, production of short-chain fatty acids (SCFAs), and alteration of gut microbiota in an early colon carcinogenesis model induced by 1,2-dimethylhydrazine (DMH) in male BALB/c mice.

The experimental design involved daily administration of DTA81 and L. rhamnosus GG (LGG) in freeze-dried skim milk via orogastric gavage over an 8-week period. A DMH injection protocol was utilized to induce pre-neoplastic lesions in all groups, including controls receiving skim milk alone or no intervention. At the end of the study, assessments included biochemical analyses of liver oxidative stress markers, flow cytometric evaluation of colonic cytokine levels, SCFA quantification in cecal samples, and characterization of gut microbiota alterations.

Overall, our findings suggest that DTA81 administration attenuates hepatic oxidative stress markers, reduces pro-inflammatory cytokine levels in the colon, enhances the abundance of beneficial gut bacteria like Ruminiclostridium, and increases SCFA production. These outcomes collectively indicate the potential of *L. paracasei* DTA81 as a promising candidate for mitigating early stages of CRC development. Further research into its mechanisms and long-term effects could pave the way for targeted probiotic interventions in CRC prevention strategies.

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Chemoprevention of DMH-Induced Early Colon Carcinogenesis in Male BALB/c Mice by Administration of *Lactobacillus Paracasei* DTA81

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Abstract: We evaluated the effects of the probiotic candidate *Lactobacillus paracasei* DTA81 (DTA81) on liver oxidative stress, colonic cytokine profile, and gut microbiota in mice with induced early colon carcinogenesis (CRC) by 1,2-dimethylhydrazine (DMH). Animals were divided into four different groups (n = 6) and received the following treatments via orogastric gavage for 8 weeks: Group skim milk (GSM): 300 mg/freeze-dried skim milk/day; Group *L. paracasei* DTA81 (DTA81): 3×10^9 colony-forming units (CFU)/day; Group *Lactobacillus rhamnosus* GG (LGG): 3×10^9 CFU/day; Group non-intervention (GNI): 0.1 mL/water/day. A single DMH dose (20 mg/kg body weight) was injected intraperitoneally (i.p), weekly, in all animals (seven applications in total). At the end of the experimental period, DTA81 intake reduced hepatic levels of carbonyl protein and malondialdehyde (MDA). Moreover, low levels of the pro-inflammatory cytokines Interleukin-6 (IL-6) and IL-17, as well as a reduced expression level of the proliferating cell nuclear antigen (PCNA) were observed in colonic homogenates. Lastly, animals who received DTA81 showed an intestinal enrichment of the genus *Ruminiclostridium* and increased concentrations of caecal acetic acid and total short-chain fatty acids. In conclusion, this study indicates that the administration of the probiotic candidate DTA81 can have beneficial effects on the initial stages of CRC development.

Keywords: probiotic; colorectal cancer; *Lactobacillus paracasei* DTA81; 1,2-dimethylhydrazine (DMH); short-chain fatty acids; 16S rRNA; oxidative stress biomarkers; cytokine levels

1. Introduction

Non-communicable diseases (NCDs) account for 63% of global deaths [1]. Among them, colorectal cancer (CRC) is the third most frequently diagnosed worldwide and ranked second in terms of mortality [2]. It is a multifactorial disease, and its increased incidence has been observed in Western countries, particularly in the last few decades. This illness is positively correlated with changes in dietary patterns such as high consumption of ultra-processed food and beverages with low nutritional value, as well as lifestyle factors [3].

Amongst the metabolic changes that precede precancerous cell development, chronic cellular damage due to an imbalance between reactive oxygen and nitrogen species (ROS; RNS) production and antioxidants may lead to DNA damages and genetic mutations [4]. Another factor contributing to CRC development concerns the continuous local inflammatory process, evident in inflammatory bowel disease (IBD), which consists of cellular and humoral responses via increased production of pro-inflammatory cytokines such as IL-6, IL-17, interferon-gamma (IFN-γ), and tumor necrosis factor (TNF-α) in an attempting to regenerate the injured tissue [5,6].

Intrinsically linked to the immune system response pattern and various metabolic processes, the intestinal microbiota and its role in CRC development have been studied in depth in the last few years under health and disease conditions [7–9]. As major outcomes, gut microbiome alterations, a process named dysbiosis, along with the identification of CRC-associated microorganisms, have made possible the discovery of diagnostic markers and the potential use of specific bacterial genera for prophylactic and/or therapeutic purposes [10,11].

In this context, several studies have been carried out to identify new chemopreventive agents or functional foods with a recognized beneficial effect towards CRC prevention [12–15]. In a systematic review conducted by Cruz et al. (2020) [16], the authors emphasize that probiotics and synbiotics can prevent colorectal cancer through different mechanisms such as intestinal microbiota and immune response modulation, reduction of inflammation, biosynthesis of compounds with anticarcinogenic activity, and improvement in redox system balance.

Overall, dairy products are considered a rich and deliverable source of lactic acid bacteria (LAB) with potential probiotic properties [17]. Among the cultures used in the manufacture of fermented dairy, the genus *Lactobacillus* includes a great number of species considered as GRAS (generally recognized as safe) such as *L. rhamnosus*, *L. casei*, *L. paracasei*, *L. plantarum*, and *L. acidophilus* [18].

Based on a recent study conducted by Tarrah et al. (2019) [19], the strain *L. paracasei* DTA81 demonstrated an interesting antiproliferative activity and high adherence ability against the human colorectal adenocarcinoma HT-29 cell line. However, no information is available concerning its prophylactic capability to prevent precancerous lesions during in vivo studies on animal models. Therefore, the present study aimed to evaluate the effects of DTA81 on the reduction of oxidative stress, colonic cytokine profile, caecal short-chain fatty acids (SCFAs) production, and gut microbiota modulation in an animal model of colorectal cancer.

2. Materials and Methods

2.1. Probiotic Strains, Fermented Milk Production, and Probiotic Dose

L. paracasei DTA81 was isolated from a Brazilian infant's stool [19]. The commercial probiotic strain *L. rhamnosus* GG was included for comparison. *Lactobacillus* strains were routinely grown in De Man, Rogosa and Sharpe (MRS) broth (Oxoid, Milan, Italy) at 37 °C for 24 h aerobically. Fermented milk was prepared following the procedures described by Iyer and Tomar (2011) [20], with some modifications. A total of 100 mL of skim milk was inoculated with each bacterial strain (single batches) at 2% (*v*/*v*) and incubated for 48 h at 37 °C. After this period, another round of fermentation (48 h at 37 °C) was performed by the addition of 100 mL of skim milk. This procedure aimed to increase cellular yield after the fermentation period. Finally, the fermented milk obtained was frozen at −80 °C and freeze-dried to reduce the volume of gavage administered to the animals. After that, freeze-dried fermented milk was

produced for each strain, total viable cell counting was performed through plating serial dilutions (1 g sample dissolved in 9 mL phosphate-buffered saline (PBS) pH 7.2 (NaCl 0.13 M, KCl 2 mM, Na_2HPO_4 9 mM, KH_2PO_4 1 mM)) on MRS agar medium. Plates were incubated at 37 °C for 48 h. Results were expressed in colony-forming units (CFU)/g. The probiotics (300 mg) were resuspended in 0.1 mL of autoclaved tap water daily and supplied to their respective group of mice as described below.

2.2. Animals and Experimental Design

2.2.1. Animals

Twenty-four male BALB/c mice (*Mus musculus*) at 12 weeks old, were obtained from the Central Animal Facility of the Center for Biological Sciences and Health at Universidade Federal de Vicosa, Minas Gerais, Brazil. In order to maintain homogeneity among the experimental groups, animals were divided according to body weight into four different groups ($n = 6$). The animals were collectively allocated in polypropylene cages with three mice each, kept under controlled conditions, at a temperature of 22 ± 2 °C and humidity of 60–70%, with a 12 h light/dark cycle. The animals received free access to a standard pellet diet (Presence®, Paulinia, Brazil) and water throughout the experimental period.

All experimental procedures using animals were performed following Directive 86/609/EEC of 24 November 1986, in compliance with the ethical principles for animal experimentation. The study protocol was approved by the Ethics Committee of the Universidade Federal de Vicosa (CEUA/UFV, protocol n° 15/2020; date of approval: 4 August 2020).

2.2.2. Study Design

After the acclimation period, animals received in the first experimental week via orogastric gavage their respective treatments. From the second week onwards, the protocol for induction of colon carcinogenesis was introduced for all animals enrolled in this study. The colon carcinogen 1,2-dimethylhydrazine (DMH) (Sigma-Aldrich, St. Louis, USA) was prepared in 0.9% saline solution containing 1 mM EDTA and 10 mM sodium citrate, pH 8, as described by Newell and Heddle (2004) [21]. A DMH single dose (20 mg/kg body weight) was injected intraperitoneally (i.p), once a week, performing a total of seven applications per animal in all groups.

The experimental treatments were organized as follows:

Group GSM (skim milk): 300 mg of freeze-dried skim milk resuspended with 0.1 mL of sterile tap water.
Group DTA81 (*L. paracasei* DTA81): 300 mg of freeze-dried fermented milk containing ~3×10^9 cells/0.1 mL of sterile tap water daily.
Group LGG (*L. rhamnosus* GG): 300 mg of freeze-dried fermented milk containing ~3×10^9 cells/0.1 mL of sterile tap water daily.
Group GNI (non-intervention): 0.1 mL of sterile tap water daily.

2.3. Food Intake, Body Weight, and Feces Collection

To evaluate the physiological effects of the prophylactic administration of probiotics in weight loss/gain of the animals, food intake and body weight were monitored regularly after the beginning of the DMH protocol. Food intake was measured daily and was calculated by the difference in the amount of diet offered (g) for each group ($n = 6$) and the remaining amount in the day after. At the end of each experimental week, body weight was measured using a digital semi-analytical scale.

The feces of each animal involved in this study were harvested at the beginning ($t0$) and one day before at the end of the experimental period ($t1$). To obtain fecal samples, individual cages were previously cleaned, sanitized, and kept with a single mouse until enough feces were spontaneously expelled. Samples were kept at −80 °C for further analysis.

2.4. Euthanasia

After the end of the experimental period (8 weeks), all animals were anesthetized (ketamine 80 mg/kg; xylazine 20 mg/kg) and blood samples were collected from the retro-orbital sinus. The mice were sacrificed by cervical dislocation. The liver and caecum were harvested, snap-frozen in nitrogen liquid, and stored at −80 °C, whereas the colon was resected, washed with cold PBS solution, and sliced into small fragments. These fragments were equally distributed in three different microcentrifuge tubes, with one of them prefilled with RNAlater (Sigma-Aldrich, St. Louis, MI, USA), and stored at −80 °C.

2.5. Evaluation of Liver Oxidative Stress Markers

In total, four oxidative stress markers were chosen to assess liver oxidant status. The liver sample was prepared by homogenizing approximately 200 mg of tissue with 800 µL of 50 mM PBS solution containing 1 mM EDTA (pH 7.4). The homogenate was centrifuged at 10,000× *g* for 10 min at 4 °C and the supernatant obtained was used for oxidative markers quantification. For the determination of total proteins in the liver homogenate, the method described by Lowry et al. (1951) [22] was adopted. Catalase (CAT) activity, determined by its ability to cleave hydrogen peroxide (H_2O_2) into the water and molecular oxygen, was evaluated according to the method described by Aebi (1984) [23] and the results are expressed as U CAT/mg protein. The superoxide dismutase (SOD) activity, defined as the enzyme amount causing 50% inhibition in pyrogallol autoxidation, was performed according to the methodology described by Marklund (1985) [24] and the results are expressed as U SOD/mg protein. The liver concentration of malondialdehyde (MDA) was determined as thiobarbituric acid reactive substances (TBARS) of lipid peroxidation and followed the methodology described by Buege and Aust (1978) [25]. The results were expressed as nmol of MDA/mg protein. Lastly, the level of liver protein oxidative damage, indicated by the levels of protein carbonyls, was measured according to the method of Levine et al. (1990) [26].

2.6. Cytokine Profile in Colon Homogenate

To determine colonic cytokines production, colon samples (100 to 200 mg) were ground using a tissue homogenizer (IKA WORKS GMBH and CO, Staufen, Germany, model T10 basic) in PBS buffer (pH 7.0), centrifuged (10,000× *g*, for 10 min at 4 °C) and the supernatant recovered. Pro- and anti-inflammatory cytokines (Interleukin-2; Interleukin-4, Interleukin-6, Interferon-γ, Tumor Necrosis Factor, Interleukin-17A, and Interleukin-10) were simultaneously determined by the Cytometric Bead Array (CBA) mouse Th1/Th2/Th17 Kit in a BD FACSVerse Flow cytometry following the manufacturer's recommendations (Becton, Dickinson, and Company, Franklin Lakes, NJ, USA). The data were processed using the FCAP Array Software v3.0 and the results were expressed in pg/g of tissue.

2.7. Fecal Short-Chain Fatty Acids (SCFAs) Quantification

The SCFA profile of acetate, propionate, butyrate, and lactate present in the caecum of all the animals was evaluated by high-performance liquid chromatography (HPLC) according to the method described by Siegfried et al. (1984) [27]. Briefly, approximately 100 mg of caecal content were thawed at room temperature, homogenized in 300 µL of distilled water, and centrifuged at 12,000× *g* for 10 min. After that, 300 µL of supernatant was recovered and transferred to a new microcentrifuge tube where 300 µL of calcium hydroxide solution (CHS) and 150 µL of cupric sulphate solution (CSR) were thawed at room temperature and then centrifuged at 12,000× *g* for 10 min. To the supernatant (500 µL), 14 µL of H_2SO_4 concentrated was added, and the tubes were frozen. Lastly, tubes were centrifuged (12,000× *g* for 10 min) and 300 µL was recovered and stored in an HPLC vial at 4 °C until the analysis. For the determination of the fatty acids, a Dionex Ultimate 3000 HPLC system (Thermo Scientific, Bremen, Germany) was used. Samples were separated in a RezexROA column–Organic acid H^+ (8%), (Phenomenex, Torrance, EUA), 300 mm × 7.8 mm at a flow rate of 0.7 mL/min. The mobile

phase was H_2SO_4 5 mM. The column temperature was set at 45 °C and the injection volume was 20 µL. Results were expressed in µmol/g feces.

2.8. Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

The relative expression level of tumor-related genes was evaluated by qPCR in an Illumina Eco® real-time polymerase chain reaction system (Illumina, San Diego, CA, USA), using the GoTaq® 1-Step RT-qPCR System (Promega, Madison, WI, USA). After extraction following Tryzol® reagent (Thermo-Fisher, Waltham, MA, USA) protocol, the RNA was quantified using Qubit 4 Fluorometer (Thermo-Fisher). The thermal cycles used were the default of the equipment: 10 min at 94 °C to polymerase activation, followed by 40 cycles of 10 sec at 95 °C to open strands and 30 sec at 60 °C to annealing and extension. The primers used are listed in Table 1. The analysis was performed by the $2^{-\Delta Ct}$ method on the EcoStudy® software (Illumina, San Diego, CA, USA), using GAPDH as an endogenous control.

Table 1. Table of primer sequences of four genes associated with colorectal cancer (CRC) development.

Gene	Sequence (5′→3′)	Reference
PCNA	F: TAAAGAAGAGGAGGCGGTAA R: TAAGTGTCCCATGTCAGCAA	[28]
Caspase-3	F: AGCAGCTTTGTGTGTGTGATTCTAA R: AGTTTCGGCTTTCCAGTCAGAC	[29]
c-myc	F: TCCTGTACCTCGTCCGATTC R: GGAGGACAGCAGCGAGTC	[30]
p53	F: GTATTTCACCCTCAAGATCC R: TGGGCATCCTTTAACTCTA	[31]
GAPDH	F: CTGCTTCACCACCTTCTTGA R: AAGGTCATCCCAGAGCTAAA	[32]

2.9. Fecal Bacterial Composition Analysis Using Next-Generation Sequencing (NGS)

2.9.1. DNA Extraction

Mice fecal samples (250 mg) were obtained before probiotics administration (*t*0) and at the end of the experimental period (*t*1). The metagenomic DNA was extracted using the DNeasy PowerLyzer PowerSoil DNA isolation kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA concentration and purity were determined via 260/280 and 260/230 ratios measured on the NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA, USA).

2.9.2. 16S rRNA Gene Amplicon Sequencing

After stool DNA extraction, samples of four different animals composing each experimental group at the times *t*0 and *t*1 (32 samples) were collected and sent for NGS at Molecular Research DNA (MR DNA, Shallowater, TX, USA). To assess the gut microbial profile, the hypervariable region V1-V2 of the 16S rRNA gene was chosen. Briefly, PCR primers 27f/338r with a barcode on the forward primer were used in a 30-cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, Germantown, MD, USA) under the following conditions: 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 53 °C for 40 s and 72 °C for 1 min, after which a final elongation step at 72 °C for 5 min was performed. After amplification, PCR products were checked in 2% agarose gel to determine the success of amplification and the relative intensity of bands. Multiple samples were pooled together in equal proportions based on their molecular weight and DNA concentrations, purified using calibrated Ampure XP beads, and then used to prepare Illumina DNA library. Sequencing was performed on a MiSeq (Illumina, San Diego, CA, USA) following the manufacturer's guidelines generating 300 bp paired-end (PE) reads.

2.9.3. Bioinformatic Data Analyses

Sequence data were processed using the Molecular Research DNA analysis pipeline (MR DNA). In summary, sequences were joined, depleted of barcodes then sequences <150 bp removed, sequences with ambiguous base calls removed. Sequences were denoised, operational taxonomic units (OTUs) generated and chimeras removed. OTUs were defined by clustering at a 97% similarity. Final OTUs were taxonomically classified using BLASTn against a curated database derived from RDPII and NCBI. Raw reads were deposited in the Sequence Read Archive (SRA) database under the BioProject PRJNA661570.

The file containing the OTU abundance information, the metadata of the experimental design, and its phylogenetic tree were imported into the MicrobiomeAnalyst web-based application [33], where statistical analyses were carried out except for alpha diversity comparison, conducted with GraphPad Prism 7 (see Item 2.10). Following data inspection, removal of low count/variance sequences, and data scaling (cumulative sum scaling–CSS) were conducted with default parameters. When appropriate, data were rarefied to the minimum library size (26,070 sequences).

Differences between gut microbial community structure (beta diversity analysis) before and after the experimental period were calculated by permutational multivariate analysis of variance (PERMANOVA). Scatter plots of the principal coordinate analysis (PCoA) were computed from the calculated unweighted and weighted UniFrac distance matrixes.

Lastly, the significant difference of the most abundant OTUs among the groups at different times was assessed using the linear discriminant analysis (LDA) effect size (LEfSe) [34] tool setting up alpha-value of 0.05 and Log LDA threshold of 2.0.

2.10. Statistical Analysis

Variables were checked for Gaussian distribution with the Shapiro–Wilk test. The intergroup variation was assessed by one-way analysis of variance (ANOVA) using Graphpad Prism Version 7.00 (San Diego, CA, USA). In all significant results, post hoc comparison was performed using Tukey's multiple comparisons test. For those variables with non-normal distribution, the non-parametric Kruskal–Wallis method was adopted with Dunn's as a post hoc multiple comparisons test. Differences were considered significant at $p < 0.05$. Unpaired *t*-test with Welch's correction was used to evaluate animal food intake before and after DMH injection. All the results were expressed as mean ± standard error of the mean (SEM).

3. Results

3.1. Food Intake and Body Weight

Four experimental groups were formed and received a commercial standard pellet diet during the entire experimental period. One group (LGG) received the strain *L. rhamnosus* GG and another group (DTA81) received the strain *L. paracasei* DTA81, both dispersed in skim milk. One group (GSM) received skim milk without bacteria and the last one neither bacteria nor skim milk. All groups were treated with DMH to induce pre-neoplastic lesions. All groups started the experimental period with homogeneous body weight (Figure S1) and there were no significant differences ($p > 0.05$) in body weight among all groups (Figure 1A). In terms of food intake, there were no significant differences among the groups neither before nor after DHM injection (Figure S2). However, a significant reduction in food consumption (intra-group comparison) on the day after DMH injection was observed in all experimental groups (Figure 1B).

3.2. Effect of L. Paracasei DTA81 on Oxidative Stress Biomarkers in the Liver

The effects of supplementation of strains *L. paracasei* DTA81 and *L. rhamnosus* GG on biomarkers of oxidative stress were evaluated by measuring the enzymes involved in the endogenous antioxidant defense system (Superoxide Dismutase–SOD; and Catalase–CAT), along with the products of lipid

and protein oxidation, MDA and protein carbonyl, respectively. The daily intake of LGG significantly decreased the hepatic level of SOD only when compared to the experimental group that received skim milk (group GSM) (Figure 2A), however, there was no significant difference among the experimental groups concerning CAT levels (Figure 2B). The determination of MDA levels in mouse liver (Figure 2C) revealed that the animals fed with the strains *L. rhamnosus* GG and *L. paracasei* DTA81 showed on average reduced levels of lipid peroxidation in liver homogenate when compared to GSM and GNI groups, although statistical significance has been observed only between LGG and GSM (Figure 2C–group GSM: mean = 0.27, coefficient of variation = 21.10%; group DTA81: mean = 0.18, coefficient of variation = 20.89%; group LGG: mean = 0.18, coefficient of variation = 15.82%; group GNI: mean = 0.26, coefficient of variation = 24.73%). Lastly, animals supplemented with LGG had a significant decrease level of carbonyl proteins when compared to the group fed with skim milk (Figure 2D).

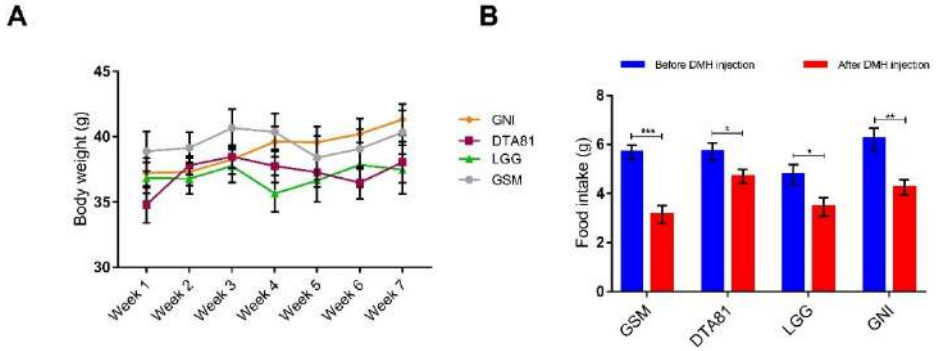

Figure 1. (**A**) Changes in BALB/c mice body weight during the experimental period in the control (skim milk, GSM, and non-intervention, GNI) and probiotic (*L. paracasei* DTA81, and *L. rhamnosus* GG, LGG) groups. (**B**) Food intake was significantly reduced in all groups following 1,2-dimethylhydrazine (DMH) injection. Data represent the mean weight during the experimental period. Data are shown as mean ± standard error of the mean (SEM, $n = 6$). *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

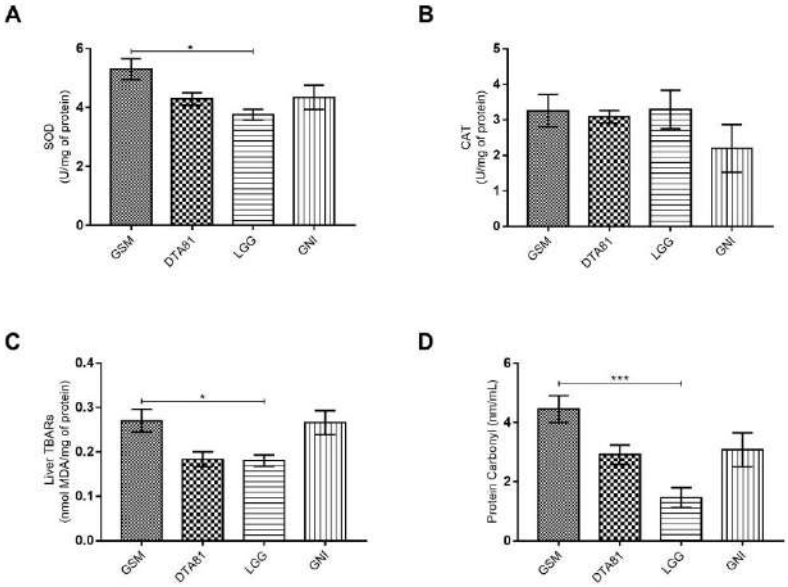

Figure 2. Oxidant status markers in the liver. Effect of *L. paracasei* DTA81 (DTA81) or *L. rhamnosus* GG (LGG) in liver tissue of male BALB/c animals subjected to 1,2-dimethylhydrazine. (**A**) superoxide dismutase (SOD) activity; (**B**) catalase (CAT) activity; (**C**) malondialdehyde (MDA); (**D**) protein carbonyls. The data are expressed as means ± SEM ($n = 6$). *, $p \leq 0.05$; ***, $p \leq 0.001$.

3.3. Cytokine Production Profile in Colon Tissue

In total, the levels of seven cytokines (IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-17, and TNF) were measured in colon tissue by flow cytometry (Figure 3). A remarkable low level of pro- and anti-inflammatory cytokines were observed in GSM and DTA81 groups, whereas the highest level was noticed in the GNI group. Animals receiving DTA81 displayed lower amounts of the anti-inflammatory cytokines interleukin IL-4 (Figure 3A) and IL-10 (Figure 3B) in colonic homogenates compared to the LGG group. Group DTA81 showed also lower levels of pro-inflammatory cytokines IL-2 (Figure 3C), IL-6 (Figure 3D), and IL-17 (Figure 3E) compared to the GNI group. Lastly, the levels of the pro-inflammatory cytokines TNF and IFN-γ were significantly higher in the LGG group when compared to the control group GSM (Figure 3F,G).

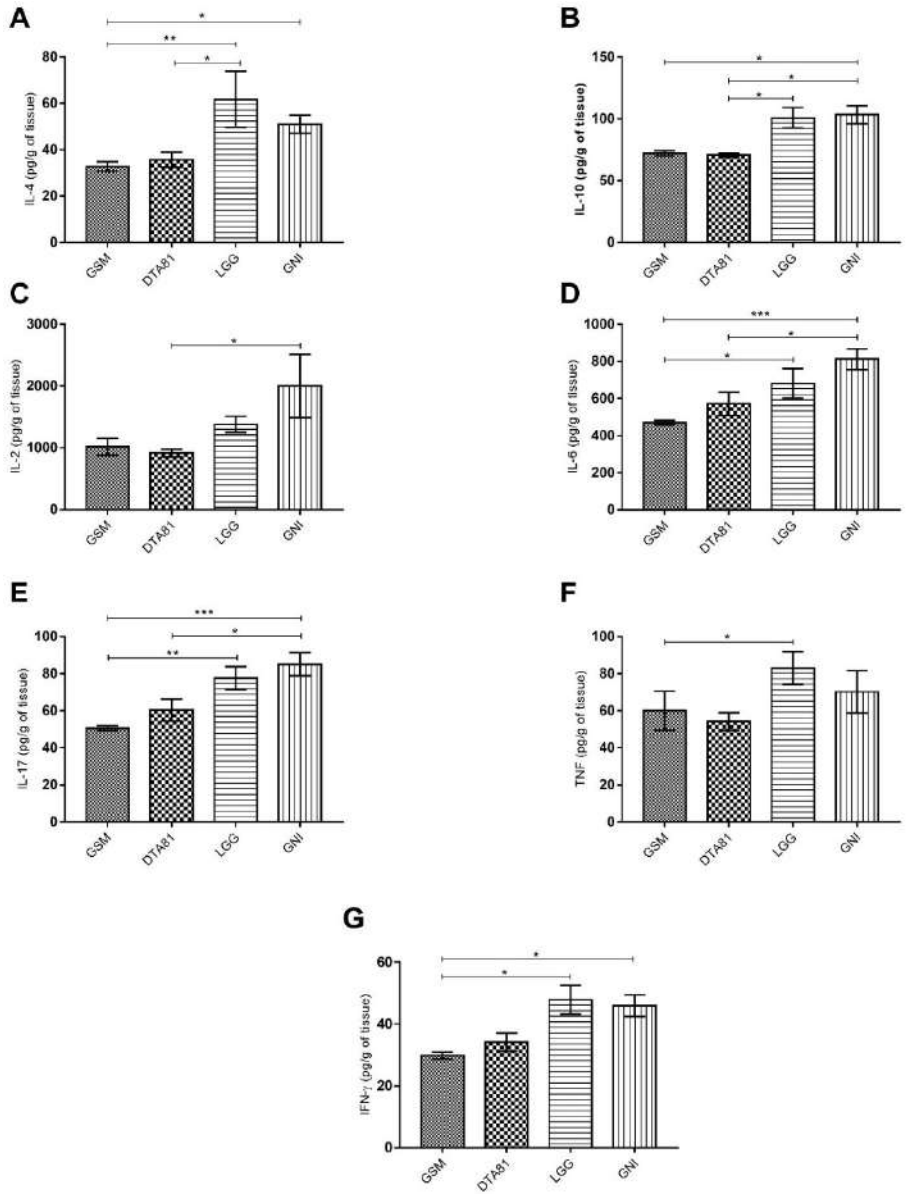

Figure 3. Pro- and anti-inflammatory cytokine profile in colon tissue using the cytometric bead array kit. (**A**) Interleukin-4 (IL-4); (**B**) IL-10; (**C**) IL-2; (**D**) IL-6, (**E**) IL-17; (**F**) tumor necrosis factor (TNF); (**G**) interferon-gamma (IFN-γ). Values are expressed as means ± SEM ($n = 6$). *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

Introduction: *Lactobacillus paracasei* DTA81, a cholesterol-lowering strain having immunomodulatory activity, reveals gut microbiota regulation capability in BALB/c mice receiving high-fat diet

Non-communicable diseases (NCDs) such as cardiovascular diseases and diabetes mellitus are major public health concerns worldwide, with dyslipidemia and hyperglycemia being significant risk factors. Diet plays a crucial role in influencing these metabolic parameters, particularly high-fat diets (HFDs), which are linked to increased incidence of metabolic disorders.

Probiotics, particularly strains of *Lactobacillus*, have gained attention for their potential health benefits, including modulation of lipid metabolism, glucose homeostasis, immune function, and gut microbiota composition. *Lactobacillus paracasei* DTA81, isolated originally from a human infant's stool, has shown promising in vitro properties such as cholesterol assimilation and absence of negative traits like antibiotic resistance genes. However, it's in vivo effects and mechanisms in modulating metabolic parameters and gut microbiota remain underexplored.

This study aimed to investigate the effects of *L. paracasei* DTA81 on lipid and glucose metabolism, immune modulation, gastrointestinal survivability, and gut microbiota in a BALB/c mouse model fed a high-fat diet. Over a 6-week period, we assessed changes in total cholesterol, fasting blood sugar (FBS), cytokine profiles in colon tissue, and alterations in gut microbiota composition using high-throughput 16S rRNA sequencing.

Results indicated significant reductions in total cholesterol and FBS levels among mice supplemented with *L. paracasei* DTA81 compared to controls on HFD. Immunomodulatory effects were observed with decreased levels of inflammatory cytokine interleukin-6 (IL-6) in colon tissues of treated animals. Furthermore, supplementation with DTA81 significantly increased taxa such as Bacteroidetes and Coprococcus in the gut microbiota, indicating a potential role in enhancing microbial diversity and metabolic health.

In addition to experimental findings, genomic analysis of DTA81 confirmed the presence of genes associated with cholesterol assimilation without harboring antibiotic resistance genes or other detrimental traits. These findings underscore the potential of *L. paracasei* DTA81 as a non-pharmacological probiotic intervention to improve metabolic homeostasis, particularly in individuals consuming high-fat diets, thereby potentially reducing the risk of coronary heart disease and other metabolic disorders.

The study contributes to understanding the mechanisms by which probiotics like *L. paracasei* DTA81 exert their beneficial effects, highlighting their potential as dietary supplements for enhancing metabolic health and modulating gut microbiota composition. Further research is warranted to elucidate the long-term effects and clinical implications of such interventions in human populations.

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ORIGINAL ARTICLE

Lactobacillus paracasei DTA81, a cholesterol-lowering strain having immunomodulatory activity, reveals gut microbiota regulation capability in BALB/c mice receiving high-fat diet

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Abstract

Aims: *In-vitro*/*In-vivo* evaluation of cholesterol-lowering probiotic strain *Lactobacillus paracasei* DTA81 and the possible connection with the gut microbiota modulation.

Methods and Results: In the present study, strain DTA81 has been evaluated for the possible influence on blood lipid and glucose concentrations, modulation of the immune system, gastrointestinal survivability and modulation of gut microbiota in BALB/c mice receiving a high-fat diet. After 6 weeks of treatment, a significant reduction of total cholesterol and fasting blood sugar (FBS) among animals treated with *L. paracasei* DTA81 has been recorded. Comparison of colon tissue levels of different cytokines revealed a significant reduction of the inflammatory cytokine interleukin-6. The comparison of gut microbiota using the 16S rRNA approach indicated that the treatment with *L. paracasei* DTA81 significantly increased the taxa *Bacteroidetes* and *Coprococcus*. Moreover, the genome of DTA81 was sequenced for the *in-silico* assessment, and the analysis indicated the presence of cholesterol assimilation-related genes as well as the absence of negative traits such as transmissible antibiotic resistance genes, plasmids and prophage regions.

Conclusion: The outcome of this study revealed the *in-vitro* and *in-vivo* properties of *L. paracasei* DTA81 and the possible mechanism between consumption of this strain, the abundance of *Bacteriodetes/Coprococcus* taxa, immunomodulatory activity and the subsequent reduction of cholesterol/FBS in BALB/c mice.

Significance and Impact of the Study: *Lactobacillus paracasei* DTA81 as a non-pharmacological potential probiotic supplement can influence metabolic homeostasis in individuals, particularly those adopting high-fat diets, and it can contribute to reduce coronary heart disease.

Introduction

According to the last definition by the FAO/WHO (Food and Agriculture Organization/World Health Organization), probiotics are 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (Hill *et al.* 2014). Probiotic foods represent a relevant portion of functional foods available on the market worldwide, projected to reach a value of US $ 46·55 billion by 2020 (Singh *et al.* 2018). During the past years, the probiotic potential of many lactic acid bacteria has been studied since they are generally recognized as safe

micro-organisms and can, therefore, be safely used in food preparations (Tarrah *et al.* 2020a, 2020b). Probiotic consumption can benefit human health by modulating the immune system, affecting the gut microbial composition and by producing antimicrobial substances that can contribute to the reduction of deleterious bacteria and promote stability of beneficial microbes (Magnusson and Schnürer 2001; Baker *et al.* 2009; Amund 2016). Many studies revealed the influence of gut microbiota on metabolic disorders and obesity in humans (Crovesy *et al.* 2020; Wang *et al.* 2020). A recent study indicated that energy homeostasis and metabolism of the human can be directly influenced by the gut microbiota (Cani *et al.* 2019). Indeed, new studies report that transferring the gut microbiota from obese to germ-free mice can result in a higher weight increase in comparison to the transfer of gut microbiota from a lean mouse (Rosenbaum *et al.* 2015; Cani *et al.* 2019). Nowadays, hypercholesterolaemia is reported to be a common human disorder, which is mostly related to cardiovascular disease (CVD) and coronary heart disease (CHD) (Dunn-Emke *et al.* 2001). Many *in-vitro* and *in-vivo* studies recently reported that probiotics such as some *Lactobacillus* and *Bifidobacterium* can have beneficial effects on serum lipid profiles (He *et al.* 2017; Mo *et al.* 2019). Probiotics can also reduce blood cholesterol in different ways, by utilizing prebiotics to produce short-chain fatty acids (SCFAs) in the human gut that can further inhibit hepatic cholesterol synthesis and will result in a reduction of blood lipids or by assimilating cholesterol directly and reduce its presence in the human gut (Pereira and Gibson 2002). Therefore, several probiotic bacteria have been proposed and used as food supplements to reduce the rate of hypercholesterolaemia in humans (Marchesi *et al.* 2016). On the other side, it has been proven that probiotic strains can modulate the human immune system in different ways. The expression of cytokines in the human body has been the most frequent approach to describe the immunomodulatory effect of probiotics. In Wang *et al.*'s (2015) study, treated people with *Bifidobacterium bifidum*, *Bifidobacterium catenulatum*, *Bifidobacterium longum* and *Lactobacillus plantarum* showed a decrease in serum levels of pro-inflammatory cytokines TNF-α, IL-5 and IL-6, while levels of serum of IL-10 significantly increased. In a previous *in-vitro* study (Tarrah *et al.* 2019), we have characterized the probiotic potential of strain *Lactobacillus paracasei* DTA81 which was found to possess interesting properties. In particular, DTA81 revealed a strong adherence ability to human cell lines. Therefore, this strain was used in this study to be further investigated regarding its immunomodulatory effects, metabolic alteration and possible gut microbiota modulation effect, using *in-vitro* and *in-vivo* approaches.

Materials and methods

Bacterial strain and growth conditions

Lactobacillus paracasei DTA81 (Guerra *et al.* 2018) was routinely grown using MRS medium (Sigma, MO) at 37°C for 24 h. For *in-vivo* assays, overnight cultures were centrifuged at 5000 ***g*** for 5 min, washed two times with sterile PBS (NaCl 8·0 g l^{-1}, KCl 0·2 g l^{-1}, Na_2HPO_4 1·44 g l^{-1}, KH_2PO_4 0·24 g l^{-1}, pH 7·4) and resuspended in skim milk (10%) to a final concentration of about 10^{10} CFU per ml.

Measurement of cholesterol assimilation by *L. paracasei* DTA81

Initially, 1% overnight culture was incubated in MRS broth (Sigma) containing 0·30% ox gall bile salt (Sigma) and 100 μg ml^{-1} filter-sterilized cholesterol (Cholesterol–methyl-β-cyclodextrin, Sigma) for 24 h at 37°C. Tubes were then centrifuged at 5500 ***g*** for 15 min at 4°C, and 1 ml of supernatant was collected for measurement of residual cholesterol using a colorimetric method (Miremadi *et al.* 2014). Cholesterol concentration was measured using a standard curve from 0 to 100 μg ml^{-1}. The experiment was repeated two times with three replicates each. The ability of *L. paracasei* DTA81 to assimilate cholesterol was calculated as a percentage of cholesterol removal after 24 h.

Animals

In all, 24 male BALB/c mice (4-week old) were obtained from the Animal House at the Biological Sciences Center of the Universidade Federal de Viçosa. All mice were housed (four animals per cage) in a controlled environment: temperature 22°C, humidity 55 ± 5%, 12 h light/-dark cycle and received food (Nuvilab, São Paulo, Brazil) and sterilized tap water *ad libitum* except at sampling time when the access to food was restricted. Mice body weight and food consumption were recorded weekly and daily respectively. The animal study design was approved by the animal ethics committee at the Universidade Federal de Viçosa (CEUA/UFV, protocol n° 15/2020) and it was in accordance with the National Research Council guide for the care and use of laboratory animals (Clark *et al.* 1997).

Diets and experimental design

All animals were fed for a week (week 0) with a conventional diet (CD). Then they were randomly divided considering the body weight and GTT (glucose tolerance test)

into three experimental groups (8 animals per group). The first one received the CD (CD group), the second one was fed with a high-fat diet (HFD group) and the third one received HFD + *L. paracasei* DTA81 (DTA81 group). The composition of CD and HFD diets is reported in Table 1 (Reeves *et al.* 1993; Zhao *et al.* 2019).

The potential probiotic strain was administered daily for 6 weeks (from weeks 1 to 6) via a unique oral administration by gavage of 100 μl, equivalent to approx. 10^9 CFU dispersed in 10% skim milk. During the same period, the remaining groups received the same amount of skim milk without cells. Then, the animals were anesthetized using ketamine (Imalgène, 200 mg kg^{-1}, Sigma) and Rompun (Xylasine, 20 mg kg^{-1}, Sigma) diluted in NaCl 0·9%, the mice were sacrificed by cervical dislocation, and the blood samples were collected from the retro-orbital sinus for the biochemical analysis.

Oral glucose tolerance test

The oral glucose tolerance test (OGTT) was performed at the end of week 0 and 6 (end of the experiment), according to Ferrere *et al.* (2016), with some modifications. A solution of 0·2% D-glucose was given to each animal via gavage after overnight (12 h) fasting conditions, then blood was collected from the tail after 0, 30, 60 and 120 min. Glucose concentration in the blood serum was measured by putting a blood drop on a Comfort Curve Strips (F. Hoffmann–La Roche, Basel, Switzerland) that was then inserted into an ACCU-CHEK Advantage Glucometer (Roche, Basel, Switzerland) and the OGT was determined according to Cardoso *et al.* (2011).

Table 1 Composition of basal diets for conventional and high-fat diet (g/100 g)

Ingredient	Conventional diet	High-fat diet
Corn starch	46.56	–
Fat (lard)	–	31.7
Casein	14	25.8
Dextrinized starch	15.5	16.2
Sucrose	10	8.9
Soybean oil	4	3.2
Microfine cellulose	5	6.5
Mineral mix	3.5	1.3
Vitamin mix	1	1.3
L-cystine	0.18	0.39
Choline bitartrate	0.25	0.3
Potassium citrate	–	2.1
Calcium phosphate	–	1.7
Calcium carbonate	–	0.7
Energy density (kcal g^{-1})	3.76	5.17

Viable bacteria enumeration after transit through the GIT

The survival of *L. paracasei* DTA81 after transit through the GIT was evaluated at the end of week 3 and at the end of the study (end of week 6). Three mice from each group (from different cages) were randomly selected and their faeces were collected, weighed resuspended in 10 ml of sterilized PBS and serially diluted using the same solution. Then they were plated on MRS medium supplemented with kanamycin (64 μg ml^{-1}; Sigma) and incubated at 37°C for 48 h. Resistance of *L. paracasei* DTA81 to kanamycin had been determined in a previous study (Tarrah *et al.* 2019). After incubation, colony forming units were counted and reported per gram of wet faeces. Besides, five colonies were also randomly taken from plates and investigated by Gram staining, catalase and oxidase tests. The same mice were used for this evaluation at week 3 and at week 6.

Determination of the lipid profile and transaminases

Blood samples collected from sacrificed animals were centrifuged at 700 ***g*** for 10 min to obtain the serums and immediately examined for total cholesterol, high-density lipoprotein (HDL), triglyceride, glutamate-oxaloacetate transaminase (GOT), and glutamate-pyruvate transaminase (GPT) using Bioclin kits (Diagnostics, Belo Horizonte, Brazil) and an auto-analyzer equipment (Analyzer BS-200; Mindray, Shenzhen, China). Low-density lipoprotein (LDL) was calculated according to the method of Friedwald et al. (Friedwald 1972).

Immunomodulatory effects on colon tissue

The commercial BD CBA Human Th1/Th2/Th17 Cytokine Kit II (BD Biosciences, San Jose, CA) and a BD FACSVers flow cytometer were used to quantitatively measure interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-17 (IL-17), tumour necrosis factor (TNF) and interferon-γ (IFN-γ) protein levels in mice colon samples. To perform local inflammatory cytokine analysis, colon tissue (100–200 mg) was ground using a tissue homogenizer (IKA WORKS GMBH & CO, Staufen, Germany, model T10 basic), washed in cold phosphate-buffered saline (pH 7·0) and centrifuged (10 000 ***g***, for 10 min at 4°C). Finally, the supernatant was collected and stored at −80°C for further analysis. The samples were prepared according to the manufacturer's instruction before being analysed by flow cytometry.

16S rRNA gene amplicon target sequencing

Four mice from each group (from different cages) were randomly chosen at the beginning and at the end of the experiment and their faeces were collected in a separated clean cage for each mouse and the samples were immediately stored at −80°C for further analysis. Later, 0·25 g of collected faeces was weighed and total DNA was extracted using the DNeasy PowerSoil Microbial Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The quality and quantity of the extracted DNA were assessed by the Spark 10 M spectrophotometer (Tecan Trading AG, Männedorf, Switzerland). The V3–V5 regions of the 16S rRNA gene were PCR amplified and sequenced using an Illumina MiSeq desktop sequencer (Eurofins Genomics Germany GmbH, Ebersberg, Germany) producing 300 bp paired-end (PE) reads.

Genome sequencing and genomic analysis of *L. paracasei* DTA81

Lactobacillus paracasei DTA81 was grown overnight in MRS broth at 37°C for 24 h and genomic DNA was isolated using the DNeasy PowerSoil Microbial Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The assessment of isolated DNA quality and quantity was done using Spark 10M spectrophotometer (Tecan Trading AG, Männedorf, Switzerland). *Lactobacillus paracasei* DTA81 genome was sequenced using the paired-end sequencing technology with NextSeq500 Illumina sequencer at the Interdepartmental Center for Research on Innovative Biotechnology CRIBI (CRIBI, Padova, Italy).

Bioinformatic analyses

The raw data of 16S rRNA gene sequencing were imported and analysed with the CLC Genomics Workbench software v.12.0.2 (Qiagen, Hilden, Germany) using the Microbial genomics module plugin as described by da Silva Duarte *et al.* (2020). In summary, quality filtering, operational taxonomic unit (OTU) clustering, taxonomical assignment (Greengenes v13_8 database), alpha- and beta-diversity indices calculation were performed with default parameters. Raw reads were deposited in the Sequence Read Archive (SRA) database (http://www.ncbi.nlm.nih.gov/sra) under the BioProject 'PRJNA638135'.

The Shannon and Chao 1 indices were compared among the experimental groups (CD, DTA81 and HFD) using ANOVA and Kruskal–Wallis tests, followed by Tukey's and Dunn's *post hoc* tests, correspondingly. The Welch's *t* test was chosen to identify significant differences inside the same group (intra-group comparison) between the time-points 't0' and 't1'. Both statistical analyses were conducted using GraphPad Prism software (version 7, GraphPad Software, Inc., San Diego, CA). For beta-diversity analysis, gut microbial dissimilarities among the groups were calculated by permutational multivariate analysis of variance (PERMANOVA) with 999 permutations using Unweighted and Weighted UniFrac diversity metrics. Principal coordinate analysis (PCoA) was chosen as the ordination method to explore and visualize the data.

After data inspection, data filtering (low count and low variance filters) and normalization (cumulative sum scaling), differential abundance analysis were carried with MicrobiomeAnalyst (Dhariwal *et al.* 2017) considering three taxonomic levels (phylum, family and genus) by applying the linear discriminant analysis (LDA) effect size (LEfSe) function setting up FDR-adjusted p value (*q* value) cutoff of 0·05 and log LDA score of 2·0. Pearson correlation with a complete linkage method was chosen to cluster the samples based on taxon abundance in a heat map graph generated with Heatmapper (Babicki *et al.* 2016).

Regarding DTA81 genome analysis, *de novo* assembly of the raw reads was done using Velvet algorithm package, ver. 1.1.04 setting on parameters as (min read length: 15, min average quality of read: 20 and min adapter match: 15) (Larsen *et al.* 2012). Rapid Annotation using Subsystems Technology (RAST) was also used for gene prediction and annotation (Aziz *et al.* 2008).

The PATRIC 3.6.3 server (Wattam *et al.* 2017) was used to construct a graphical genome map/annotation after scaffolding the related contigs using the Medusa web server (Bosi *et al.* 2015) and *L. paracasei* ATCC 334 as the reference genome.

The presence of prophage regions was predicted using the PHASTER server (Arndt *et al.* 2016). The detection of plasmid and transmissible antibiotic resistance genes was assessed using PlasmidFinder 2.0 and ResFinder 3.2 servers, respectively (Zankari *et al.* 2012; Carattoli *et al.* 2014).

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JAAVWK000000000. The version described in this paper is version JAAVWK010000000.

Statistical analyses

Data were analysed using one-way analysis of variance (ANOVA). Tukey's test was used as *post hoc* analysis by the GraphPad Prism software (version 7, GraphPad Software, Inc., San Diego, CA). In general, results were considered significantly different when *P* values were lower than 0·05. The number of asterisks is used to indicate the

level of confidence of the statistical analyses results: $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$.

Results

In-vitro cholesterol assimilation

Cholesterol reduction by *L. paracasei* DTA81 grown in MRS supplemented with 0·3% ox bile was measured *in-vitro*. DTA81 was able to reduce 16·16 ± 2·0% of the cholesterol presents in the medium after 24 h.

Weight gain and oral glucose tolerance

At the end of the experimental period (6 weeks), mice treated with DTA81 did not evidence a significant difference in weight gain (Fig. 1a); the weekly monitoring of animal weight (Fig. 1b) showed a ponderal increase of HFD during the last 2 weeks while DTA81 group had the same trend of CD. The influence of DTA81 supplementation on plasma glucose is indicated in Fig. 1c,d. Regarding the fasting blood sugar (FBS), a significant glucose reduction was recorded in the animals treated with *L. paracasei* DTA81 compared with the CD and HFD groups ($P < 0{\cdot}05$) (Fig. 1c). However, after receiving the glucose, no significant difference was recorded regarding the glucose tolerance among the groups, neither at the beginning nor at the end of the experiments (Fig. 1d).

Determination of the lipid profile and transaminases

Results of mice blood biochemical analyses at the end of the study (week 6) are reported in Fig. 2. Total cholesterol (TC), HDL and LDL evidenced a significant ($P < 0{\cdot}01$) reduction in the DTA81 group compared with the HFD group, whereas triglycerides, GOT and GPT did not show significant differences between any group. In addition, a significant difference ($P < 0{\cdot}05$) was detected for TC and HDL between groups HFD and CD while no statistically significant difference was seen for LDL.

Immunomodulatory activity in the colon tissue

Comparison of colon tissue levels of Th1 (IL2, TNF and IFN-γ), Th2 (IL4, IL6 and IL10) and Th17 (IL-17) at the end of the experiment (week 6) is presented in Fig. 3. The outcome revealed a significant reduction of IL-6 and IL-10 in the group treated with *L. paracasei* DTA81 when compared with CD and HFD groups. However, the level of cytokines IL-2, IL-4, IL-17, TNF and INF-γ did not show any significant difference between DTA81 and HFD or CD groups, except for INF-γ that showed a significant difference with the CD group.

16S rRNA sequencing analysis of the gut microbiota

A high-throughput 16S rRNA sequencing was performed to assess possible changes in the gut microbiota composition caused by the administration of high-fat diet or by *L. paracasei* DTA81 supplementation. A total of 536 330 reads from mice faecal microbiota were analysed with a mean of 22 621 (±8·191) sequences for each sample (8 samples for each experimental group; $n = 24$). Shannon index and phylogenetic diversity curves (Fig. S1) confirmed that the total number of reads obtained from 16S rRNA gene amplicon sequencing covered most of the microbial diversity and that the majority of bacterial phylotypes inside each group were sampled.

At the end of the experimental period (t1), a significant reduction in faecal microbial diversity was observed inside all groups (Fig. 4a), as evidenced by Shannon's diversity index, compared to the same groups at the beginning of the experiment (week 0; t0). A reduced Chao1 value (richness diversity index) was also detected at the end of the experimental period in faecal samples of all animals regardless the experimental group. However, only animals of the HFD group displayed a significant reduction in diversity compared to the same group at the beginning of the trial (Fig. 4b).

The investigation of community structures of faecal samples of BALB/c mice was done using the phylogenetic distance-based measurements weighted and unweighted UniFrac. As shown in Fig. 4c,d, scatter plot of the PCoA using both distance metrics revealed a significant microbial shift (PERMANOVA weighted UniFrac: $P = 0{\cdot}001$, Pseudo-*f* statistic = 4·87; PERMANOVA unweighted UniFrac: $P = 0{\cdot}001$, Pseudo-*f* statistic = 2·24) after the experimental period in all groups enrolled in this study, although a more pronounced difference was observed when weighted UniFrac distance metric was considered, which accounts for the relative abundance of OTUs. Supplementation of strain DTA81 significantly changed the intestinal microbial composition when compared to the groups receiving a CD (PERMANOVA weighted UniFrac: $P = 0{\cdot}029$, Pseudo-f statistic = 4·49; PERMANOVA unweighted UniFrac: $P = 0{\cdot}031$, Pseudo-*f* statistic = 1·50) and an HFD (PERMANOVA unweighted UniFrac: $P = 0{\cdot}027$, Pseudo-*f* statistic = 1·60). A significant difference in terms of beta-diversity of the intestinal microbiota was also observed between CD and HFD groups (PERMANOVA weighted UniFrac: $P = 0{\cdot}023$, Pseudo-*f* statistic = 3·11; PERMANOVA unweighted UniFrac: $P = 0{\cdot}025$, Pseudo-*f* statistic = 1·60). In total, weighted and unweighted UniFrac components (PCoA 1 and PCoA 2) accounted, respectively, for 53 and 66% of the total variance.

The relative distributions of bacteria at the phylum, family and genus level identified by 16S rRNA gene

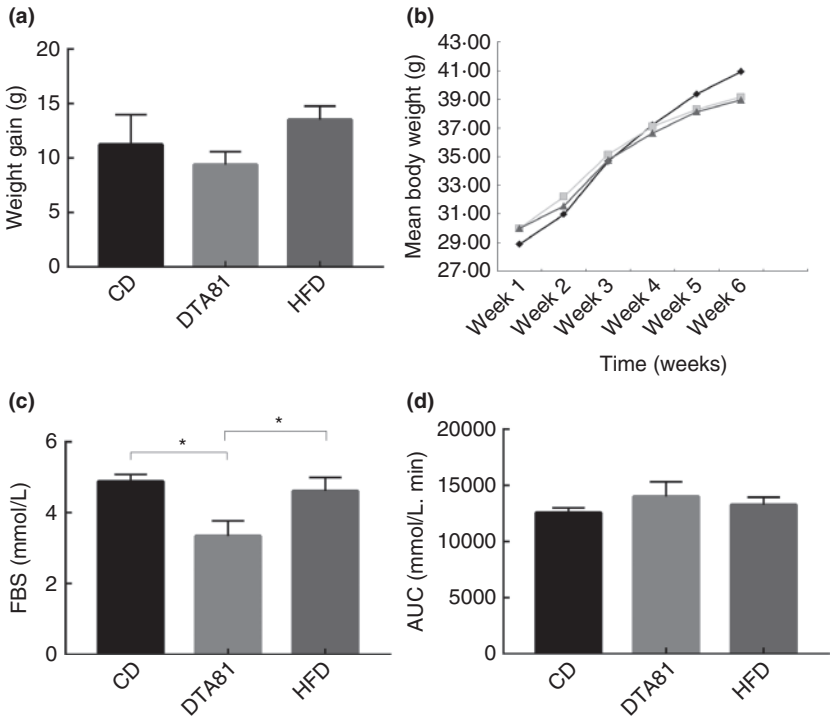

Figure 1 Effect of probiotic consumption on body weight, fasting blood sugar (FBS) and glucose tolerance test. (a) Mean body weight; (b) weight gain at the end of the experiment; (c) fasting blood sugar (FBS); (d) glucose tolerance test. Results are expressed as means ± SEM (n = 8). The number of asterisks is used to indicate the level of confidence of the statistical analyses results: *statistically significant $P < 0{\cdot}05$, (—♦—) HFD; (—■—) DTA81; (—▲—) CD.

amplicon sequencing are reported in Fig. S2. Prevalent phyla considering all groups were *Firmicutes* (65%), *Proteobacteria* (16%) and *Bacteroidetes* (6%). At t1, the consumption of a high-fat diet (HFD group) significantly increased *Firmicutes* (LDA = 3·9), *Actinobacteria* (LDA = 3·3), *Deferribacteres* (LDA = 2·8) and *Spirochaetes* (LDA = 2·2), whereas the supplementation of DTA81 significantly increased *Bacteroidetes* (LDA = 3·1) (Fig. 5). Considered important in the development of obesity, the *Firmicutes*/*Bacteroidetes* (F/B) ratio was calculated and results show that the potential probiotic intervention improved on average the proportion F/B (7·8 ± 4·65) when compared to the groups that received a CD (11·11 ± 3·95) or a high-fat diet (18·71 ± 12·65).

At family level, Lachnospiraceae, Ruminococcaceae, Erysipelotrichaceae, Lactobacillaceae and Desulfovibrionaceae were the top five taxa (Fig. S2b). At the end of the experimental period, major changes were observed mainly in samples from the HFD group, where four families (Bifidobacteriaceae, LDA = 3·3; Desulfovibrionaceae, LDA = 3·2; Ruminococcaceae, LDA = 3·1; Deferribacteraceae, LDA = 2·8) resulted overrepresented in this group (Fig. 5). Only the family Lactobacillaceae increased in the CD group (LDA = 3·7), while in the DTA81 group there were no statistically significant biomarkers (LDA score <2 and/or q value >0·05) after the potential probiotic supplementation.

With regard to the genera observed in faecal samples across the groups, *Lactobacillus*, *Oscillospira*, *Allobaculum*, *Helicobacter* and *Ruminococcus* were identified as the top five taxa (Fig. S2c). Linear discriminant effect size (LEfSe) analysis revealed an enrichment of 13 biomarkers (Fig. 5), among which a higher relative abundance of the genera *Oscillospira* (LDA = 3·0) and *Coprococcus* (LDA = 2·2) were associated with *L. paracasei* DTA81 administration. In stool samples of mice of the CD group, an enrichment of the genera *Lactobacillus* (LDA = 3·7) and *SMB53* (LDA = 2·6) was observed at the end of the experimental period. Lastly, the HFD was associated with a higher proportion of *Allobaculum* (LDA = 3·4), *Bifidobacterium* (LDA = 3·3) and *Mucispirillum* (LDA = 2·8) in faeces samples.

L. paracasei DTA81 cell enumeration after mice GI transit

Survival of *L. paracasei* DTA81 after passage through mice GIT was evaluated after 21 (week 3) and 42 (week

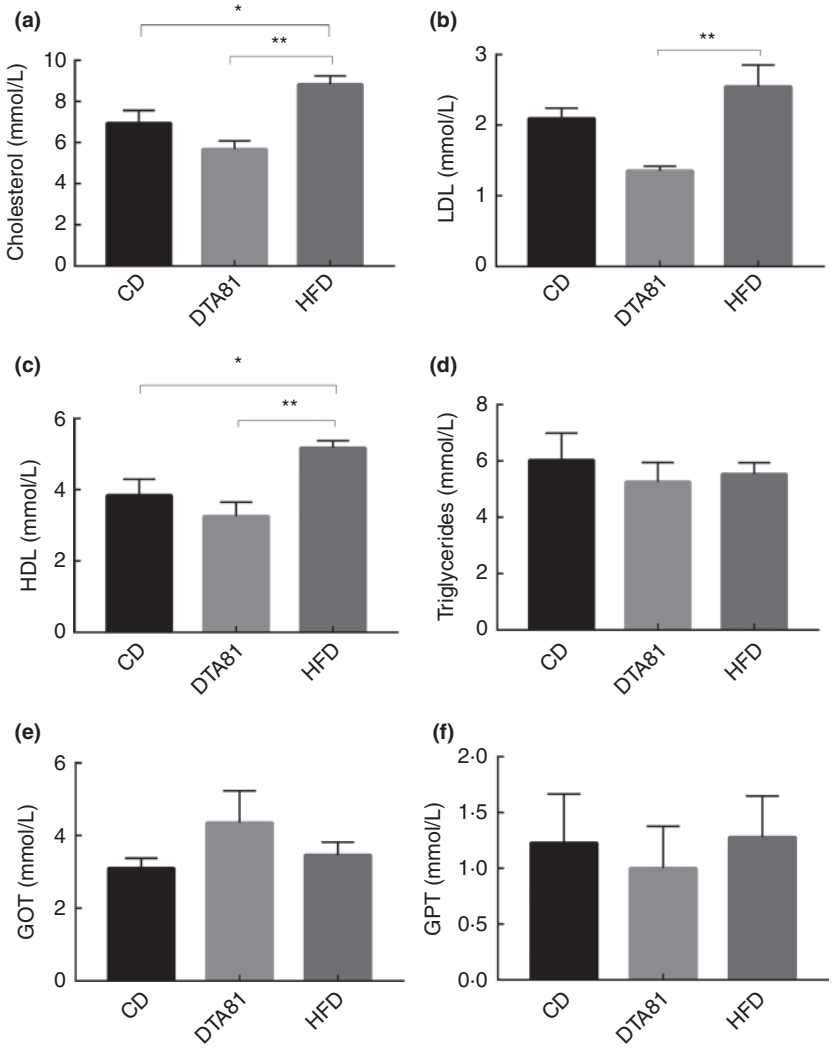

Figure 2 Effect of different treatments on blood parameters. (a) Total cholesterol; (b) high-density lipoprotein (HDL); (c) low-density lipoprotein (LDL); (d) triglyceride; (e) glutamate-oxaloacetate transaminase (GOT); (f) glutamate-pyruvate transaminase (GPT); Results are expressed as means ± SEM (n = 8). The number of asterisks is used to indicate the level of confidence of the statistical analyses results: *statistically significant $P < 0{\cdot}05$, **statistically significant $P < 0{\cdot}01$.

6) days. The strain was administered to the animals at the dose of approx. 10^9 CFU per day and viable cells were then enumerated from collected faeces. After week 3, potential probiotic-treated animals revealed 8·50 ± 0·16 log CFU per g of wet faeces while no colony was recovered from CDC and HFD groups. After week 6, no significant difference in the number of retrieved cells (8·67 ± 0·23 log CFU per g) was recorded.

Genome sequencing and genomic analysis of *L. paracasei* DTA81

Sequencing of *L. paracasei* DTA81 genome produced 1 443 422 reads with an average size of 150·5 bp. The assembled genome of *L. paracasei* DTA81 produced 39 scaffolds, giving a genome size of 3·00 Mb with a GC content of 46·1% (Table 2). RASTtk server predicted a total number of 3077 protein-coding sequences (CDSs) classified into 236 different subsystems. The largest part of this subsystem is allocated to the carbohydrate metabolism (22·03%) followed by amino acids and derivatives (13·45%) and protein metabolism (11·86%). A total number of 59 structural RNAs including 3 complete rRNAs (5S, 16S and 23S) and 56 tRNAs were predicted, too. The average nucleotide identity (ANI) of 98·7% with the closest neighbour (*L. paracasei* ATCC 334) allowed a precise taxonomical placement of strain DTA81 inside the species *L. paracasei*. In Fig. 6, a circular graphical

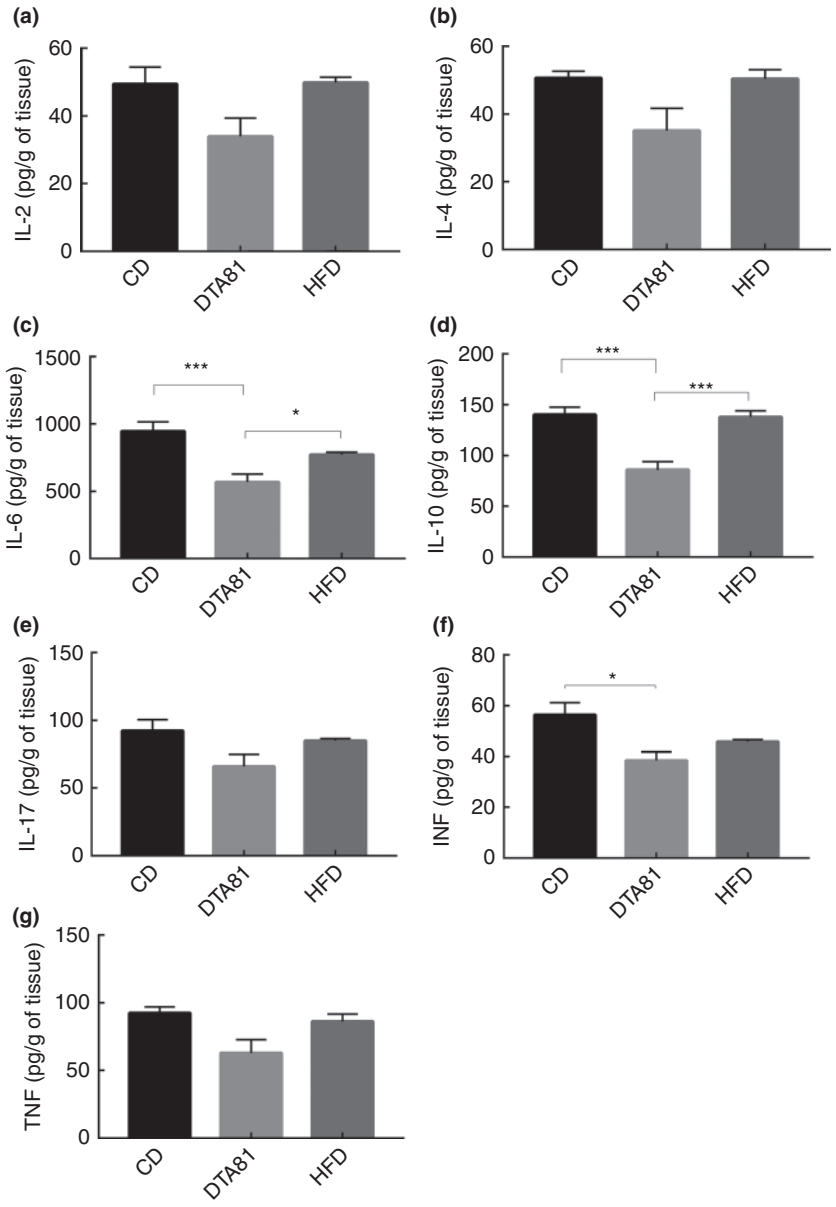

Figure 3 Effect of different treatments on local cytokines. (a) Interleukin-2; (b) interleukin-4; (c) interleukin-6; (d) interleukin-10; (e) interleukin-17; (f) interferon gamma; (g) tumour necrosis factor. Results are expressed as means ± SEM (n = 8). The number of asterisks is used to indicate the level of confidence of the statistical analyses results: *statistically significant $P < 0{\cdot}05$, ***statistically significant $P < 0{\cdot}001$.

map of the distribution of the genome annotations of *L. paracasei* DTA81 is provided which indicates the location of CDS on the forward and reverse strands, RNA genes, antimicrobial resistance genes and virulence factors. A deep analysis within the genome of DTA81 has also revealed the presence of cholesterol assimilation-related genes namely, *ccpA*, *fba*, *lbpg_rs09895*, *lbpg_rs11190* and *lbpg_rs10085* (Table 3) (Lee *et al*. 2010). Finally, the absence of mobile elements such as prophage regions, acquired antibiotic resistance genes and plasmid sequences on DTA81 genome was confirmed using PHASTER, PlasmidFinder and ResFinder servers respectively.

Discussion

It has been reported that the presence of probiotics inside the gut microbiota can be considered as a potential therapeutic strategy for some metabolic disorders such as hyperglycaemia, hypercholesterolemia and obesity (Janssen and Kersten 2015).

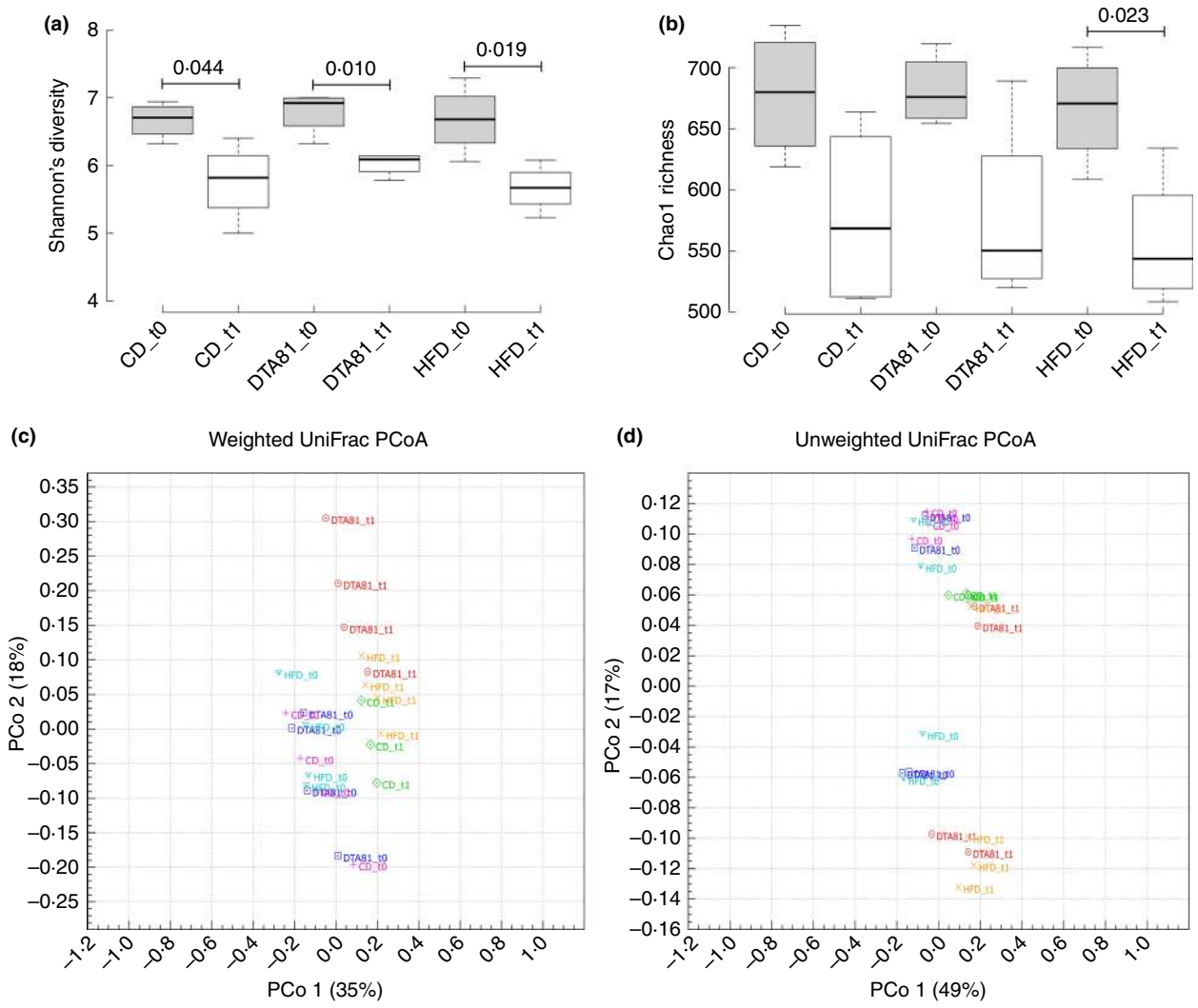

Figure 4 Alpha- and beta-diversity analysis of faecal samples. Box and whisker plots comparing the alpha-diversity indices Shannon diversity (a) and Chao1 richness (b) among the three groups (CD, DTA81 and HFD) before (t0) and after (t) their respective intervention. Horizontal bold lines show the median values. The bottom and top of the boxes show the 25th and the 75th percentiles respectively. The whiskers extend up to the most extreme points within 1·5 times the interquartile ranges (IQR). Level of significance: $P \leq 0{\cdot}05$. Principal coordinate analysis (PCoA) based on Weighted (c) and Unweighted (d) UniFrac distances for CD, DTA81 and HFD in two different time-points (t0 and t1). PERMANOVA with 999 permutations was used to detect significant microbial community structure dissimilarity in the different experimental groups enrolled in this study. , (▽) HFD_t0, (⊡) DTA81_t0, (◇) CD_t1, (×) HFD t1, (⊙) DTA81 t1, (+) CD_t0. [Colour figure can be viewed at wileyonlinelibrary.com]

In this study, mice treated with DTA81 did not evidence a significant difference in weight gain. It should be noted that the use of a high-fat diet does not always cause changes of total body weight, and metabolic changes related to obesity, such as hyperlipidaemia, hyperglycaemia, diabetes and low-grade inflammation, should also be observed. In a recently published study, the GTT was recorded significantly higher in BALB/c mice that had received a high-fat diet compared to BALC/c mice that received a control diet. This indicates that the high-fat diet interfered with the glucose metabolism in these mice; besides, serum levels of total cholesterol, LDL-c, triglycerides/HDL-c ratio, liver and adipose tissue were higher in BALC/c mice that had received the high-fat diet. However, there was no change in body weight (Li *et al.* 2020).

After 6 weeks of treatment, a significant fasting blood glucose reduction was recorded in the animals treated with *L. paracasei* DTA81 compared with the CD and HFD groups. The reduction of fasting blood glucose by DTA81 compared to the group receiving a CD is very interesting since the CD (control) contains higher

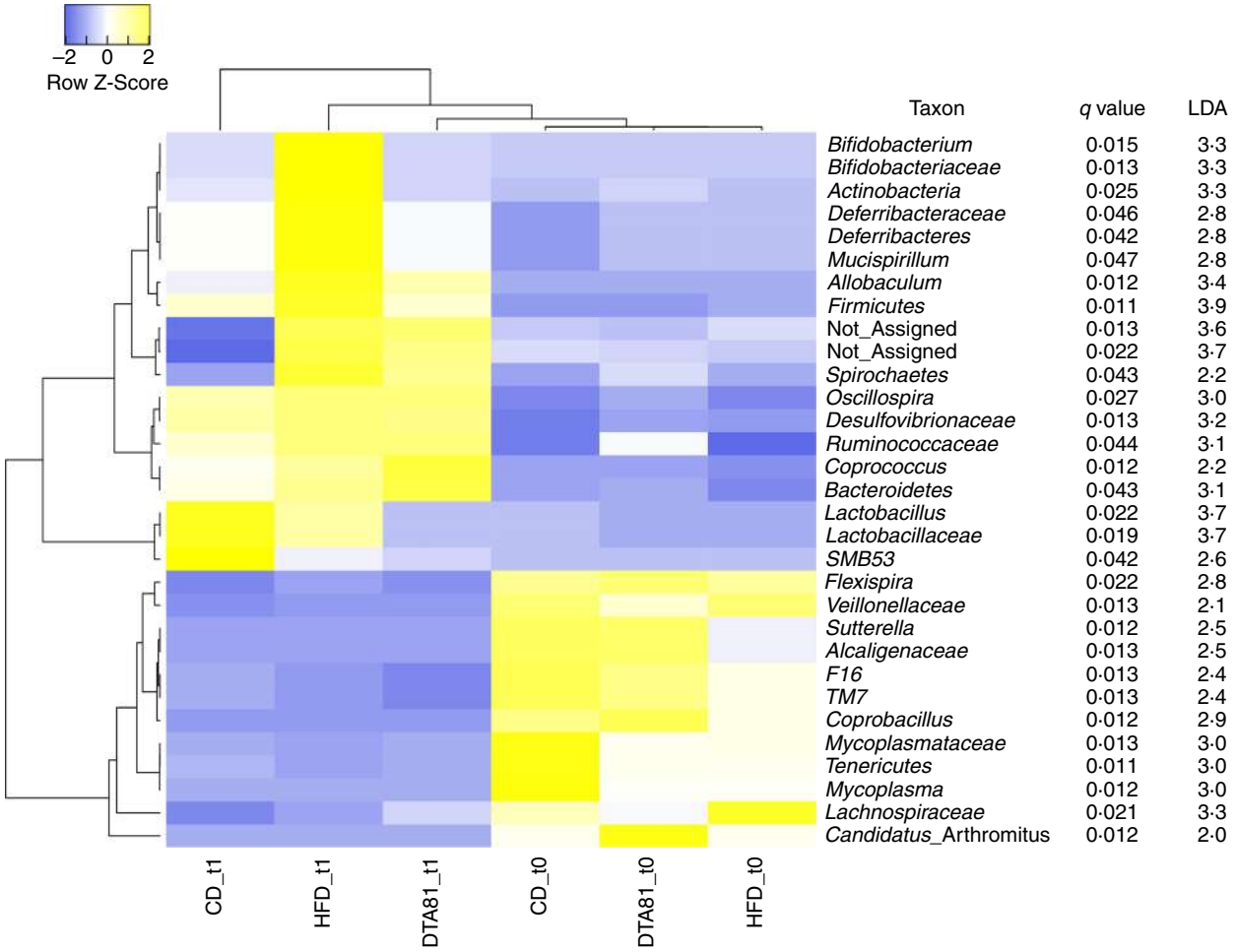

Figure 5 Heat map based on linear discriminant analysis effect size (LEfSe) at phylum, family and genus levels. An FDR adjusted *P* value (or *q* value) less than 5% and an LDA score greater than 2 were used to determine taxa that are significantly enriched among the groups CD, DTA81 and HFD before (t0) and after (t1) the experimental period. In the heat map, an enrichment trend is reported in yellow while depletion is represented in blue. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 2 Main characteristics of *L. paracasei* DTA81 genome

Feature	Value
Genome size	3 002 945
G+C content (%)	46.2
Contig N50	156 284
Contig L50	7
Number of scaffolds	39
Number of protein coding sequences (CDSs)	3077
Number of rRNAs	3
Number of tRNAs	56
Number of genes related to Virulence, disease, and defense	0

carbohydrate concentrations than that of the high-fat diet; therefore, our findings demonstrate the possible benefit of DTA81 in this condition too. The mechanism of glucose-lowering by probiotics is not well understood, but probiotics can modulate the human immune system which can influence glucose metabolism. Moreover, probiotics can reduce the inflammatory cytokines and regulate the immune system (de LeBlanc and Perdigón 2010).

Laitinen *et al.* (2008) demonstrated that the immunomodulatory effect of probiotics can lead to glucose reduction. In our study, *L. paracasei* DTA81, with its fasting blood-sugar-lowering activity, induced significantly lower values for interleukin 6 and 10.

Probiotics can also lower blood cholesterol in different ways, indirectly by fermentation of prebiotics and consequent production of SCFA in the human gut that can further inhibit hepatic cholesterol synthesis and will result in a reduction of blood lipids (Ashaolu *et al.* 2020). Alternatively, probiotics can assimilate cholesterol

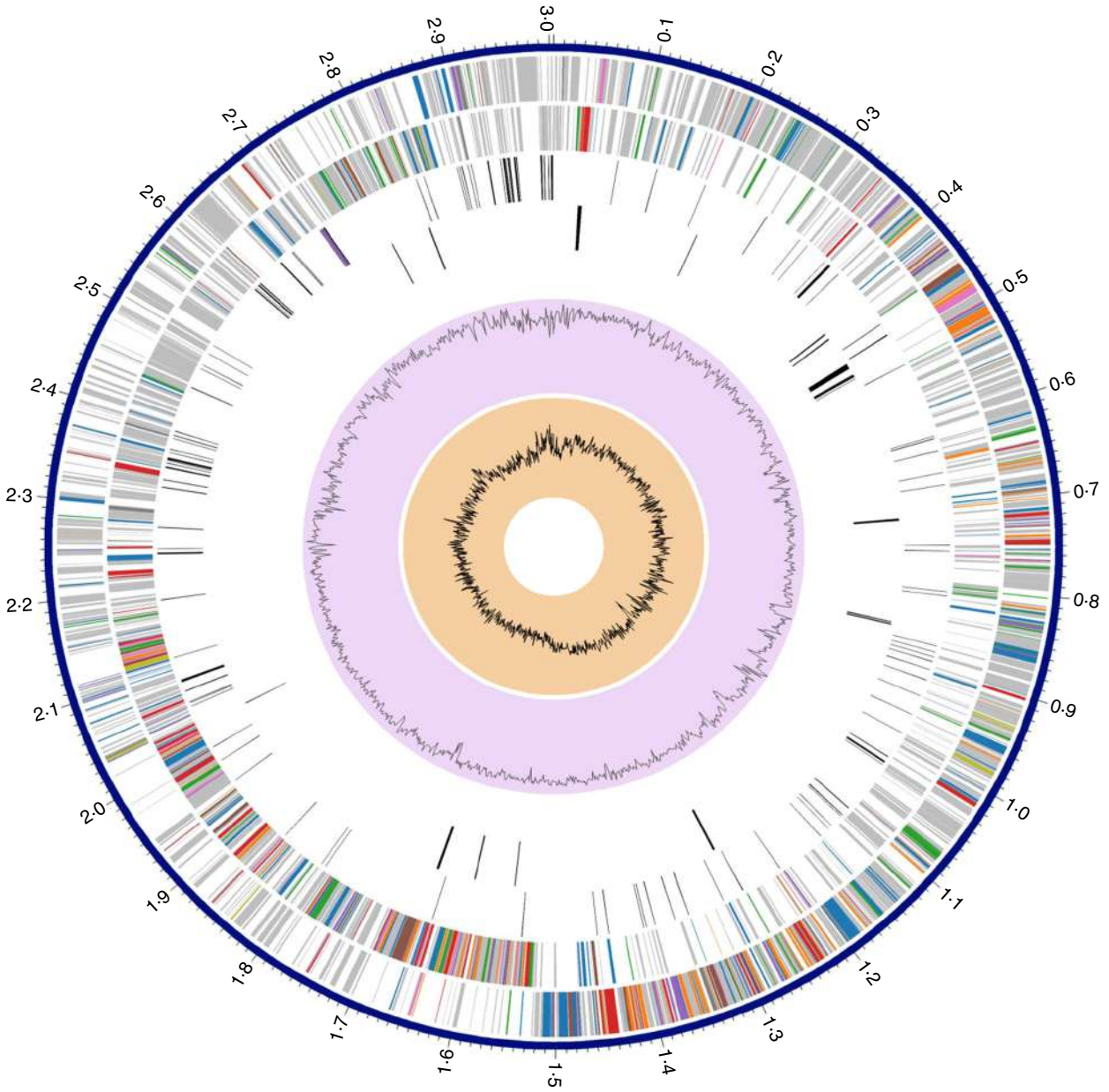

Figure 6 Circular graphical display of the distribution of the genome map and annotations. This includes, from outer to inner rings, CDS on the forward strand, CDS on the reverse strand, RNA genes, CDS with homology to known antimicrobial resistance genes, CDS with homology to known virulence factors, GC content and GC skew. The colours of the CDS on the forward and reverse strand indicate the subsystem that these genes belong to. (●) Metabolism, (●) Protein processing, (●) Stress response, Defence, Virulence, (●) DNA processing, (●) Energy, (●) Cellular processing, (●) RNA processing, (●) Membrane transport, (●) Cell envelope, (●) Regulation and cell signalling, (●) Miscellaneous. [Colour figure can be viewed at wileyonlinelibrary.com]

directly, thus eliminating it from the human gut (Pereira and Gibson 2002; Pan *et al.* 2011; Öner *et al.* 2014). In their study, Shimizu *et al.* (2015) reported that consumption of probiotics by elderly and hypercholesterolaemic patients could be more effective than in youngsters and individuals with normal lipid levels. Besides, probiotics can reduce cholesterol levels by assimilating and entrapping this molecule into bacterial membranes (Castorena-Alba *et al.* 2018; Bhat and Bajaj 2020). There are numerous species of *Lactobacillus* and *Bifidobacterium* that have shown cholesterol assimilation in *in-vitro* experiments and it has been reported that this ability is strictly strain-

Table 3 Identification of cholesterol assimilation-related genes in *Lactobacillus paracasei* DTA81

Gene	Gene description	Organism	Identity score (%)	Accession number
ccpA	Catabolite control protein A	*L. paracasei* AO356	100.00	CP025499.1
fba	Class II fructose-1,6-bisphosphate aldolase	*L. paracasei* N1115	100.00	CP007122.1
lbpg_rs09895	Glycogen phosphorylase	*L. paracasei* AO356	100.00	CP025499.1
lbpg_rs11190	FMN-binding protein	*L. paracasei* W56	100.00	HE970764.1
lbpg_rs10085	MFS transporter	*L. paracasei* N1115	100.00	CP007122.1

dependent (Costabile *et al.* 2017; Castorena-Alba *et al.* 2018). Taking into account the published data in the literature, cholesterol assimilation by probiotic strains can range from 0·86% to more than 40%; however, we usually do not see the same reduction when we use probiotics in the *in-vivo* conditions, which can be due to the effect of gastrointestinal conditions of the strains, microbial competition, colonization on the epithelial cells, etc. (Belviso *et al.* 2009; Tokatlı *et al.* 2015; Castorena-Alba *et al.* 2018). The outcome of our study indicates that consumption of *L. paracasei* DTA81 can lead in mice to a statistically significant reduction of total cholesterol and LDL and HDL, which appears very interesting and useful in people who suffer from CVD and CHD. This ability by *L. paracasei* DTA81 could be connected to the presence of cholesterol assimilation-related genes, coding membrane-associated proteins that can adhere to the cholesterol molecule and further incorporate it inside the cell (Lee *et al.* 2010). Besides, in our previous study (Tarrah *et al.* 2019), DTA81 had revealed a great adhesion capability to human cell lines, which could play a significant role in its potential beneficial activity.

Gut microbiota dysbiosis can also promote the occurrence of metabolic syndromes in diverse ways, such as low-grade inflammation, through increased production of bacterial lipopolysaccharide. In the present study, we detected an increase in *Bacteroidetes* and *Coprococcus* after 6 weeks of treatment with strain DTA81. The phylum *Bacteroidetes* belongs to Gram-negative bacteria that normally colonize the human lower gastrointestinal tract during infancy, due to the abundance of non-digestible oligosaccharides in mother's milk which support their growth (Marcobal *et al.* 2011). Since colonization, they play an essential role by breaking down complex sugars and degrading proteins in the human gut, as well as by exerting an immunomodulatory effect (Rakoff-Nahoum *et al.* 2004; Rajilić-Stojanović and de Vos 2014). Another important function of this bacterial group in the human gut is related to the deconjugation of bile acids which is linked to cholesterol-lowering activity (Narushima *et al.* 2006; Leitch *et al.* 2007). Also, it has been reported that, due to the broad metabolic potential of *Bacteroidetes*, their reduced abundance could be linked to obesity in humans (Ley 2010). On the other side, it was proven that the increase in the genus *Coprococcus,* an anaerobic genus which is normally present in the human faecal microbiota, can have an anti-carcinogenic and anti-inflammatory effect in the human gut, due to butyric acid production (Hamer *et al.* 2008; Ai *et al.* 2019).

As determinants and modulators of immune pathology, cytokines play a key regulatory role among the many components of the animal immune system (Lin and Karin 2007). A broad spectrum of cells such as fibroblasts, endothelial cells, neuronal cells, macrophages and mast cells can produce cytokines; however, production is mainly dependent from the differentiation state of T cells, which can be divided into three different types according to the pattern of cytokine production (Saito *et al.* 2010; Yang *et al.* 2017). Among the cytokines considered in the present study, IL-2, TNF-α and IFN-γ are produced by T helper 1 cells and play an important role in the cell-mediated immune response. By contrast, IL-4, IL-6 and IL-10 are secreted by T helper 2 cells and enhance humoral immunity (Kikuchi and Crystal 2001). Moreover, we have studied the IL-17 secreted by T helper 17 cells which is involved in allergic responses by inducing and mediating the proinflammatory responses (Korn *et al.* 2009). Overproduction or inappropriate production of certain cytokines by the body can result in inflammatory diseases.

Occasionally, it has been reported that the insertion of external bacterial cells inside the human body leads to inappropriate production of certain cytokines which can cause inflammatory diseases (Percoco *et al.* 2013; Cattaneo *et al.* 2017). In our study, the group of mice treated with *L. paracasei* DTA81 showed lower average values for IL-6 and IL-10 when compared to CD and HFD groups. Cytokine IL-6 is locally produced in response to infection or injury and delivered to other body parts by the bloodstream, activating immunological defences. IL-6 also stimulates intestinal epithelial proliferation and repair after injury (Kuhn *et al.* 2014); however, excessive or prolonged production of IL-6 is involved in various diseases (Narazaki and Kishimoto 2018). Several studies have reported that IL-6 is a pro-inflammatory cytokine that is detected in higher amounts in obese individuals and

contributes to the occurrence of type 2 diabetes, insulin resistance and CVDs (Higa and Panee 2011; Shi *et al.* 2019). The IL-6 level observed in this work does not differ between CD and HFD groups and can be explained by the reduced duration of the experiment when compared to other studies in the literature assessing the immunomodulatory effect of probiotics (8–14 weeks) (Sheil *et al.* 2006; Antunes *et al.* 2020). Moreover, it can be related to a potential pro-inflammatory property of the commercial diet, which has approximately 56% carbohydrate, a component that at high concentrations induces inflammation in skeletal muscle (Antunes *et al.* 2020). As regards the reduction of IL-10, it could be explained considering the strong positive correlation between IL-6 and IL-10 normally found in the human body, which contributes to homeostasis maintenance (Dizdarević-Hudić *et al.* 2009; Sapan *et al.* 2016). The IL-10 level was reduced by DTA81 intake, and a significant difference was observed compared to the other two groups. Although the increase in IL-10 level is referred to as the immunomodulatory effect of probiotics, this is not the unique form of probiotics action, which depends on the bacterial strain, concentration and administration method. The reduction level of IL-10 may decrease the immunostimulatory effect of this cytokine in innate immunity (de Moreno de LeBlanc *et al.* 2011).

It is well known that probiotic traits are strain-specific and this gives strong motivation to keep seeking new potentially better strains (Senok *et al.* 2005). In our study, L. paracasei DTA81 indicated a good resistance to the gastrointestinal environment in-vivo. The resistance of lactobacilli to the harsh GIT conditions reported previously (Noriega *et al.* 2004; Burns *et al.* 2010) seems to be linked to the preservation of cell internal pH, functionality and integrity of cell membrane, and to the existence of bile salt efflux pumps (Bustos *et al.* 2011; Wu *et al.* 2012, 2014).

The outcomes of this study revealed the possible mechanism that started from the gut microbiota regulation by *L. paracasei* DTA81 with increasing the abundance of *Bacteriodetes* and *Coprococcus* taxa followed by a significant reduction of inflammatory cytokine interleukin 6 which has subsequently led to a decrement of FBS. On the other side, total cholesterol reduction in the group treated with DTA81 could be possibly related to the above-mentioned gut microbiota modulation as well as the direct cholesterol assimilation by *L. paracasei* DTA81. Overall, considering the results of this study alongside the previous findings, *L. paracasei* DTA81 has a great potential to be used as a commercial promising probiotic with great influence on metabolic homeostasis in individuals, particularly those adopting high-fat diets.

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Conflict of Interest

No conflict of interest declared.

Author Contributions

Conceptualization: A.T., B.C.S.C. and V.S.D.; investigation: A.T., B.C.S.C., V.S.D., R.S.D., S.P., L.L.O.; data curation: A.T. and V.S.D.; writing—original draft preparation: A.T.; writing—review and editing: A.T. and A.G.; supervision: A.G., S.O.P., M.C.G.P., L.L.O.; funding acquisition: A.G., V.C., S. O. P. and M.C.G.P.; All authors have read and agreed to the published version of the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Rarefaction curves of Shannon entropy (a) and phylogenetic diversity (b) of stool samples before (t0) and after (t1) 6 weeks of experimental period.

Figure S2. Relative abundance of bacterial phyla (a), top-20 families (b), and top-20 genera (c) identified in feces samples of BALB/c mice in groups CD, DTA81 and HFD before (t0) and after (t1) the experimental period.

BioConserv Market Potential: Analysis of Distribution Opportunities and Strategies

Market Potential

BioConserv, developed by BRC Ingredientes, is a revolutionary bioaroma with antimicrobial and probiotic properties, offering significant advantages over conventional food preservatives. With a drastic reduction in sodium content and enhanced efficacy, BioConserv meets the rising consumer demand for healthier, safer, and more natural food products. The increasing awareness of food safety and health benefits associated with probiotic and postbiotic products positions BioConserv as a promising solution in the global food preservative market, which is expected to grow significantly in the coming years.

1. **International Markets:**

    - **Asia-Pacific and Europe:** These regions, known for their high demand for natural food products and stringent food safety regulations, offer substantial market potential. BRC can leverage its existing certifications, like Halal and FSSC 22000, to build trust and enter these markets. BioConserv's efficacy in extending shelf life, reducing product returns, minimizing losses, and preventing financial setbacks for industries will resonate strongly.

    - **Middle East:** The Halal certification specifically opens doors to markets in the Middle East, where adherence to Islamic dietary laws is essential. Additionally, promoting BioConserv's ability to extend shelf life can help reduce product waste and financial losses for businesses in these regions.

    - **Latin America, China, and Africa:** In markets such as Mexico, China, and some African countries, where cost efficiency is crucial, BioConserv's capability to extend shelf life is particularly valuable. By reducing product returns, minimizing losses, and preventing financial setbacks for industries, BioConserv can be a significant asset.

    - **North America and Japan:** In more premium markets, the focus should be on BioConserv's health benefits, such as its postbiotic properties that contribute to consumer well-being. Highlighting these health benefits can attract health-conscious consumers who prioritize high-quality, health-enhancing food products. This approach can help BRC differentiate BioConserv in markets where shelf life issues are less prevalent.

By tailoring marketing strategies to the specific needs and priorities of different international markets, BRC can maximize BioConserv's impact and expand its global reach.

Strategies to Maximize BioConserv’s Market Impact

1. **Partnerships and Collaborations:**

- Scientific and Academic Institutions: Continue and expand collaborations with universities and research institutions to further validate BioConserv’s benefits and applications. This can lead to new product innovations and stronger scientific backing.

- Industry Partnerships: Partner with established food manufacturers to incorporate BioConserv into their product lines, providing case studies and testimonials that highlight its effectiveness.

2. **Marketing and Branding:**

- **Health and Safety Campaigns:** Emphasize the health benefits, such as reduced sodium intake and the presence of postbiotic properties, through targeted marketing campaigns. Highlighting these benefits can resonate with health-conscious consumers.

- **Shelf Life Extension:** Promote the significant shelf life extension capabilities of BioConserv. By ensuring longer preservation of meats and related products, BioConserv not only reduces waste but also provides a cost-effective solution for both retailers and consumers. This shelf life extension helps diminish product returns, reduce losses, and prevent consequent financial setbacks for industries. Highlighting these advantages can appeal to businesses aiming to enhance their supply chain efficiency and profitability.

- **Certifications and Quality Assurance:** Utilize the Halal and FSSC 22000 certifications in marketing materials to build trust and credibility. Display these certifications prominently on product packaging and marketing collateral. These certifications not only assure quality and safety but also expand market reach to include consumers with specific dietary requirements.

3. **Sustainability Focus:**

- Highlight Environmental Benefits: Market BioConserv's 100% yield process and lack of effluent generation as a significant environmental benefit. Position the product as a sustainable choice for eco-conscious consumers and companies.

- Align with Global Sustainability Goals: Align BioConserv's marketing strategy with global sustainability initiatives and trends, such as the reduction of food waste and the promotion of sustainable agriculture.

4. **Innovation and Product Development:**

- New Applications: Invest in research and development to explore new applications of BioConserv in different food products and industries. This can include ready-to-eat meals, snacks, and beverages.

- Enhanced Formulations: Develop enhanced formulations of BioConserv that cater to specific industry needs, such as tailored antimicrobial properties for different food matrices.

BioConserv holds significant market potential due to its unique properties and alignment with current consumer trends toward health and sustainability. By strategically expanding distribution, forming key partnerships, and emphasizing its certifications and health benefits, BRC Ingredientes can maximize BioConserv's impact in the market. Through continuous innovation and a strong marketing strategy, BioConserv can become a leading natural preservative in the global food industry.

Conclusion

Recap of Key Points

1. **BioConserv's Unique Properties and Benefits:**

- BioConserv is a groundbreaking bioaroma derived from natural sources with antimicrobial and probiotic properties.
- It offers significant health benefits, including anti-inflammatory, immunomodulatory, antioxidant, and antimicrobial effects.

- BioConserv contains about ten times less sodium than conventional preservatives and is approximately five times more effective.

2. **Scientific Validation and Research:**

- Extensive in-vitro and in-vivo studies have demonstrated BioConserv's efficacy in reducing cholesterol and fasting blood sugar levels, modulating immune responses, and altering gut microbiota favorably.

- Research indicates BioConserv's potential as a postbiotic, showing benefits regardless of probiotic viability and contributing to gut-brain axis health.

3. **Market Potential and Distribution Opportunities:**

- Growing consumer demand for natural, healthier, and safer food preservatives aligns perfectly with BioConserv's properties.

- Significant opportunities exist in international markets, particularly in regions with strict food safety regulations and a preference for natural products.

- Distribution channels include direct sales to food manufacturers, retail health food stores, online platforms, and international markets.

4. **Recognition and Certifications:**

- BioConserv has received prestigious industry awards, such as the Fi Innovation Awards, for its innovative and sustainable properties.
- BRC's commitment to quality and safety is further validated by certifications like Halal and FSSC 22000, ensuring products meet high standards for both Muslim and non-Muslim consumers.

5. **Sustainability and Social Responsibility:**

- BioConserv's production process is sustainable, with no effluent generation and the use of natural/organic inputs.
- The product supports reduced sodium intake, decreased reliance on chemically synthesized preservatives, and minimized food waste.

Future Outlook for BioConserv and BRC

BioConserv:

- Expansion and Market Penetration: BioConserv is well-positioned to penetrate new markets, especially in regions with high demand for natural preservatives. Efforts will focus on leveraging certifications and unique benefits to gain a competitive edge.

- Continuous Innovation: Ongoing investment in research and development will lead to new applications, enhanced formulations, and broader use across various food categories. BioConserv will remain at the forefront of food preservation technology.

- Sustainability Focus: As global sustainability concerns grow, BioConserv's eco-friendly production and health benefits will become increasingly attractive. BRC will emphasize these advantages in marketing strategies.

BRC:

- Leadership in Food Science and Technology: BRC will continue to be a leader in food science and technology, driven by innovative products like BioConserv. The company's commitment to high-quality research and standards will support this leadership.

- Strengthening Partnerships: BRC will deepen collaborations with scientific institutions and industry partners, enhancing credibility and scientific backing while expanding market reach.

- Customer-Centric Approach: BRC will maintain its focus on meeting the specialized needs of clients, offering tailored solutions that enhance food safety and quality. This approach will help build long-term relationships and trust.
- Sustainable Practices: BRC will prioritize sustainability in operations, aligning products and processes with global sustainability goals. This commitment will appeal to environmentally conscious consumers and businesses.

In conclusion, BioConserv and BRC have a bright future, driven by innovative solutions, scientific validation, and a strong commitment to sustainability and customer satisfaction. By capitalizing on BioConserv's unique properties and market potential, and through strategic initiatives and partnerships, BRC is poised for significant growth and a lasting impact in the food industry.

BRC

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The Revolution in Scientific Conservation