**The Lepidoptera Galleria mellonella “in vivo” model: a preliminary pilot study on oral administration of Lactobacillus plantarum (now Lactiplantibacillus plantarum)**

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**Running Title:** probiotic potential, Galleria mellonella, L. plantarum

**SUMMARY**

This work aims to assess the *in vitro* adhesion of two type strains of *Lactobacillus plantarum* (ATCC 14917 and ATCC BAA-793) (now *Lactiplantibacillus plantarum*). The experiments were conducted both *in vitro* on colon cells lines (Caco-2 and HT-29) and *in vivo* by adopting *Galleria mellonella*, a well-known alternative preclinical model.

Data comparison obtained from *in vitro* and *in vivo* assays showed that adhesion performance is comparable in both models. Moreover, the type strain BAA-793, originally isolated from human saliva, showed enhanced adhesion performance, either *in vitro* to the low mucus-producing cell line (HT-29) or *in vivo* into the *G. mellonella* gut. These results suggest a possible adaptation of this strain to its ecological niche compared to ATCC 14917.

This preliminary pilot study, once again, showed the reliability of *G. mellonella* oral administration model as a first-line screening tool for *in vitro* to *in vivo* translation.

Also, for the first time, the permanence of *Lactobacillus* strains into *G. mellonella* gut has been reported, reinforcing the claim that this preclinical model can be used, together with standardised *in vitro* and *in vivo* procedures already accepted across the community, for the evaluation and investigation of new probiotic potentials.

**Keywords:** animal model, adhesion, survival, probiotic potential, L. plantarum and Galleria mellonella

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INTRODUCTION

Intestinal microbiota plays a crucial role in human health (O’Hara and Shanahan 2006). Indeed, the gastrointestinal tract (GIT) is the most colonized organ of the human body: it hosts more than 70% of all microbes, with biomass of 0.2 kg (Sender et al., 2016). In physiological conditions, bacterial communities are in a condition of eubiosis, but when the resilience mechanisms fail, i.e., due to an increase in fermentative or putrefactive bacteria, the balance is altered and "intestinal dysbiosis" is set up (Conlon and Bird 2015). The etiopathogenesis of microbiota alteration can be linked to a sedentary lifestyle, poor dietary choices, the abuse of xenobiotic substances, smoking, infections and stress. Recent studies show a strong correlation between dysbiosis and health conditions such as inflammatory bowel disease (IBD), obesity, diabetes, allergies, cystic fibrosis, autoimmune diseases (multiple sclerosis and rheumatoid arthritis), neurodegenerative diseases, autism, psychiatric disorders and colon cancer (Buford 2017).

The manipulation of the intestinal microbial community can, therefore, represent a useful strategy in the treatment of these pathologies, considering that current pharmacological therapies are often insufficient and have several side effects. In this regard, the focus on gut microflora composition (O’Hara and Shanahan 2006) for preventive and therapeutic purposes is continuously growing (Blandino et al., 2008).

Advances in molecular biology techniques have played a crucial role in identifying the biodiversity of the intestinal microflora, especially the sequencing of bacterial 16S rRNA and metagenomic analysis, that allows the study of microbial communities directly in their natural habitat, avoiding the problem of isolation and laboratory cultivation (Pham and Lawley 2014).

Therefore, modulate intestinal dysbiosis through novel therapeutic strategies is the new key clinical goal in the treatment of many diseases. To re-establish a balanced microbiota, different therapeutic approaches can be evaluated according to the severity of the dysbiosis (Koboziev et al., 2014). In this context, the use of probiotics has shown promising results in restoring the gut flora homeostasis. Indeed, their action is carried out at different levels, including competition with enteropathogens for intestinal epithelial cells adhesion sites and nutrients (García-Cayuela et al., 2014); improvement of the barrier function of the epithelial coating; immunomodulation (Mason et al., 2008; Inturri et al., 2017); influence on other organs through the connection between the enteric and the central nervous systems (Carabotti et al., 2015); production of antimicrobial substances (bacteriocins) against pathogenic bacteria. Among these, the adhesion and interaction process to the host intestinal epithelium constitutes the first line of resistance against colonization of exogenous and commensal opportunistic pathogenic microbes by competing for the receptor sites and therefore preventing their penetration and colonization (Rescigno 2011). For this reason, adhesion to intestinal mucosa is considered one of the main selection criteria for potential probiotics strains as it can increase persistence in the intestine, thereby allowing health-promoting bacteria to exert its effects(García-Cayuela et al., 2014).

Therefore, probiotics administration has been assessed in the treatment and prevention of gastrointestinal diseases such as infectious diarrhoea, IBD such as Crohn’s disease and ulcerative colitis (Celasco et al., 2008; Sánchez et al., 2017) and to prevent the invasion of colon cancer cells (Motevaseli et al., 2017). The applications of probiotics are much broader in all phases of life (Gibson et al., 2017) to prevent type 1 diabetes
(Heeney et al., 2018), obesity and atherosclerosis (Duranti et al., 2017); to modulate the immune system in an anti-atopic way in case of allergies (Ouwehand 2007), atopic dermatitis and psoriasis (Abrahamsson et al., 2012), rheumatoid arthritis and multiple sclerosis (de Oliveira et al., 2017; Heeney et al., 2018); neurodegenerative diseases such as Alzheimer’s and Parkinson’s disease (Akbari et al., 2016; Barichella et al., 2016).

In this context, there is considerable scientific evidence on the therapeutic applications of potentially probiotic strains belonging to the Lactic Acid Bacteria (LAB) and especially regarding Lactobacillus spp., which can adhere along the entire human crypt-villus axis even under pH stress in the human gastrointestinal tract (Costello et al., 2014; Zhang et al., 2018).

Indeed, in the last two years, numerous studies of L. plantarum (now Lactiplantibacillus plantarum) strains have highlighted several positive effects on human health. The most significant ones concern the protective role against weight gain (Crovesy et al., 2020), possible anti-hypertensive effect (Lewis-Mikhael et al., 2020), improved clinical severity scores in children with atopic dermatitis (Adler-Neal et al., 2019), significant promotion of iron absorption in humans (Vonderheid et al., 2019) and anti-atherosclerotic effect in mice (Fijan et al., 2019). In addition, L. plantarum has demonstrated a remarkable ability to adapt to the intestinal ecosystem, so it is clear that the probiotic potential is strictly strain-dependent (Marco et al., 2010).

The human health benefits of L. plantarum ATCC 14917, isolated from a food matrix, are already well known. A recent metabolomic analysis has highlighted the adhesion mechanisms of this strain under initial acid and alkali stress (Wang et al., 2018); moreover, the daily consumption of L. plantarum ATCC 14917 significantly inhibited atherosclerotic lesion formation in an atherosclerosis-prone apolipoprotein E-deficient (ApoE−/−) mice model (Hassan et al., 2020).

On the other hand, L. plantarum BAA-793 (WCFS1), isolated from human saliva, can induce nuclear-κB-dependent pathways (Van Baarlen et al., 2009) and express specific cellular products able to modulate anti-inflammatory responses in vivo (Grangette et al., 2005). Recent work on global gene expression profiling has shown that this strain has adapted specifically to the human intestinal ecosystem, providing new insights into microbial-host interactions and their influence on human health (Marco et al., 2010).

Given the increasing interest observed for probiotic strains, it is essential to have the opportunity to study these microorganisms in a reliable and reproducible in vivo model that allows the translation of results from in vitro to in vivo setting. Greater ethical awareness and stricter control for in vivo studies have led the scientific community to propose numerous alternatives to rodents. Among these, the greater wax moth Galleria mellonella has played a predominant role. Due to many of its characteristics, such as, i.e., the ability to grow at a temperature of 37°C (the physiological temperature in mammals), G. mellonella has proved to be an excellent in vivo preclinical, experimental model for host/microbial interaction, bacterial and fungal pathogenetic mechanism and preliminary toxicity studies (Cutuli et al., 2019; Petronio Petronio et al., 2020). Previous findings on intestinal microbiota of G. mellonella have identified a predominant presence of microorganisms belonging to Enterococcus spp. and Staphylococcus spp., but details are still unclear (Johnston and Rolff 2015b; Krams et al., 2017).
This work aims to evaluate the strain-dependent in vitro adhesion of two well-known *L. plantarum* (ATCC 14917 and ATCC BAA-793), isolated from food matrix and human saliva, respectively, to Caco-2 and HT-29 (human colon adenocarcinoma) cells lines. In addition, the in vivo permanence in *Galleria mellonella* gut as a novel oral administration model for the screening, evaluation and investigation of new potential probiotic strains has been evaluated for the first time.

**MATERIALS AND METHODS**

*Chemicals and reagents*

Vancomycin hydrochloride (S-114359), bromocresol green (S-114359), hydrochloride (HCl) (320331), hydrogen peroxide (H1009) and L -Cysteine hydrochloride (S-C1276), agarose for molecular biology (A4718), were all purchased from Sigma-Aldrich (Milan, Italy). De Man, Rogosa and Sharpe (MRS) (CM0359B Oxoid), bacteriological agar (LP0011B Oxoid), SYBR Safe DNA Gel Stain (S-7585 Invitrogen), Dulbecco’s Modified Eagle's Medium (DMEM 21969035 Gibco), fetal bovine serum (FBS 10270106 Gibco), penicillin/streptomycin (p/s) (Gibco 15070063), L-glutamine 200 mM (25030081 Gibco) and Dulbecco’s phosphate-buffered saline (DPBS Gibco 14190250) were purchased from Thermo Fisher Scientific, US. Trypsin EDTA 1X was purchased from Corning (25-051-CI Corning, NY). EmeraldAmp GT PCR Master Mix (RR310A Takara BIO INC., Japan). Gram staining kit (17PL8055/25 Biolife, Italy), API® 50 CH (50 300 Biomerieux, France), Genomic DNA Extraction Kit Blood/Bacteria/Cultured Cells (YGB100 RBCBioscience, Taiwan), Ladder Dye Plus (3421A Takara BIO INC., Japan).

*Bacterial strains, cell lines and Galleria mellonella larvae*

*Lactobacillus plantarum* subsp. *plantarum* ATCC® 14917™ (isolated from food matrix), *Lactobacillus plantarum* ATCC® BAA-793™ (isolated from the saliva of human oral cavity) purchased from the American Type Culture Collection (Rockville, MD). All strains were cultured in MRS (De Man, Rogosa and Sharpe) at 37°C under microaerophilic conditions.

Human Caucasian colon adenocarcinoma cells (Caco-2 ATCC® HTB-37™) and Human colon adenocarcinoma cells (HT-29 ATCC® HTB-38™) were purchased from the American Type Culture Collection (Rockville, MD). The cell lines were cultured in complete DMEM medium, with the addition of 10% FBS, 1% penicillin/streptomycin and 1% L-glutamine 200 mM and incubated at 37°C in 95% humidified atmosphere containing 5% CO₂. The cell culture medium was changed every two days, and the cells were subcultured at 80% confluence every week.

*G. mellonella larvae* were purchased from SA.GI. P. s.a.s. (Ravenna, Italy) and stored at 15°C in the dark until use.

**In vitro adhesion on Caco-2 and HT-29 cell lines**

The adhesion assay of *L. plantarum* strains ATCC 14917 and ATCC BAA-793 on human intestinal epithelial cells (IECs), CaCo-2 and HT-29 was conducted, according to Cammarota et al. (Cammarota et al., 2009) and Nicolosi et al. (Nicolosi et al., 2020) with some modification.

Overnight cultures of *L. plantarum* strains were harvested by centrifugation at 5000 rpm for two minutes, washed three times in DPBS, diluted by the concentration of 10⁴ to 10⁸ CFU/ml in the same medium. Bacterial
suspensions were plated in MRS agar plate at 37°C for 48h with 5% CO₂ to determine the bacterial loads inoculated into the cells.

Cell line monolayers grown to approximately 95% confluence (1.2 x10⁶ cells) were washed three times in DPBS to remove antibiotics before adding 2 ml of each bacterial suspension. All media used in this assay were free of antibiotics and serum. Bacterial suspensions were inoculated in triplicate into cell lines at the ratio between bacterial and human cells (CFU/IEC) of 1/10, 1/1, 10/1, and 100/1, adding 1.2 x10⁵, 1.2 x10⁶, 1.2 x10⁷ and 1.2 x10⁸ CFU/ml of each strain, respectively. Plates were incubated with each ratio for 1 hour at 37°C, 5% CO₂.

After the incubation period, supernatants were removed, and wells were gently washed three times with DPBS buffer to remove non-attached bacteria. Finally, Caco-2 and HT-29 monolayers were trypsinized with 0.25% trypsin-EDTA solution 1X and bacterial counts were carried out by serial dilution on MRS agar plates with the same incubation condition previously described. Adhesion data were expressed as the percentage of recovered viable bacteria adhered (% adhesion) compared to the initial inoculum to the monolayers.

**In vivo permanence on G. mellonella**

In order to assess whether oral administration of the two *L. plantarum* strains under study affected the viability of *G. mellonella larvae*, preliminary larval survival experiments were conducted, up to 72h, by forced feeding of serial inocula dilutions (from 10³ to 10⁸ CFU/larvae) and no significant difference between infected larvae and control were recorded (data not shown). Larvae standardization, challenge, dosing by oral gavage together with standardised scoring were performed according to Champion et al. with appropriate modifications (Champion *et al.*, 2018).

**In vivo** permanence assay was conducted according to Lange et al., with some modification (Jackson *et al.*, 2002; Lange *et al.*, 2019). The larvae were selected by weight (0.2-0.4 g), light-coloured and free of dark spots and/or cuticle pigments.

Overnight bacterial suspensions of *L. plantarum* strains cultured in MRS broth at 37°C with 5% CO₂ were harvested by centrifugation at 5000 rpm for ten minutes, washed three times in saline solution, and bacteria concentration (expressed in CFU/ml) was evaluated by spectrophotometric determination (OD 600).

To evaluate in vivo permanence, forty *G. mellonella larvae* were fed through gavage with 10μl of each bacterial suspension and twenty larvae with DPBS (control to assess the hypothetical presence of *L. plantarum* strains in *G. mellonella* gut). Each bacterial suspension contained approximately 1x10⁵ CFU/larvae. Bacterial cultures were administered using a syringe (0.5 ml, 29 G x 12.7 mm) into a microsyringe pump. The larvae were incubated in Petri dishes at 37°C for 24 hours in the dark.

**Recovery and isolation of L. plantarum from G. mellonella larvae**

Immediately after the gavage (T0) and after 24 hours (T24) larvae were disinfected with ethanol solution (70% v/v) and after a short thermal shock (freezing for 5 min) to minimize painful stimulation, were sacrificed. Their digestive channels were removed aseptically and homogenized in 1 ml of DPBS.

To evaluate the persistence of *L. plantarum* after gavage administration to *G. mellonella*, five digestive channel homogenates for each group of larvae were incubated on LAMVAB (*Lactobacillus Anaerobic MRS with
Vancomycin and Bromocresol green) agar plates (chromogenic medium for differentiation of *Lactobacillus* spp) at 37°C with 5% CO₂ for 48h. For bacterial CFU/larvae determination, only green colonies (*Lactobacillus* spp) were taken into account.

LAMVAB agar is composed of 104.4 g/l MRS broth, 0.05 % (w/v) Cysteine hydrochloride, 0.005% (w/v) Bromocresol green, 4% (w/v) Bacteriological Agar No., and 0.0002 % (w/v) Vancomycin hydrochloride. The pH was adjusted to 5.0±0.1 using 4 M HCl before autoclaving. The medium was prepared according to Jackson et al., (Jackson et al., 2002)

LAMVAB agar outperforms other media in this context because of the low pH of the medium and the presence of vancomycin, which inhibits the competing Gram-positive bacteria, and bromocresol green, an acid-base indicator with a pH range of 3.8-5.4 (yellow-blue) so that organic acid produced by LAB made the surrounding agar turn yellow and the bacterial colonies green (Yun et al., 2009).

**Morphological and phenotypic identification of *L. plantarum* strains from *G. mellonella's* gut**

Surrounding green colonies on LAMVAB plates (see Supplementary Materials Figure S1A and S1B) were subjected to microscopic examination by Gram's stain. Also, the catalase test was performed on the Gram-positive bacilliform colonies to verify the absence of the enzyme. *Lactobacillus* spp. were identified by API® 50 CH gallery (Biomerieux, France). The biochemical profile obtained was identified using the ApiwebTM identification software (http://apiweb.biomerieux.com) (see Supplementary Material Figure S2).

**L. plantarum molecular identification by 16S rDNA**

All DNA samples were extracted by Genomic DNA Extraction Kit (Blood/Bacteria/Cultured Cells) (RBC Bioscience, Taiwan), according to manufacturer's instructions. The analyses were carried out by isolating the genetic material from different tested samples. Briefly, bacterial DNA was extracted from LAMVAB green colonies isolated from *G. mellonella* larvae fed with the two *L. plantarum* strains tested immediately after the gavage (T0) and at 24 hours (T24). DNA from *L. plantarum* ATCC 14917 and *L. plantarum* ATCC BAA-793 grown on MRS plates were used as positive controls, while no DNA (replaced with water) PCR mix as negative ones.

DNA purity and concentration were evaluated by absorbance method using NanoDrop 2000 Thermo Scientific™ (Massachusetts, USA) spectrophotometer.

*L. plantarum* 16S rDNA PCR was conducted according to Berthier et al. (Berthier and Ehrlich 1998). Oligonucleotides used were 16F forward (GCTGGATCACCTTTC) and reverse (ATGAGGTATTCAACTTATG). 50μl PCR reactions were performed with EmeraldAmp GT PCR Master Mix (2X Premix), 1μl DNA, 0.2μM Primer Forward, 0.2μM Primer Reverse and sterile water. PCR reactions were carried out in a Heal Force Thermal Cycler. Amplification consisted of 30 cycles of 1 min at 94°C and 30sec at 50°C, then 1 min at 72°C. The first cycle was preceded by incubation for 5 min at 94°C. PCR products were electrophoresed in a 1.5% (w/v) agarose gel and were subsequently visualized by UV illumination after SYBR Safe staining.
DNA Ladder (Dye Plus) (Takara BIO INC., Japan) of 50bp was used to determine the molecular weight of the PCR amplicons. Each molecular analysis was performed three times in triplicate.

Statistical analysis
Data are expressed as mean ± standard deviation (S.D.) for three replicates of three independent experiments (i.e., biological and technical triplicates). Prism GraphPad Software 6 was used for all statistical analyses. Differences between the adhesion of two strains of L. plantarum to human cell lines Caco-2 and HT-29 were assessed by two-way ANOVA, followed by Bonferroni multiple testing corrections (Figure 1). Multiple t-test with Holm-Sidak correction was performed on data belonging to bacteria recovery from the G. mellonella larvae gut assay (Figure 2).

RESULTS

In vitro adhesion assay
The two strains of L. plantarum tested exhibit a comparable in vitro adhesion trend on both cell lines (Figure 1).
As regards the bacteria inoculation/cell number ratios evaluated (Figure 1), L. plantarum ATCC 14917 shown an adhesion percentage of 5.3%, 13.6%, 16.9% and 17.8% on the Caco-2 cell line and 9.7%, 15.8%, 22.6% and 24.1% on HT-29. On the other hand, L. plantarum ATCC BAA-793 showed a percentage of 7.2%, 13.8%, 17.4% and 20.0% on the Caco-2 and 13.7%, 18.8%, 23.5% and 26.9% on the HT-29 cell line.
A proportional increase in bacteria inoculation/cell number ratios was observed, and was dependent on the type of human cell line tested. Indeed, both strains of L. plantarum showed higher adhesion when the starting inoculum was 100/1 and lower adhesion when it was 1/10.

Both L. plantarum strains showed statistically greater adhesion values on the HT-29 cell line. Moreover, HT-29 and Caco-2 in vitro cell line adhesion had maximum values of 24.1% and 17.8% for ATCC 14917 compared with 26.9% and 20.0% for ATCC BAA-793. Conversely, the minimum adhesion values were 9.7% and 5.3% for ATCC 14917 and 13.7% and 7.2% for ATCC BAA-793 for HT-29 and Caco-2, respectively. These data showed an adhesion rate of L. plantarum ATCC BAA-793 to both Caco-2 and HT-29 cell lines and a more effective percentage difference in adhesion rate on HT-29 (Table 1).

Recovery and phenotypic identification of L. plantarum in G. mellonella gut
Survival test results showed no significant change in G. mellonella larvae mortality force-fed with the two bacterial strains tested, compared to the untreated control group (DPBS) (data not shown).
Cultural analysis of homogenized G. mellonella larvae gut administered with DPBS displayed no growth on LAMVAB agar plates both after gavage (T0) and at 24 hours (T24). These data demonstrate the physiological absence of Lactobacillus spp. in G. mellonella gut, as already pointed out by Johnston et al. (Johnston and Rolff 2015a)

Immediately after G. mellonella administration (T0) with the bacterial inocula (1x10⁴ CFU/larvae), the presence of the L. plantarum strains tested was confirmed by bacterial plate count on LAMVAB agar plates (see Supplementary Materials Figure S1A and S1B). As shown in Figure 2, at T0 9.2x10³±0.6x10³ CFU/larvae
were counted for *L. plantarum* ATCC 14917 and 9.2x10³±0.7x10³ CFU/larvae for *L. plantarum* ATCC BAA-793, with a recovery percentage of 92% for both strains.

At 24 hours, 1.03x10³±0.2x10² CFU/larvae were counted for *L. plantarum* ATCC 14917 and 1.72x10³±0.3x10² CFU/larvae for *L. plantarum* ATCC BAA-793, with a recovery percentage of 1.03% for *L. plantarum* ATCC 14917 and 1.72% for ATCC BAA-793.

These data showed a statistically significant difference between the two tested strains, in particular a higher permanence ability of *L. plantarum* ATCC BAA-793 compared to *L. plantarum* ATCC 14917 into *G. mellonella* gut (Figure 2).

Morphological examination of green colonies on LAMVAB agar plates derived from the gut of the *G. mellonella* larvae fed with the two strains of *L. plantarum* ATCC 14917 and ATCC BAA-793 showed bacilliform catalase-negative Gram-positive bacteria. Biotyping analysis with API® 50 CH identified these colonies as *L. plantarum* (see Supplementary Materials Figure S2).

**L. plantarum 16S rDNA amplification**

Data obtained by the bacterial plate counting method were confirmed by 16S rRNA gene PCR amplification with specific *L. plantarum* primer (Berthier and Ehrlich 1998).

Figure 3 shows the electrophoresis results. All bacterial genomic DNA extracted from the four different biological samples tested revealed a band of 205bp corresponding to the *L. plantarum* 16S rRNA gene.

**DISCUSSION**

The administration of probiotic strains with the aim of bringing the intestinal microbiota back to eubiosis represents a therapeutic choice in constant growth for diseases where intestinal dysbiosis is involved. The intestinal microbiota is the first line of defence against opportunistic or commensal pathogens because it competes for intestinal cell adhesion sites that contribute to the elimination of harmful microorganisms from the GIT (O'Hara and Shanahan 2006; Blandino et al., 2008; Claesson et al., 2009a; Claesson et al., 2009b; Sommer and Bäckhed 2013).

The adhesion ability of bacteria is often considered a virulence factor, not only in the GIT but also in the urogenital tract (Genovese et al., 2018). In this context, one of the most remarkable peculiarities of probiotics is that they adhere to intestinal cells to prevent enteropathogen adhesion (Markowiak and Śliżewska 2017).

The most commonly studied bacteria with probiotic potential belong to LAB, in particular those of *Lactobacillus* genus (Wedajo 2015).

Currently, some strains of *L. plantarum* are used in many food preparations, e.g., olive preparations, as they have the essential characteristics to make it a probiotic, such as the “GRAS” status, and the ability to survive in the gastrointestinal tract at acidic pH and bile salts (De Vries et al., 2006).

As concerns safety, *L. plantarum* is safe for human use, but there are limited data for other strains. These findings highlight that probiotics are heterogeneous and should be evaluated “strain by strain” to certify their safety and efficacy (Hoffmann et al., 2014).

Due to the beneficial effects that this strain might have on human health, *L. plantarum* ATCC 14917 (Mantzourani et al., 2019) and ATCC BAA-793 (Gu et al., 2019) strains, isolated from food matrix and human
saliva, respectively, have been chosen as "pilot test strains" to evaluate in vitro adhesion and in vivo permanence in G. mellonella larvae.

The authors are fully aware that the use of standard plating technology to assess microbiota composition cannot be considered sufficient and should be supported by a genomic approach. Indeed, this preliminary pilot study will also need to be expanded to analyse molecular aspects, both for the quantification of the bacterial load and for the immunomodulatory effects of these strains on the G. mellonella in vivo models, such as the haemocyte count and the expression of antimicrobial peptides.

Nonetheless, here, for the first time, the probiotic potential of in vivo evaluation of two L. plantarum type strains by the preclinical, orally-administered Galleria mellonella model have been reported. In particular, permanence in the insect gut and the ability to affect larvae survival have both been studied. Despite these differences, G. mellonella larvae gut also has numerous similarities with human GIT in some histological, anatomical and physiological features (Maguire et al., 2016).

Although the adhesion mechanisms of these bacteria to G. mellonella gut are still unknown, the presence of these functional and structural analogies between the mammalian and insect gut has allowed us to report that L. plantarum can remain inside the digestive channel of the insect with a behaviour similar to that shown in in vitro adhesion experiments on two well-known human cellular lines (Caco-2 and HT-29). Indeed, in vitro adhesion results showed that L. plantarum strains had excellent adhesion to human intestinal cell lines Caco-2 and HT-29 in a strain-dependent manner. The adhesion rate to both Caco-2 and HT-29 cells was higher for ATCC BAA-793. These data are in agreement with previous studies where probiotic adhesion was demonstrated to be strain- and species-dependent (Tallon et al., 2007; García-Ruiz et al., 2014).

Moreover, for each bacterial inoculum, the percentage of adhesion was higher for HT-29, a low mucus producer cell line, compared to the non-mucus-producing Caco-2 cell model (Figure 1). These different behaviours have already been observed by several authors (Anderson et al., 2010; García-Ruiz et al., 2014; Sharma and Kanwar 2017) who have shown that adhesion to the mucus-producing matrix is a necessary preliminary step for probiotic organisms to interact with host IECs that are covered by a mucus layer.

The in vitro adhesion trend overlapped the in vivo results, allowing the evaluation of G. mellonella as a potential first-line screening in vivo model for the evaluation of probiotic potential.

It is also interesting to note that the ATCC BAA-793 strain, isolated from human saliva, has better adhesion performance both in vitro to the low mucus producer cell line (HT-29) and in vivo. These results suggest a possible adaptation of this strain to its biological niche compared to the food-borne strain ATCC 14917.

Indeed, in the larvae group fed with DPBS, there was no bacterial growth on LAMVAB agar plates, confirming the absence of Lactobacillus spp, already demonstrated by Johnston et al. (Johnston and Rolff 2015a). The permanence in G. mellonella gut of each L. plantarum administrated by gavage was confirmed by cultural, phenotypical and molecular analysis.
In particular, 24 hours after gavage, the greater ability of permanence in *G. mellonella* gut was observed for *L. plantarum* ATCC BAA-793 (1.72% of recovery) compared to ATCC 14917 (1.03%), with a statistically significant difference (Figure 2).

Moreover, compared to other insects such as *Caenorhabditis elegans* and *Drosophila melanogaster*, the ability of *G. mellonella* to grow at 37°C (Cutuli *et al.*, 2019) allows its use as a model for oral bacterial infection studies (Fedhila *et al.*, 2010). In this context, our results proven that the two strains of *L. plantarum* tested do not affect larvae survival when administered orally.

In conclusion, the data obtained in this study confirmed that both bacterial strains tested can adhere to intestinal cells *in vitro* and persisted in *G. mellonella* gut, at least in a 24-hour window. Also, when administered orally, they can be recognized as safe for this insect without effect on larvae mortality. These results confirmed the *in vitro* and *in vivo* difference between the two strains, showing that *L. plantarum* ATCC BAA-793 has a higher adhesion capacity than *L. plantarum* ATCC 14917.

Therefore, this preliminary pilot study, once again (Lange *et al.*, 2018; Lange *et al.*, 2019) showed the reliability of the *G. mellonella* oral administration model as a first-line screening tool for *in vitro* to *in vivo* translation, reinforcing the claim that *G. mellonella* can be used, together with standardised *in vitro* and *in vivo* procedures already accepted across the community (Cozzolino *et al.*, 2020), as an alternative animal model for probiotic strain evaluation.
REFERENCES


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Table 1 Adhesion percentage difference (Δ %) *L. plantarum* ATCC 14917 and *L. plantarum* ATCC BAA-793 *in vitro*. 1/10, 1/1, 10/1 and 100/1 are the ratios between bacterial and human cells that have been inoculated into human cell lines.

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<th>Bacteria/Human Cells</th>
<th>( \Delta % \text{ ATCC BAA-793 vs ATCC 14917} )</th>
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<td>Caco- 2</td>
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<td>1/10</td>
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Figure 1 L. plantarum in vitro adhesion assay. Black histograms: adhesion on HT-29 cell line. Dark grey histograms: adhesion on Caco-2 cell line. a) L. plantarum ATCC 14917. b) L. plantarum ATCC BAA-793. 1/10, 1/1, 10/1 and 100/1 are the ratios between bacterial and human cells that have been inoculated on the human cell lines. The data are expressed as the number of adhering bacteria compared to the number of bacteria seeded (% adhesion). The bars represent means ± SD of three independent experiments performed in triplicate. Statistical analysis was performed using the two-way ANOVA with Bonferroni multiple comparison post-test. **P < 0.01, *P < 0.05.
Figure 2 CFU/larvae of *L. plantarum* strains recovered from *G. mellonella* larvae’s gut administrated by gavage with $1 \times 10^4$ CFU/larvae. Black squares: larvae fed with DPBS. Light grey histograms: larvae fed with *L. plantarum* ATCC 14917. Dark grey histograms: larvae fed with *L. plantarum* ATCC BAA-793. Data expressed as the mean ± standard deviation (CFU/larvae) of three independent experiments. Statistical analysis was performed using Multiple t-test with Holm-Sidak correction. *P < 0.05* was considered to be significant.
Figure 3 Agarose gel electrophoresis of 16S rRNA gene amplification of *G. mellonella* homogenized gut fed with the two *L. plantarum* strains and plated on LAMVAB agar plates. From left to right. Column 1 ladder DNA; column 2, LAMVAB1 T0: *L. plantarum* ATCC 14917 DNA extracted from *G. mellonella* gut homogenized immediately after administration; column 3, LAMVAB2 T0: *L. plantarum* ATCC BAA-793 DNA extracted from *G. mellonella* gut homogenized immediately after administration; column 4, LAMVAB1 T24: *L. plantarum* ATCC 14917 DNA extracted from *G. mellonella* gut homogenized after 24h administration; column 5, LAMVAB2 T24: *L. plantarum* ATCC BAA-793 DNA extracted from *G. mellonella* gut homogenized after 24h administration. Column 6 DNA extracted from *L. plantarum* ATCC 14917 (MRS1) and column 7 DNA from ATCC BAA-793 (MRS2) both growth on MRS agar plates and used as positive controls. Lastly, column 8 PCR mix without DNA as negative control and column 9 DNA ladder.
Supplementary material

**Figure S1A** Bacterial colony evaluation of *G. mellonella* digestive channels homogenates administered with *L. plantarum* strains on MRS agar plates. 6.3X total magnification by Leica MZ6© Stereomicroscope.

<table>
<thead>
<tr>
<th>Agar medium</th>
<th>Plate/Bacterial colony</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty plate</td>
<td></td>
<td>After agarization plate are clear, brown colored with a pH-value (25 °C), 5.6 - 5.8.</td>
</tr>
<tr>
<td><em>L. plantarum</em> ATCC 14917</td>
<td></td>
<td>After incubation, both <em>L. plantarum</em> strain tested showed typical white circular/entire raised colonies. No change in color of the surrounding areas have been observed</td>
</tr>
<tr>
<td>MRS</td>
<td><em>L. plantarum</em> ATCC BAA-793</td>
<td>The morphologies of the other colonies (not belonging to <em>Lactobagillus</em> spp.), although varius for different microorganisms, are very similar to that of <em>L. plantarum</em>: from white to pale yellow, circular/ovoid and flat/raised. Also in this case, no change in color of the surrounding areas have been observed</td>
</tr>
</tbody>
</table>

Other
**Figure S1B** Bacterial colony evaluation of *G. mellonella* digestive channels homogenates administered with *L. plantarum* strains on LAMVAB agar plates. 6.3X total magnification by Leica MZ6© Stereomicroscope.

<table>
<thead>
<tr>
<th>Agar medium</th>
<th>Plate/Bacterial colony</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty plate</td>
<td><img src="empty_plate.png" alt="Image" /></td>
<td>After agarization plate are clear, dark green colored with a pH-value (25 °C), 5.</td>
</tr>
<tr>
<td><em>L. plantarum</em> ATCC 14917</td>
<td><img src="lplantarum_14917.png" alt="Image" /></td>
<td>After incubation, both <em>L. plantarum</em> strain tested showed pale green circular/entire raised colonies. A change in color of the surrounding areas have been observed (from dark green to bright yellow), due to the presence of bromocresol green as pH indicator.</td>
</tr>
<tr>
<td>LAMVAB</td>
<td><img src="lamvab.png" alt="Image" /></td>
<td></td>
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<tr>
<td><em>L. plantarum</em> ATCC BAA-793</td>
<td><img src="lplantarum_baa-793.png" alt="Image" /></td>
<td>Compared to <em>L. plantarum</em> colonies, those belonging to other microorganisms do not show the pale green coloration, but remain white. Furthermore, it was not observed the color change of the surrounding areas, due to the lack of acidification.</td>
</tr>
<tr>
<td>Other</td>
<td><img src="other.png" alt="Image" /></td>
<td></td>
</tr>
</tbody>
</table>
Table S1 API 50CH results for *L. plantarum* ATCC 14917 and *L. plantarum* ATCC BAA-793 strains: 99.9% similarity match with *L. plantarum*.

<p>| Test Number | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 |
|-------------|---|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| <strong>Biochemical assay</strong> | Control | Glycerol | Erythritol | D-arabinose | L-arabinose | Ribose | D-xylose | L-xylose | Adonitol | β-methyl-D-xyloside | Galactose | D-glucose | D-fructose | D-mannose | L-sorbose | Rhamnose | Dulcitol | Inositol | Manitol | Sorbitol | Methyl-D-mannoside | Methyl-D-glucoside | N-acetyl-glucosamine | Arbutine | Escaline | Salicine | Cellobiose | Maltose | Lactose | Melibiose | Sucrose | Trehalose | Imuline | Melitose | D-raffinose | Starch | Glycogene | Xyliol | β-Cellobios e | | |
| <em>L. plantarum</em> ATCC | - | - | - | - | + | - | - | - | - | - | + | + | + | + | + | - | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| <em>L. plantarum</em> ATCC-BAA | - | - | - | - | + | - | - | - | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |</p>
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</table>

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<thead>
<tr>
<th><strong>Biochemical assay</strong></th>
<th>D-turanose</th>
<th>D-lyxose</th>
<th>D-tagalose</th>
<th>D-fucose</th>
<th>L-fucose</th>
<th>D-arabitol</th>
<th>L-arabitol</th>
<th>Gluconate</th>
<th>2-keto-gluconate</th>
<th>5-keto-gluconate</th>
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</thead>
<tbody>
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<tr>
<td><strong>L. plantarum ATCC-BAA</strong></td>
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