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Qualification of tropical fruit-derived *Lactobacillus plantarum* strains as potential probiotics acting on blood glucose and total cholesterol levels in Wistar rats

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ABSTRACT

Tropical fruit and their industrial processing byproducts have been considered sources of probiotic *Lactobacillus*. Sixteen tropical fruit-derived *Lactobacillus* strains were assessed for growth-promoting effects using a host-commensal nutrient scarcity model with *Drosophila melanogaster* (*Dm*). Two *Lactobacillus* strains (*L. plantarum* 49 and *L. plantarum* 201) presenting the most significant effects ($p \leq .005$) on *Dm* growth were selected and evaluated for their safety and beneficial effects in adult male Wistar rats during 28 days of administration of 9 log CFU/day, followed by 14 days of wash-out. Daily administration of *L. plantarum* 49 and *L. plantarum* 201 did not affect ($p > .05$) food intake or morphometric parameters. Both strains were associated with reduction ($p \leq .05$) in blood glucose levels after 28 days of administration and after wash-out period; glucose levels remained reduced only in the group that received *L. plantarum* 49. Both strains were able to reduce ($p \leq .05$) total cholesterol levels after 14 days of administration; after the wash-out period these levels remained reduced only in the group that received *L. plantarum* 201. *L. plantarum* 49 and *L. plantarum* 201 were detected in the intestine and did not cause alteration or translocate to spleen, kidneys or liver during the experimental or wash-out period. These results indicate that *L. plantarum* 49 and *L. plantarum* 201 present potential for use as probiotics with intrinsic abilities to modulate biochemical parameters of interest for the management of metabolic diseases.

1. Introduction

Probiotics are live microorganisms, which exert a positive health benefit on the host when ingested in an adequate amount (FAO/WHO, 2006). Tropical fruit and their industrial processing byproducts have been considered sources of *Lactobacillus* with the required features for a probiotic because this genus represents great part of the autochthonous raw fruit microbiota (Garcia et al., 2016). Some physicochemical parameters of fruit or fruit-by-products, such as acidity and presence of competitive microbiota, may resemble traits of the human gastrointestinal tract (Vitali et al., 2012). Previous studies have suggested that previous adaptation to these conditions might help fruit-derived *Lactobacillus* to survive in the human gastrointestinal tract (Albuquerque et al., 2017; Vitali et al., 2012).

In addition to the experimental evaluation of beneficial effects, the safety of use is a critical factor to establish the probiotic potential of new strains (Park et al., 2017; Shanahan, 2012). Primarily, the possible translocation of bacteria to extra intestinal organs should be assessed using in vivo models, which are also relevant to select promising strains with prophylactic or therapeutic effects (Daniel et al., 2006; Sanders

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et al., 2010). Furthermore, the identification of a new probiotic strain when administrated to animal models is important to avoid to be confounded with strains of the same species in host microbiota (Kechagia et al., 2013; Park et al., 2017).

Recently, a scarcity nutrient model using *Drosophila melanogaster* flies has been proposed for screening of new beneficial strains for growth-promoting effects (Schwarzer et al., 2016; Storelli et al., 2011). Once *D. melanogaster* larval growth is fully dependent on food richness environment, poor-nutrient environment severely affects both its systemic growth and maturation rate and consequently influences the timing of adult emergence. Under nutrient shortage, *D. melanogaster* microbiota is necessary for optimal larval development (Storelli et al., 2011). The use of monoxenic model (one microbe-one host) has been considered effective to reveal if a such strain exerts promoting effects on *D. melanogaster* growth (Erkosar et al., 2015). Since the growth promoting ability of lactobacilli in *D. melanogaster* can be translated to mice (Schwarzer et al., 2016), the model may represent a fast and straight-forward screening method for selection of beneficial strains.

Among the *Lactobacillus* species assessed for probiotic features, *L. plantarum* has been considered as a highly versatile species able to promote distinct health effects (Karasu, Simsek, & Con, 2010; Siezen & Vlieg, 2011). Earlier studies have reported that specific strains of *L. plantarum* can inhibit obesity development by reducing mesenteric adipose tissue (Park et al., 2017) and favoring lipid metabolism in a diet-induced obesity murine model (Kim, Hong, Choi, & Kim, 2014). Anti-diabetic and hypoglycemic effects have also been reported for strains of *L. plantarum* isolated from distinct sources (Li et al., 2016a, 2016b). Previous studies have described features that characterize tropical fruit-derived *Lactobacillus* strains as potential probiotics using in vitro approaches (Albuquerque et al., 2017; Costa et al., 2018; Garcia et al., 2016). However, the safety and health-promoting effects of such strains remains unknown.

This study evaluated the growth-promoting effects of sixteen fruit-derived potentially probiotic *Lactobacillus* strains using the host-commensal *D. melanogaster* nutrient scarcity model. The strains *L. plantarum* 49 and *L. plantarum* 201, which presented the most significant growth-promoting effects, were further evaluated for their safety and effects on murinometric, biochemical and histopathological parameters in healthy male Wistar rats.

2. Materials and methods

2.1. Tested strains and inoculum preparation

Sixteen strains comprising different *Lactobacillus* species previously isolated from the pulp of *Mangifera indica* L., or from industrial fruit pulp processing byproducts of *Malphigia glabra* L., *M. indica* L., *Annona muricata* L. and *Fragaria ananassa* L. that previously presented characteristics compatible with probiotic use were included in this study (Table 1) (Garcia et al., 2016). The strains *Lactobacillus plantarum* WJL and *L. plantarum* NIZO2877 were used as controls in a growth promoting screen assay in situation of nutrient scarcity in *Drosophila melanogaster* (Schwarzer et al., 2016; Storelli et al., 2011). Stock cultures of *Lactobacillus* strains were maintained in cryovials at −80 °C in de Man, Rogosa and Sharpe (MRS) broth (HiMedia, Mumbai, India) containing glycerol 20% (v/v).

Each inoculum was obtained by preparing suspensions in sterile saline solution from overnight cultures grown on MRS broth (HiMedia, Mumbai, India) and incubated anaerobically (Anaerobic System Anaerogen, Oxoid Ltda., Wade Road, UK) at 37 °C. Cells were harvested by centrifugation (4500 ×*g*, 15 min, 4 °C), washed twice with sterile saline solution, re-suspended and homogenized using a vortex (30 s) in sterile saline solution to obtain standard cell suspensions with optical density (OD) reading at 660 nm (OD_{660}) of 1.0, which provided viable counts of approximately 9 log CFU/mL.

Table 1
Lactobacillus strains included in the study and their respective source (fruit or byproduct of pulp processing) of isolation.

Cepas teste	Fonte de isolamento
Lactobacillus plantarum WJL*	*Drosophilla melanogaster*
Lactobacillus plantarum Nizo 21*	*Drosophilla melanogaster*
Lactobacillus plantarum 40	Byproduct of guava (*Psidium guajava* L.)
Lactobacillus plantarum 49	Pulp of barbados cherry (*Malphigia glabra* L.)
Lactobacillus plantarum 53	Byproduct of barbados cherry (*M. glabra* L.)
Lactobacillus plantarum 54	Byproduct of barbados cherry (*M. glabra* L.)
Lactobacillus brevis 59	Byproduct of barbados cherry (*M. glabra* L.)
Lactobacillus paracasei 62	Byproduct of barbados cherry (*M. glabra* L.)
Lactobacillus paracasei 106	Byproduct of soursop (*Annona muricata* L.)
Lactobacillus paracasei 108	Byproduct of soursop (*A. muricata* L.)
Lactobacillus fermentum 111	Byproduct of soursop (*A. muricata* L.)
Lactobacillus fermentum 129	Byproduct of mango (*Mangifera indica* L.)
Lactobacillus fermentum 139	Byproduct of mango (*M. indica* L.)
Lactobacillus fermentum 141	Byproduct of mango (*M. indica* L.)
Lactobacillus plantarum 198	Byproduct of mango (*M. indica* L.)
Lactobacillus plantarum 201	Byproduct of mango (*M. indica* L.)
Lactobacillus fermentum 210	Pulp of mango (*M. indica* L.)
Lactobacillus fermentum 296	Byproduct of strawberry (*Fragaria ananassa* L.)

2.2. Screening of Lactobacillus strains using a nutrient scarcity model

To screen the *Lactobacillus* strains based on their growth promoting effects, a biological host-commensal nutrient scarcity model with *Drosophila melanogaster* mono colonized flies was used following previously described procedures (Storelli et al., 2011). *D. melanogaster* stocks were kept at 25 °C using a rich diet comprising yeast extract and corn meal medium. The rich diet comprised corn-meal flour (Westhove, Farigel maize H1; 80 g/L), inactivated dried yeast (Springaline; 50 g/L) and agar (VWR; 8.2 g/L) cooked for 10 min in boiling water; methylparaben sodium salt (Merck; 5.2 g/L) and propionic acid (99% v/v Carlo Erba; 4 mL/L) were added when the food formulation had cooled down. The diet used for nutrient scarcity (poor diet) was obtained by reducing the amount of inactivated yeast to 6 g/L. The diets were prepared weekly to avoid desiccation. Conventionally reared (CR) *D. melanogaster* stocks carry a conventional microbiota, which was removed in germ-free (GF) *D. melanogaster* individuals by bleaching and cultivating embryos on autoclaved conventional medium. GF *D. melanogaster* stocks were maintained on a rich diet supplemented with a cocktail of four antibiotics (ampicillin/kanamycin/tetracyclin at 50 µg/mL final each and erythromycin at 10 µg/mL final). GF females laid GF embryos, which were grown on appropriate culture medium (rich or poor diet). Bacterial suspensions of test *Lactobacillus* (150 mL, 10^7 CFU/mL) were then added directly on the embryos and the food after the egg-laying period. *L. plantarum* WJL and *L. plantarum* NIZO2877 were used as control strains and analyzed as previously described (Schwarzer et al., 2016;Storelli et al., 2011). Emerging larvae were allowed to develop for seven days on the inoculated media. Larvae were frozen and mounted on a slide in 80% glycerol in PBS. Pictures were taken on a black background using a ProgResC5 CCD camera (JenOptik) mounted on a stereomicroscope. The body length of each larva was measured using ImageJ (2015). The assays were performed in at least three biological replicates, including at least 30 individuals each.

2.2.1. Genome sequencing and design of strain-specific primers for growth-promoting L. plantarum strains

The strains *L. plantarum* 49 and *L. plantarum* 201, which were selected considering their performance in scarcity model assay, were submitted to whole genome sequencing to design primers that allow their molecular identification in samples collected during the experimental evaluation in rats. Bacterial genomic DNA was extracted from cultures grown to stationary phase in MRS broth using the UltraClean Microbial DNA isolation kit (Mo Bio, Qiagen, USA). The sequencing was

performed following the procedures described by Kim, Park, Lee, and Lee (2013). Genomic libraries were prepared following Ion Xpress Plus gDNA Fragment Library construction protocol for 400 bp reads. The strains were sequenced using the Ion Torrent PGM platform. The DNA library construction and sequencing were performed on the IGFL sequencing platform (Lyon, France). Gap closing and resequencing of low-quality regions were conducted by Sanger sequencing to reach high-quality finished genome sequence. Gaps were closed by designing primer pairs between contigs using the Primer3 software (Untergrasser et al., 2012). The endpoint PCRs were conducted on a Veriti Applied Biosystems thermocycler (Life Technologies, Carlsbad, CA). Functional annotations of the predicted genes were performed using the RAST Server (Aziz et al., 2008). The genomic regions specific to *L. plantarum* 49 and 201 strains have been manually identified through alignment of each strain of interest with all the *L. plantarum* strain genomes available in NCBI at the time of the analysis (August 2015). The strain-specific primer pairs were designed using Geneious 7 (Kearse et al., 2012).

PCR amplifications were performed in a final volume of 20 µL and the optimized conditions for the strain-specific primers were: 95 °C for 2 min, followed by 35 cycles of denaturation (95 °C, 30 s), annealing (58 °C, 30 s and 72 °C, 1 min 30 s) and elongation (72 °C, 7 min). The following *L. plantarum* strains have been used as negative controls of amplification in both PCRs: *L. plantarum* NIZO2877, *L. plantarum* WJL, *L. plantarum* WCFS1 and *L. plantarum* NC8. After electrophoresis in agarose gel 1%, the gels were treated with ethidium bromide, visualized under ultraviolet light (UV) and documented.

2.3. Evaluation of safety aspects and beneficial effects of Lactobacillus using Wistar rats

Forty-eight male Wistar rats at 21 days were used in the study. All experiments were previously approved by the Animal Research Ethics Committee (Federal University of Pernambuco, Recife, Brazil; protocol number 23076.024378/2015-13 CEUA/UFPE). Experimental procedures were performed in accordance with revised guide for the care and use of laboratory animals (Bayne, 1996).

Rats were kept in individual cages (22 ± 1 °C; 12 h photoperiod; 50–55% relative humidity) with food (AIN 93 M diet) and water provided ad libitum (Reeves, Nielsen, & Fahey, 1993) and randomly distributed into three groups of 16 animals as follow: group Lp49, which received 9 log CFU/mL of strain *L. plantarum* 49; group Lp201, which received 9 log CFU/mL of strain *L. plantarum*; and control group, which received PBS daily by orogastric gavage during four weeks. Before the administration (baseline values), after 14 and 28 days of administration and during the wash-out period (14 days after the end of the administration of lactobacilli strains or PBS), four rats per group were fasted for 12 h and anesthetized by intraperitoneal injection of 1 mL of ketamine hydrochloride (75 mg) and 1 mL of xylazine hydrochloride (5 mg) per kg body weight. At each evaluation point, feces were collected and the murinometric parameters were measured. Blood samples were collected by cardiac puncture in the left ventricle and after the euthanasia via aortic transection, organs (intestine, kidneys, spleen and liver) were removed, weighted, subdivided and randomly distributed for microbiological and histopathological analysis (Batista et al., 2018).

2.3.1. Determination of murinometric parameters

Using a tape measure, the abdominal circumference (AC) immediately preceding the front leg, the thoracic circumference (TCi) immediately behind the foreleg and the body length from the nose to the base of the tail were measured. Body weight was obtained and body mass index (BMI) was calculated by dividing body weight (g) by body length squared (cm^2) (Novelli et al., 2007). The Lee index (LI) was calculated by dividing the cube root of body weight (g) by length (cm). Food intake was recorded daily (Lien et al., 2001).

2.3.2. Serum analysis and lipid profile

Four milliliters of blood was collected via direct cardiac puncture and centrifuged (807 × *g*, 10 min, 4 °C) from anesthetized animals. The serum levels of glucose were measured using the Glucose PAP Liquiform kit (Labtest®, Minas Gerais, Brazil). The serum levels of aspartate transaminase (AST) and alanine transaminase (ALT) were analyzed using the AST and ALT kits (Bioclin®, Minas Gerais, Brazil), respectively. Serum concentrations of total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-c) were measured using the Trinder enzymatic method and the accelerator selective detergent method using Liquiform Cholesterol and HDL LE kits (Labtest®, Minas Gerais, Brazil), respectively. Triglycerides (TG) levels were determined using the Trinder method with a TAG Liquiform kit (Labtest®, Minas Gerais, Brazil). All analyses followed the manufacturers recommendations and absorbance was determined using a LabMax 240 Premium automatic analyzer (Labtest ®, Minas Gerais, Brazil) at 505 nm (TAG), 500 nm (TC) or 600 nm (HDL) (Batista et al., 2018).

2.4. Histopathological evaluation

To assess if the tested strains can translocate to the liver, spleen and kidneys, fragments of these organs as well as of intestine were collected from different animals of each group. The tissue samples were washed in saline solution (0.9% NaCl, *w/v*), fixed in 10% (v/v) buffered formalin for 48 h and processed according to the routine histological technique. The obtained slides were stained using the technique of Hematoxylin-Eosin (H&E) as previously described (Batista et al., 2018).

Morphological analysis of liver included evaluation of the occurrence of degenerative processes by fatty degeneration and inflammatory parameters, including leucocyte migration, edema, hyperemia, hemorrhage, necrosis, preservation of liver parenchyma and presence of micro thrombi. The analysis of spleen included evaluation of inflammatory processes presence, such as stasis, leucocyte migration, hemorrhage, vasodilation and necrosis, as well as evaluation of epithelial preservation, hypertrophy and hyperplasia of the white or red pulp. The morphological analysis of kidney included evaluation (presence) of glomerulonephritis and interstitial nephritis, necrosis and degeneration. The intestine was evaluated for inflammatory processes presence, such as stasis, leucocyte migration, hemorrhage, vasodilation and necrosis, as well as for epithelial preservation, hypertrophy and hyperplasia of the outer muscular layer (Batista et al., 2018; Erben et al., 2014; Rigo et al., 2013). To confirm the observations, the slides were re-evaluated by the same pathologist after being randomized by an independent person and the general agreement between the two analyses was considered as an evaluation criterion (Wang, Li, Li, Zhang, & Li, 2009).

2.5. Cultivation of Lactobacillus from organs and fecal samples

At each evaluation point, samples of liver, kidneys, spleen and intestine were mixed (1:10) in sterile saline solution, washed twice (4500 × *g*, 15 min, 4 °C) with sterile saline solution and vortexed (30 s) to break down the clusters of bacteria and to remove bacteria weakly bound to the tissues. The vortexed solution was washed (4500 × *g*, 15 min, 4 °C) again, the supernatant was removed and the organs were aseptically fractionated and directly plated onto MRS agar (Wang, Li, et al., 2009). For enumeration of *Lactobacillus* in feces, at each evaluation point, feces were collected, weighed, diluted (1:10) in sterile saline solution and homogenized for 30 s using a vortex. The mixture was serially diluted (10^{-1}–10^{-6}) in the same diluent and 20-µL aliquots of each dilution were dispensed onto MRS agar using a microdrop inoculation technique (Herigstad, Hamilton, & Heersink, 2001). The plates were incubated at 37 °C for 48 h under anaerobiosis (Anaerobic System Anaerogen, Oxoid) and the results were expressed as log CFU/g.

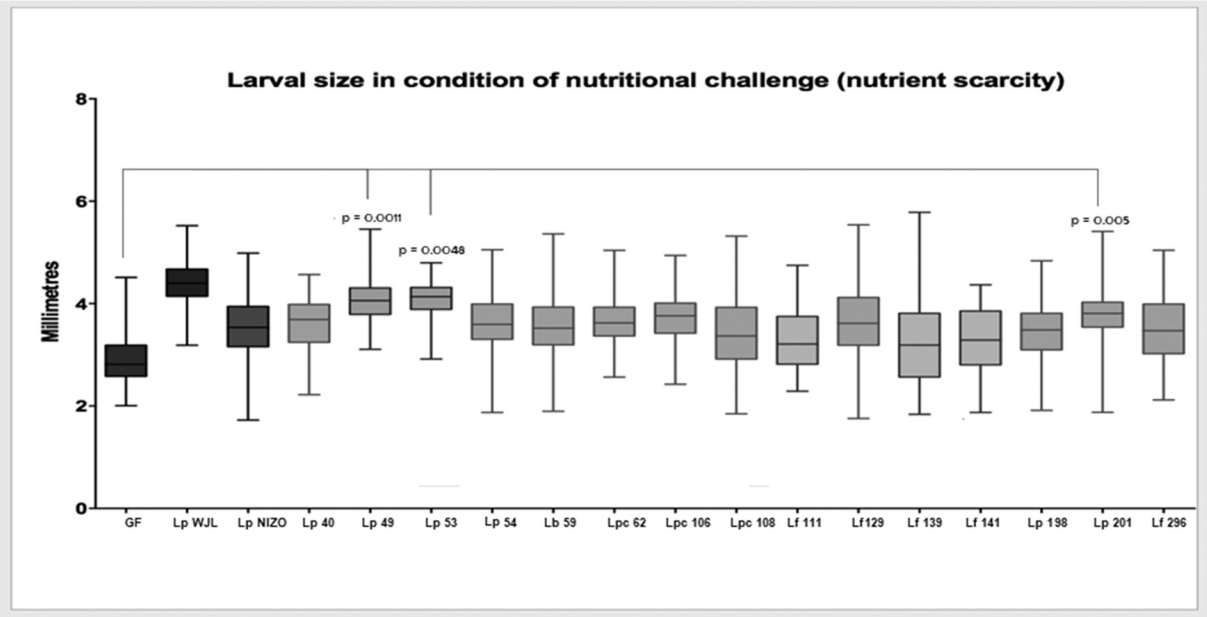

Fig. 1. Longitudinal size of larvae (n > 60 larvae/group) measured 7 days after egg deposition on poor nutrient. Larvae were kept germ-free (GF) or associated with the respective strain of *Lactobacillus*: GF – germ-free; Lp WJL: *L. plantarum* WJL; Lp NIZO: *L. plantarum* NIZO2877; Lp 40: *L. plantarum* 40; Lp 49: *L. plantarum* 49; Lp 53: *L. plantarum* 53; Lb 54: *Lactobacillus plantarum* 54; Lb 59: *Lactobacillus brevis* 59; Lpc 62: *L. paracasei* 62; Lpc 106: *L. paracasei* 106; Lpc 108: *L. paracasei* 108; Lf 111 *L. fermentum* 111; Lf 129: *L. fermentum* 129; Lf 139: *L. fermentum* 139; Lf 141: *L. fermentum* 141; Lp 198: *L. plantarum* 198; Lp 201: *L. plantarum* 201; Lf 210: *L. fermentum* 210. Indicative lines show the fruit-derived *Lactobacillus* strains with most relevant results based on Student's *t*-test (considering the difference between the larval size of *D. melanogaster* larvae colonized and larvae of germ free (GF) *D. melanogaster*).

2.6. PCR of organs and feces

Bacterial DNA extraction from the tissues (liver, spleen, kidneys and intestine) and feces samples was performed using phenol-chloroform method (Chomczynski & Sacchi, 1987). Briefly, each sample was frozen with liquid nitrogen and grounded to form a powder. An aliquot (20 mg) was mixed with 500 µL of lysis buffer (Tris-HCl/EDTA/SDS/water) and 25 µL protein kinase K (10 mg/mL) and maintained for 2 h at 55 °C in water bath. Then, 500 µL of phenol/chloroform/isoamyl alcohol (25:24:1) was added and the resulting mixture was sequentially vortexed (30 s), placed on the rotary shaker (25 rpm, 15 min) and after centrifugation (13,000 ×*g*, 27 °C, 5 min) the upper aqueous phase was recovered to a new tube. The process was repeated twice. Finally, sodium acetate 3 mM/L (1:10) and ethanol (100%; 2.5:1, stored at −20 °C) were added to the aqueous phase, centrifuged (13,000 ×*g*, 4 °C, 5 min) and the supernatant removed. An 800 µL-aliquot of ethanol (70% v/v; stored at −20 °C) was added to the remaining pellet, centrifuged (13,000 ×*g*, 4 °C, 5 min) and the supernatant was removed. The final pellet was resuspended with RNAse free water. DNA concentration and purity (260/280 nm absorbance ratio) was determined using a Nanodrop 2000 (Thermofisher). The samples were prepared using the PCR mix with GoTaq Promega reagents. The PCR reactions were performed in a final volume of 20 µL using the specific primers designed for *L. plantarum* 49 or *L. plantarum* 201 and analyzed as described in Section 2.2.1.

2.7. Reproducibility and statistical analysis

For *D. melanogaster* assays, statistical difference between the larval size of *D. melanogaster* larvae colonized with strains tested and larvae of germ free (GF) *D. melanogaster* was assessed using Student's *t*-test. Samples with values of $p \leq .05$ were considered statistically different. For assays in rats, statistical power of 1.0 (100%) was obtained using the software SigmaPlot 12.5 for Windows (Systat Software Inc.) by estimating forty-eight male Wistar rats (sixteen per group) when the minimally detectable effect size was 0.8 and the significance level was 0.05 ($p \leq .05$). The results were expressed as means and standard deviation of three independent experiments performed in triplicate. Statistical analyses were performed to determine significant differences ($p \leq .05$) using ANOVA followed by post hoc Turkey test or Student's *t*-test. Sigma Stat 3.5 computer software (Jandel Scientific Software, San Jose, California) was used for the statistical analyses of the data.

3. Results

3.1. Screening of Lactobacillus strains using a nutrient scarcity model

The strains *L. plantarum* WJL, *L. plantarum* 49, *L. plantarum* 53 and *L. plantarum* 201 showed the most significant ($p \leq .005$) growth promoting effects on *D. melanogaster* upon the nutrient scarcity environment test (Fig. 1). The larvae colonized with these strains exhibited sizes approximately two-fold higher ($p \leq .05$) than those observed for larvae of germ free (GF) *D. melanogaster* when submitted to the same nutrient reduction. *L. plantarum* 40, *L. plantarum* 189, *L. fermentum* 296, *L. paracasei* 62, *L. paracasei* 106, *L. paracasei* 108, *L. brevis* 54, *L. brevis* 59 and *L. fermentum* 129 strains also promoted *D. melanogaster* larval growth. Otherwise, *L. fermentum* 111, *L. fermentum* 139 and *L. fermentum* 144 strains did not show any growth-promoting effects, with results similar ($p > .05$) to GF *D. melanogaster*. Phylogenetic analysis on their 16S rRNA gene sequences revealed that *L. plantarum* 49 (isolated from pulp of barbados cherry) and *L. plantarum* 53 (isolated from byproduct of barbados cherry) were closely related, while *L. plantarum* 201 (isolated from pulp of mango) showed higher distance in the evolutionary chain (Supplementary Fig. 1). Therefore, *L. plantarum* 49 and *L. plantarum* 201 strains were selected for genome sequencing and in vivo studies.

Based on the results of whole genome analysis of *L. plantarum* 49 (GeneBank number QBKW00000000) and *L. plantarum* 201 (GeneBank number QBKX00000000), specific primers were designed. *L. plantarum* 49 specific feature is the presence of a different domain (800 bp) in lp_0946 (mucus binding protein) compared to the other lactobacilli (Fig. 2A-B). Based on this domain, the primers 5′-GGCATCGACCTCCG

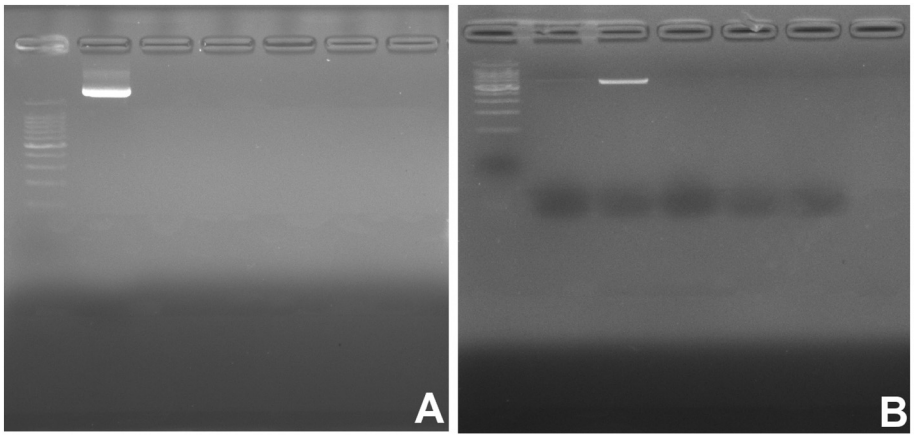

Fig. 2. Fragments resulting polymerase chain reaction (PCR) products using the designed primers to *L. plantarum* 49 and *L. plantarum* 201 separated by 1.0% agarose gel electrophoresis (A). Line 1 corresponds to molecular size marker of 100 bp.; Line 2: fragments observed for *L. plantarum* 49 (Lp 49); Line 3 *L. plantarum* 201 (Lp 201); Line 4: *L. plantarum* NIZO2877 (Lp NIZO); Line 5: *L. plantarum* WJL (Lp WJL); Line 6: *L. plantarum* WCFS1 (Lp WCFS1); Line 7: *L. plantarum* NC8 (Lp NC8). (B) Line 1 corresponds to molecular size marker of 100 bp.; Line 2: *L. plantarum* 49 (Lp 49); Line 3: fragments observed for *L. plantarum* 201 (Lp 201); Line 4: *L. plantarum* NIZO2877 (Lp NIZO); Line 5: *L. plantarum* WJL (Lp WJL); Line 6: *L. plantarum* WCFS1 (Lp WCFS1); Line 7: *L. plantarum* NC8 (Lp NC8).

Table 2
Food intake and murine parameters of Wistar male rats during 28 days of administration of *L. plantarum* 49 and *L. plantarum* 201 and wash-out.

Food intake and murine parameters				
Indicator	Period	Groups		
		Control	*L. plantarum* 49	*L. plantarum* 201
Food intake (g)	Before	23.18 ± 3.43 ^{Aa}	21.30 ± 2.19 ^{Aa}	22.11 ± 2.54 ^{Aa}
	14 days	22.56 ± 2.74 ^{Aa}	22.44 ± 1.62 ^{Aa}	21.19 ± 2.91 ^{Aa}
	28 days	24.46 ± 3.26 ^{Aa}	22.59 ± 1.57 ^{Aa}	21.87 ± 1.58 ^{Aa}
	Wash-out	19.64 ± 3.28 ^{Aa}	22.05 ± 3.61 ^{Aa}	22.94 ± 3.49 ^{Aa}
Body weight (g)	Before	228.12 ± 22.30 ^{Aa}	218.75 ± 16.01 ^{Aa}	219.37 ± 19.62 ^{Aa}
	14 days	258.12 ± 22.27 ^{Aa}	261.87 ± 17.26 ^{Aa}	259.69 ± 16.68 ^{Aa}
	28 days	287.45 ± 19.44 ^{Aa}	290.48 ± 17.32 ^{Aa}	290.25 ± 16.18 ^{Aa}
	Wash-out	311.63 ± 20.21 ^{Aa}	318.44 ± 11.29 ^{Aa}	319.06 ± 15.65 ^{Aa}
Body length (cm)	Before	22.37 ± 1.11 ^{Aa}	21.37 ± 1.49 ^{Aa}	22.00 ± 1.22 ^{Aa}
	14 days	22.37 ± 0.75 ^{Aa}	22.37 ± 1.49 ^{Aa}	23.12 ± 1.03 ^{Aa}
	28 days	24.12 ± 1.31 ^{Aa}	23.5 ± 0.71 ^{Aa}	23.00 ± 0.82 ^{Aa}
	Wash-out	24.37 ± 0.75 ^{Aa}	24.00 ± 1.15 ^{Aa}	23.50 ± 0.87 ^{Aa}
Body mass index (g/cm^2)	Before	0.46 ± 0.0 ^{Aa}	0.48 ± 0.05 ^{Aa}	0.46 ± 0.07 ^{Aa}
	14 days	0.52 ± 0.05 ^{Aa}	0.48 ± 0.05 ^{Aa}	0.49 ± 0.03 ^{Aa}
	28 days	0.55 ± 0.06 ^{Aa}	0.53 ± 0.05 ^{Aa}	0.55 ± 0.02 ^{Aa}
	Wash-out	0.53 ± 0.05 ^{Aa}	0.55 ± 0.04 ^{Aa}	0.58 ± 0.02 ^{Aa}
Lee index (g/cm)	Before	0.27 ± 0.02 ^{Aa}	0.28 ± 0.01 ^{Aa}	0.27 ± 0.02 ^{Aa}
	14 days	0.28 ± 0.01 ^{Aa}	0.27 ± 0.02 ^{Aa}	0.28 ± 0.01 ^{Aa}
	28 days	0.27 ± 0.01 ^{Aa}	0.28 ± 0.01 ^{Aa}	0.29 ± 0.01 ^{Aa}
	Wash-out	0.28 ± 0.01 ^{Aa}	0.29 ± 0.01 ^{Aa}	0.29 ± 0.01 ^{Aa}
Thoracic circunference (cm)	Before	13.12 ± 1.25 ^{Aa}	13.12 ± 0.75 ^{Aa}	13.62 ± 1.8 ^{Aa}
	14 days	13.62 ± 1.03 ^{Aa}	13.87 ± 1.65 ^{Aa}	14.25 ± 1.5 ^{Aa}
	28 days	14.50 ± 0.82 ^{Aa}	14.62 ± 1.03 ^{Aa}	14.05 ± 0.42 ^{Aa}
	Wash-out	14.62 ± 0.95 ^{Aa}	14.12 ± 0.25 ^{Aa}	14.37 ± 1.49 ^{Aa}
Abdominal circunference (cm)	Before	14.25 ± 1.25 ^{Aa}	14.62 ± 0.48 ^{Aa}	16.00 ± 1.82 ^{Aa}
	14 days	15.25 ± 1.32 ^{Aa}	14.75 ± 1.71 ^{Aa}	15.62 ± 1.8 ^{Aa}
	28 days	15.87 ± 1.03 ^{Aa}	15.62 ± 1.03 ^{Aa}	15.00 ± 0.41 ^{Aa}
	Wash-out	16.00 ± 1.78 ^{Aa}	14.62 ± 0.48 ^{Aa}	15.50 ± 2.12 ^{Aa}

A: Represent the differences within the column for the same indicator during the period assayed denotes differences ($p \leq .05$), based on ANOVA one way followed by post hoc Turkey test.
a: Represent the differences within the line for the same indicator during the assayed phase denotes differences ($p \leq .05$), based on ANOVA one way followed by post hoc Turkey test.

TTAAAT-3′ (forward) and 5′-CAATCAACACCAACCACCTT-3′ (reverse) were designed as strain-specific for *L. plantarum* 49. They do not amplify the genome of any other of the tested *L. plantarum* strains (Fig. 2B).

L. plantarum 201 specific feature was a large insertion (about 10 kb) of an unknown protein between lp_0045 (ribosomal RNA large subunit methyltransferase H) and lp_0046 (transcription regulator, TetR family) (Fig. 2C). The primers 5′-GGTTTATCGGGCGTTTATGA-3′ (forward) and 5′-CCAAACTCCACCAATTAGCA-3′ (reverse) were designed on this region to specifically amplify *L. plantarum* 201 strain. They did not amplify the genome of any other *L. plantarum* strain tested.

3.2. Murinometric parameters

During the experimental or wash-out period, animals of group *Lp49*, *Lp201* and control showed similar ($p > .05$) daily food intake. No differences ($p > .05$) were observed in the murinometric parameters (AC, TCi, body length, body weight, BMI and LI) of *Lp49* or *Lp201* group compared to those of control group (Table 2).

3.3. Serum analysis and lipid profile

No change was observed in glucose levels of control group over the assayed period (intervention and wash-out). After 28 days of

Table 3
Biochemical parameters of Wistar male rats during 28 days of administration of *L. plantarum* 49 and *L. plantarum* 201 and wash-out.

Biochemical parameters				
Marker	Period	Groups		
		Control	*L. plantarum* 49	*L. plantarum* 201
Glucose (mg/dL)	Before	92.75 ± 1.71 ^{Aa}	91.25 ± 1.89 ^{Aa}	92.75 ± 0.50 ^{Aa}
	14 days	93.00 ± 1.15 ^{Aa}	93.75 ± 0.96 ^{Aa}	93.50 ± 0.58 ^{Aa}
	28 days	92.75 ± 0.96 ^{Aa}	80.25 ± 1.50 ^{Bb}	82.50 ± 1.12 ^{Bb}
	Wash-out	92.25 ± 0.96 ^{Aa}	80.75 ± 1.26 ^{Bb}	92.75 ± 1.29 ^{Aa}
Total cholesterol (mg/dL)	Before	63.00 ± 0.20 ^{Aa}	62.25 ± 1.50 ^{Aa}	63.75 ± 0.96 ^{Aa}
	14 days	62.25 ± 1.26 ^{Aa}	50.62 ± 0.75 ^{Bb}	42.75 ± 1.50 ^{Bb}
	28 days	64.00 ± 1.41 ^{Aa}	51.25 ± 0.96 ^{Bb}	42.25 ± 0.96 ^{Bb}
	Wash-out	62.75 ± 1.26 ^{Aa}	63.00 ± 0.82 ^{Aa}	42.50 ± 1.29 ^{Bb}
Triglycerides (mg/dL)	Before	99.50 ± 1.29 ^{Aa}	99.50 ± 1.73 ^{Aa}	99.50 ± 0.58 ^{Aa}
	14 days	99.25 ± 1.71 ^{Aa}	98.75 ± 0.96 ^{Aa}	99.00 ± 1.41 ^{Aa}
	28 days	99.12 ± 0.58 ^{Aa}	98.75 ± 0.50 ^{Aa}	99.00 ± 0.82 ^{Aa}
	Wash-out	99.75 ± 0.96 ^{Aa}	99.50 ± 1.29 ^{Aa}	99.25 ± 1.71 ^{Aa}
HDL cholesterol (mg/dL)	Before	24.00 ± 0.82 ^{Aa}	24.25 ± 0.96 ^{Aa}	24.25 ± 1.26 ^{Aa}
	14 days	24.50 ± 0.58 ^{Aa}	25.00 ± 0.82 ^{Aa}	24.25 ± 0.50 ^{Aa}
	28 days	24.29 ± 0.85 ^{Aa}	24.70 ± 0.68 ^{Aa}	25.00 ± 0.72 ^{Aa}
	Wash-out	24.97 ± 0.84 ^{Aa}	25.30 ± 0.53 ^{Aa}	25.07 ± 0.76 ^{Aa}
ALT (U/L)	Before	152.75 ± 1.26 ^{Aa}	151.50 ± 1.29 ^{Aa}	152.75 ± 0.96 ^{Aa}
	14 days	153.87 ± 0.85 ^{Aa}	153.25 ± 1.50 ^{Aa}	153.10 ± 0.67 ^{Aa}
	28 days	153.01 ± 1.32 ^{Aa}	152.62 ± 0.82 ^{Aa}	153.11 ± 1.53 ^{Aa}
	Wash-out	153.67 ± 1.05 ^{Aa}	153.93 ± 0.82 ^{Aa}	153.67 ± 0.83 ^{Aa}
AST (U/L)	Before	51.62 ± 1.38 ^{Aa}	51.75 ± 0.96 ^{Aa}	52.51 ± 1.08 ^{Aa}
	14 days	52.67 ± 0.79 ^{Aa}	52.21 ± 0.92 ^{Aa}	52.40 ± 0.98 ^{Aa}
	28 days	52.84 ± 0.99 ^{Aa}	53.00 ± 1.15 ^{Aa}	52.16 ± 0.89 ^{Aa}
	Wash-out	51.75 ± 0.95 ^{Aa}	52.51 ± 1.09 ^{Aa}	52.85 ± 0.92 ^{Aa}

A–C: Represent the differences within the column for the same biochemical parameter during the period assayed denotes differences ($p \leq .05$), based on ANOVA one way followed by post hoc Turkey test;
a–b: Represent the differences within the line for the same biochemical parameter during the assayed phase denotes differences ($p \leq .05$), based on ANOVA one way followed by post hoc Turkey test.

administration, *Lp49* and *Lp201* groups showed a reduction ($p \leq .05$) in glucose levels. Only in *Lp49* group, glucose levels remained reduced during the wash-out period (Table 3).

No change was observed in TC levels of control group during the intervention or wash-out periods. *Lp49* and *Lp201* groups showed a reduction ($p \leq .05$) of TC levels after 14 days of intervention. In both groups, no additional changes ($p > .05$) were observed after 28 days of intervention. However, after the wash-out period, the TC levels remained reduced in the *Lp201* group, while in *Lp49* group, they returned to the baseline levels. No difference ($p > .05$) was observed in TG, ALT and AST serum levels among the control, *Lp49* or *Lp201* groups throughout the period monitored (intervention or wash-out).

3.4. Histopathological evaluation

No translocated cells were observed in intestine, spleen, kidneys or liver collected from animals of *Lp49* and *Lp201* groups. No morphological differences ($p > .05$) were observed among *Lp49*, *Lp201* and control groups (Fig. 3). The weight of organs and abdominal fat were similar ($p > .05$) between *Lp49*, *Lp201* and control groups during the experimental and wash-out periods (Supplementary Table 1).

3.5. Microbiological and PCR analysis of organs and feces

No viable cell count of *Lactobacillus* spp. was observed by plating suspension of liver, spleen or kidneys of animals from *Lp49*, *Lp201* or control groups in MRS agar. No amplification of the fragments of 800 bp, corresponding to *L. plantarum* 49, or 600 bp corresponding to *L. plantarum* 201 was observed (no band was visualized in the electrophoresis) when these same organs were submitted to PCR analysis using the strain-specific primers.

No change ($p > .05$) in counts of *Lactobacillus* spp. was observed in intestine or feces of control group over the period monitored (experimental and wash-out) (Table 4). Otherwise, *Lactobacillus* counts increased ($p \leq .05$) approximately 1.2 log CFU/g after 14 days of intervention in feces and intestine of *Lp49* and *Lp201* groups. An additional increase ($p \leq .05$) of approximately 2 log CFU/mL was observed in these groups after 28 days of intervention. After the wash-out period, *Lactobacillus* counts decreased ($p \leq .05$) to levels similar ($p > .05$) to those observed at 14 days of intervention in both *Lp49* and *Lp201* groups. PCR analysis of the colonies grown in MRS agar inoculated with suspensions of intestine and feces revealed the amplification of the 800 bp and 600 bp fragments in *Lp49* and *Lp201* groups, respectively, identical to those presented on Fig. 3. No amplification of these same fragments was observed in feces of control group.

4. Discussion

D. melanogaster mono- or poly-associated with lactobacilli strains constitutes a powerful model to evaluate the complex interplay between lactobacilli and host biologic traits (Matos & Leulier, 2014). The effects on *D. melanogaster* growth varied among the *Lactobacillus* strains tested. Strains of the same or distinct *Lactobacillus* species may be phenotypically heterogeneous regarding the guanine/cytosine contents, type of cell wall peptidoglycan, and, most importantly, regarding the metabolic profile, defined in terms of types of fermented sugars and fermentation end-products, upon which traditional taxonomic analysis is based (Salvetti, Torriani, & Felis, 2012). The sum of these factors probably defined the ability of each strain to promote *D. melanogaster* growth in a specific manner under nutrient scarcity.

The greater ($p \leq .005$) growth-promoting effects of *L. plantarum* 49, *L. plantarum* 53 and *L. plantarum* 201 when compared to other tested strains could be also related to the specific capability of these strains of using the available nutrients and produce a variety of metabolites that may influence *D. melanogaster* larval growth (Park et al., 2017; Shanahan, 2012). A previous study demonstrated that *L. plantarum* WJL

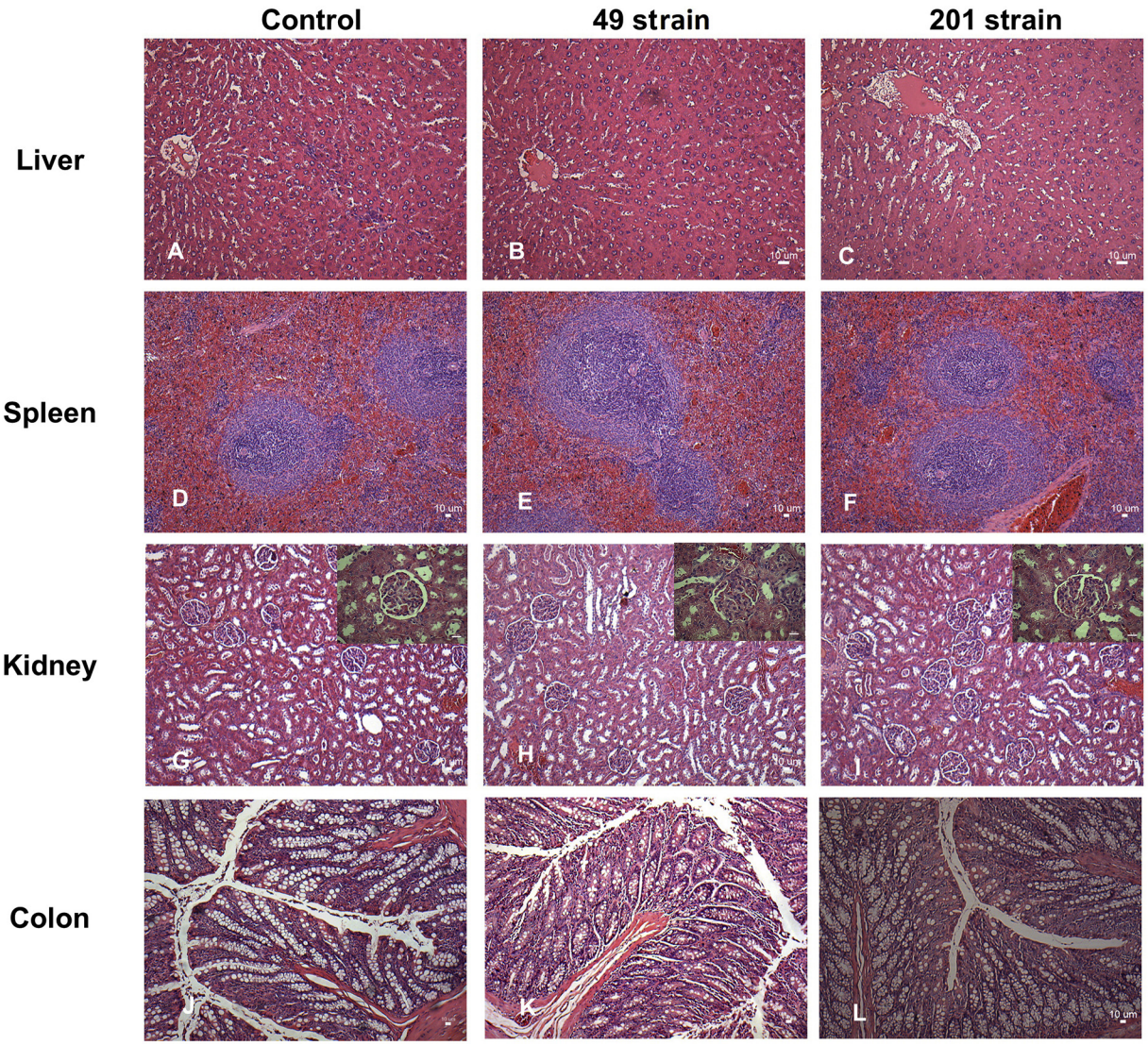

Fig. 3. Hematoxylin–eosin (H&E) staining (4 ×) for histopathological examination of the liver (A, B, C), spleen (D, E, F), kidney (G, H, I) and from the colon (J, K, L) of the control group and groups receiving *L. plantarun* 49 or *L. plantarum* 201 during the 28 days.

Table 4
Viable counts (log CFU/g) of *Lactobacillus* spp. in intestine and feces from Wistar male rats of control, *L. plantarum* 49 and *L. plantarum* 201 group through the experimental designed period.

Lactobacillus spp. counts				
Source	Period	Groups		
		Control	*L. plantarum* 49	*L. plantarum* 201
Intestine	Before	2.88 ± 0.57 ^{Aa}	2.86 ± 0.41 ^{Aa}	2.85 ± 0.27 ^{Aa}
	14 days	2.96 ± 0.69 ^{Aa}	4.69 ± 0.52 ^{Bb}	5.05 ± 0.33 ^{Bb}
	28 days	3.09 ± 0.52 ^{Aa}	6.62 ± 0.45 ^{Cb}	7.06 ± 0.39 ^{Cb}
	Wash-out	3.18 ± 0.59 ^{Aa}	4.59 ± 0.58 ^{Bb}	5.03 ± 0.52 ^{Bb}
Feces	Before	9.04 ± 0.19 ^{Aa}	9.03 ± 0.41 ^{Aa}	9.11 ± 0.22 ^{Aa}
	14 days	9.08 ± 0.59 ^{Aa}	10.62 ± 0.35 ^{Bb}	10.86 ± 0.45 ^{Bb}
	28 days	8.99 ± 0.52 ^{Aa}	12.55 ± 0.72 ^{Cb}	12.45 ± 0.33 ^{Cb}
	Wash-out	8.96 ± 0.69 ^{Aa}	10.56 ± 0.48 ^{Bb}	10.06 ± 0.30 ^{Bb}

A–C: Represent the differences within the column for the same source of strains counts during the period assayed denotes differences ($p \leq .05$), based on ANOVA one way followed by post hoc Turkey test;
a–b: Represent the differences within the line for the same source of strains counts during the assayed phase denotes differences ($p \leq .05$), based on ANOVA one way followed by post hoc Turkey test.

was capable of stimulating larval *D. melanogaster* growth under nutrient scarcity by promoting an upstream step of TOR-dependent pathway that controls hormonal growth signaling (Storelli et al., 2011). In agreement with these previous results, our findings indicate that *L. plantarum* 49, *L. plantarum* 53 and *L. plantarum* 201 may also influencing the *D. melanogaster* growth and modulate its physiological processes.

Administration of *L. plantarum* 49 and *L. plantarum* 201 did not change food intake and morphometric parameters in healthy rats. These finding are in accordance with results reported for healthy female Wistar rats receiving *L. plantarum* Lp62 during 27 days (Messias et al., 2018). However, reduction in body weight gain was observed in rats fed with a high-fat diet receiving a mixture of *L. plantarum* (*L. plantarum* CECT 7527, 7528, and 7529; approximately 9 log CFU/day) during 8 weeks (Kim et al., 2014). Difference in findings among studies supports strain-specific effects of *L. plantarum* (Park et al., 2017; Shanahan, 2012).

L. plantarum 49 and *L. plantarum* 201 decreased the serum glucose levels in rats after 28 days of administration. The α-glucosidase inhibitory activity, already identified in *Lactobacillus* (Chen et al., 2014a), has been suggested as one possible mechanism underlying the antidiabetic effects of some probiotic strains (Chen et al., 2014a, 2014b). *L.*

plantarum CCFM0236 (approximately 10 log CFU/day for 7 weeks) has shown able to control glucose and ameliorate insulin resistance in high-fat and streptozotocin induced diabetes in rats (Li et al., 2016a). Nevertheless, the hypoglycemic effect of *L. plantarum* X1 (9 log CFU/day for 10 weeks) was cited as strongly associated with changes in gut microbiota and short-chain fatty acids production (Li et al., 2016b). Further studies using diabetic rats should clarify possible hypoglycemic effects caused by *L. plantarum* 49 and *L. plantarum* 201 and the underlying mechanisms. Overall, during the wash-out period the serum glucose levels returned to baseline values in *Lp201,* but not in *Lp49* group, indicating an intrinsic ability of *L. plantarum* 49 to induce long lasting effects on glycemic control.

L. plantarum 49 and *L. plantarum* 201 decreased TC serum levels after 14 days of administration. Similar results were already observed in rats fed with a high-fat diet receiving *L. plantarum* (approximately 9 log CFU/day/rat) for 8 weeks (Kim et al., 2014). High TC serum levels could indicate a higher atherogenic risk (Grundy et al., 2014). An earlier study suggested the inhibition of cholesterol reabsorption in the intestine as a possible mechanism involved in the decrease of serum TC and TG levels in rats fed a cholesterol-rich diet receiving *L. plantarum* MA2 (11 log CFU/day) (Wang, Xu, et al., 2009). Administration of probiotics to modulate the intestinal microbiota has been also considered as a protective strategy for dyslipidemia and non-alcoholic fatty liver disease (Kim et al., 2016). Previous study has shown that a probiotic-supplemented diet inhibited the increase of TC and TG levels, as well as promoted the increase in HDL-c level in vitro and in type 2 diabetic C57 BL/6 J mice (Chen et al., 2014a, 2014b). However, it is important to consider that the reduction of glucose and cholesterol levels in the present study was observed in healthy rats, and thus does not necessarily imply the same reduction in a disease model. After the wash-out period, TC returned to the baseline levels in *Lp49*, but not in *Lp201* group, indicating that strain specific features, such as ability to reduce lipid accumulation or disturbing bile acid reabsorption already proposed as mechanism for induction of these effects by probiotics (Delgado, Tamashiro, & Pastore, 2010), could be implicated with the observed results.

L. plantarum 49 and *L. plantarum* 201 did not translocate or promote morphological changes in rat organs, being in accordance with findings of previous studies in healthy female Wistar rats after administration of *L. plantarum* L2 (9 log CFU/day) for 28 days (Wang, Li, et al., 2009) or *L. plantarum* Lp62 (approximately 9 log CFU/day) for 27 days (Messias et al., 2018).

Lactobacillus ssp. counts increased over the administration period in *Lp49* and *Lp201* groups and the presence of *L. plantarum* 49 and *L. plantarum* 201 in feces was confirmed by PCR assays. Despite the decreases observed during the wash-out period, the counts of *Lactobacillus* in *Lp49* and *Lp201* groups were higher than those found at the beginning of the experimental period. These are important results indicating that *L. plantarum* 49 and *L. plantarum* 201 are able to survive and colonize the rat gastrointestinal tract (Tuohy et al., 2007). A previous study observed increase in counts of *Lactobacillus casei* Shirota in feces of healthy Chinese adults (100 mL of beverage; 8 log CFU/mL) after a 14 day administration period and a sharp decrease in these counts −21 days after the end of the administration (Wang et al., 2015). Overall, only the recovery of *L. plantarum* 49 and *L. plantarum* 201 in feces did not give complete information to indicate the site of colonization, but the positive results observed in PCR assays with DNA extracted from epithelial surface cultures indicate the occurrence of epithelial colonization by the tested strains (Wang, Li, et al., 2009).

5. Conclusion

The screening based on growth-promoting effects in *D. melanogaster* was efficient to identify, among several fruit-derived *Lactobacillus* strains, those presenting potential probiotic features with beneficial effects on health. Using this innovative strategy, the strains *L. plantarum* 49 and *L. plantarum* 201 displayed the highest growth-promoting effects in *D. melanogaster* test, and were selected to use in further experiments using healthy adult male Wistar rats. *L. plantarum* 49 and *L. plantarum* 201 reduced glucose and TC serum levels in rats during the administration period. Interestingly, these effects remained after 14 days of the wash-out period on glucose levels only for *L. plantarum* 49 and on TC levels only for *L. plantarum* 201. These results clearly pointed that health benefits and probiotic effects of lactobacilli are strain-specific and indicate *L. plantarum* 49 and *L. plantarum* 201 as potential candidates for use in management of biochemical parameters of interest in metabolic diseases.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2018.08.035.

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