Manuscript
Click here to download Manuscript: Arena et al 2014_corrDK.doc
Click here to view linked References

Running title: probiotic activities and riboflavin-overproducing Lactobacillus strains

Probiotic abilities of riboflavin-overproducing *Lactobacillus* strains: a novel promising application of probiotics

Mattia P. Arena[1,3], Pasquale Russo[1^], Vittorio Capozzi[1^], Paloma López[2], Daniela Fiocco[3] and Giuseppe Spano[1]*

[1]*University of Foggia, Department of Agricultural Sciences, Food and Environmental, Via Napoli 25, 71100 Foggia, Italy.*

[2]*Centro de Investigaciones Biológicas (CIB), Department of Molecular Microbiology and Infection Biology, Madrid, Spain.*

[3]*Department of Clinical and Experimental Medicine, University of Foggia, Foggia, Italy.*

[^]*Promis Biotech srl, via Napoli 25, 71122 Foggia, Italy*

*Author to whom correspondence should be addressed; e-mail: giuseppe.spano@unifg.it

Key words: Lactobacillus plantarum, Lactobacillus fermentum, probiotics, riboflavin

Abstract

The probiotic potential of *Lactobacillus plantarum* and *Lactobacillus fermentum* strains, capable of overproducing riboflavin, was investigated. The riboflavin production was quantified in co-cultures of lactobacilli and human intestinal epithelial cells, and the riboflavin overproduction ability was confirmed. When milk and yogurt were used as carrier matrices, *L. plantarum* and *L. fermentum* strains displayed a significant ability to survive through simulated gastrointestinal transit. Adhesion was studied on both biotic and abiotic surfaces. Both strains adhered strongly on Caco-2 cells, negatively influenced the adhesion of *Escherichia coli* O157:H7 and strongly inhibited the growth of three reference pathogenic microbial strains. Resistance to major antibiotics and potential hemolytic activity were assayed. Overall, this study reveals that these strains of *Lactobacillus* are endowed with promising probiotic properties and thus are candidates for the development of novel functional food which would be both enriched in riboflavin and induce additional health benefits, including a potential *in situ* riboflavin production, once the microorganisms colonize the host intestine.

Keywords: Probiotic, *Lactobacillus fermentum*, *Lactobacillus plantarum*, riboflavin

Introduction

Probiotics are defined as live microorganisms which confer health benefits on the host when ingested in adequate amounts (FAO/WHO 2002; Morelli et al. 2000). The health-promoting activities exerted by probiotics on humans include the modulation of immunological, digestive and respiratory functions and the prevention of infectious disease (FAO/WHO 2001). Most of the probiotics so far identified belong to *Bifidobacterium* and *Lactobacillus* genera, and selected strains of such groups are present in many probiotic and functional food products and in dietary supplementations (Vinderola et al. 2005; Holzapel et al. 1998). Members of the *Lactobacillus* genus occur in a variety of niches, including plant material, fermented foods, and diverse parts of the animal body. In particular, lactobacilli are part of the natural human gut microbiota and several commensal species of such groups have gained attention for their positive impact on the health of the host (FAO/WHO 2001; van Baarlen et al. 2013).

In order to be defined as a probiotic, a microorganism must possess various attributes, including safety for human consumption and also the capability to survive the gastrointestinal tract, to adhere to enterocytic cells and colonize the intestine, to reinforce the intestinal epithelial barrier, and to modulate gut associated lymphoid tissue (GALT) (Marteau et al. 2001; Patel and Lin 2010). Resistance to the high gastric acidity and to the intestinal environment allows a greater number of viable, beneficial microorganisms to reach the lower gut, which is the main target organ of probiotic action. Furthermore, the ability to adhere to intestinal epithelial cells guarantees an intimate contact between enterocytes and probiotic microorganisms, and thus positively influences i) intestinal cell functions, ii) gut microbiota composition, and iii) host immune response. Microbial survival in the human gastrointestinal tract can be predicted and evaluated *in vitro* using models that mimic the gastrointestinal transit of ingested food (Bove et al. 2013; van Bokhorst-van de Veen et al. 2012). Cultures of enterocyte-like cells, such as Caco-2 cells, from human colon adenocarcinoma, are

routinely used as *in vitro* model to study the mechanisms underlying host-probiotic interaction at the intestinal level, including bacterial adhesion to the gut epithelium (Lee et al. 2000). Several studies on potential probiotic strains also consider their ability to modulate the expression of host genes involved in the immune response, apoptosis and inflammatory processes (Delcenserie et al. 2008; Maccaferri et al. 2012). A beneficial effect on the microbial equilibrium in the human intestine is another valuable probiotic trait. Indeed, some probiotics can directly compete against pathogenic bacteria for host epithelial cell receptors and/or produce antimicrobial compounds, i.e. bacteriocins, thus controlling colonization and limiting the proliferation of detrimental microbial species in the gut (Riley and Gordon 1999; Koo et al. 2012; Bove et al. 2013). A further desirable feature of probiotics is their capability to synthetize compounds, such as vitamins or short chain fatty acids, that can be helpful both to human and microbiota metabolism (Dunne et al. 2001; Hooper et al. 2002), by supplementing insufficient food intakes of these compounds and preventing the negative consequences of unbalanced diets (LeBlanc et al. 2011; Rossi et al. 2011).

Riboflavin (vitamin B_2) plays a key role in metabolism as it is the precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (Capozzi et al. 2012; Burgess et al. 2006a). Since humans cannot synthesize riboflavin, its dietary supplementation is essential (LeBlanc et al. 2010b). Nevertheless, a certain amount of riboflavin can even be supplied endogenously by some microbes residing in the large intestine (Hill et al. 1997). The identification of microbial strains that exhibit a combination of probiotic characteristics and riboflavin overproduction would represent the opportunity to develop functional foods possessing probiotic properties, high riboflavin contents, and with the potential to deliver viable microorganisms that can synthesize riboflavin directly in the intestine (Burgess et al. 2004; van Loon et al. 1996).

In this study, we assayed the potential probiotic activity of *Lactobacillus plantarum* CETC 8328 and *Lactobacillus fermentum* CECT 8448 which were selected for their ability to over-produce riboflavin in a chemically defined medium (CDM) and in bread (Russo et al., 2014). Firstly, their

riboflavin overproduction ability was tested in an *in vitro* model of the intestinal environment, i.e. co-cultures of the lactobacilli strains and Caco-2 cells. Then, the survival of the tested strains was evaluated by exposure to an *in vitro* system simulating passage through the human gastrointestinal tract. Adhesion on Caco-2 monolayers and on an abiotic surface were also analyzed. Moreover, the strains were investigated for their ability to counteract pathogen growth and pathogen adhesion, on epithelial cells. The probiotic potential of the isolates was compared with that of reference probiotic lactobacilli.

Material and methods

Bacterial strains, human cells and growth conditions

The lactobacilli used in this work were *Lactobacillus plantarum* WCFS1 (Kleerebezem et al. 2003), *Lactobacillus acidophilus* LA5 (Chr. Hansen, Hörsholm Denmark) and the previously described riboflavin over-producing *L. fermentum* strain (Russo et al. 2014). In addition, a new overproducing strain of *L. plantarum* previously selected from sourdough (Russo et al. 2014) was used. Strains were deposited to the Spanish Type Culture Collection (CECT, Valencia, Spain) and named CETC 8328 (*L. plantarum*) and CECT 8448 (*L. fermentum*). Lactobacilli were propagated in de Man Rogosa Sharpe (MRS, Oxoid, UK) (pH 6.2) and incubated at 30°C. For *L. acidophilus* LA5, MRS medium was supplemented with 0.1 % Tween and 0.05 % L-cysteine (Merck, Darmstad, Germany) and incubation was performed at 37°C. Bacteria were routinely harvested when cultures reached the mid-exponential phase (OD_{600nm} 1, corresponding to a concentration of 2-8×10^8 CFU mL^{-1}). Then, the cultures were centrifuged (1,500×*g*, 10 min) and resuspended in appropriate solutions, depending on the assay, as described below.

The bacterial pathogens used in this study were *Listeria monocytogenes* CECT 4032, *Salmonella enteritidis* CECT 409, and *Escherichia coli* O157:H7 CECT 4267. All pathogens were growth in tryptone soy broth (TBS, Oxoid, UK) and incubated at 37 °C.

Human adenocarcinoma colon cells Caco-2 were cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 2mM L-glutamine, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin, at 37°C, in an atmosphere containing 5% CO_2. Caco-2 cells were seeded at the concentration of 1.2×10^4 cells/well in 96-wells plates for the adhesion assay, and at the concentration of 2×10^5 cells/well in 24-well plates for the riboflavin production assay. The growth medium was replaced three times per week and, 24 h before each assay, it was replaced with absolute DMEM, without any supplements. Cells were grown to form differentiated and steady epithelial/enterocyte-like monolayers for 15 days.

Identification of L. plantarum CETC 8328 as a new riboflavin overproducing strain and production of riboflavin in a co-culture approach

Selection of *L. plantarum* CETC 8328 riboflavin overproducing strain, riboflavin extraction and quantification in a CDM medium were performed as already reported (Russo et al. 2014).

The ability of *L. plantarum* CETC 8328 and *L. fermentum* CECT 8448 to over-produce riboflavin in co-culture of lactobacilli and Caco-2 cells, was examined according to the procedure of Russo et al. (2012b). Bacterial cells (OD_{600nm} 1) were resuspended in absolute DMEM and incubated with Caco-2 (ratio 1000:1, bacteria to Caco-2 cells) for 8 h, at 37°C, in a humidified atmosphere with 5% CO_2. Caco-2 cells incubated in DMEM without any microbial inocula were used as a control. After incubation, the medium was collected and the riboflavin was extracted and quantified according to Jakobsen et al. (2008). Briefly, 5 mL samples were mixed to 25 mL 0.1 M HCl, autoclaved at 121 °C for 30 min and cooled at room temperature. Then, pH was adjusted to 4.5 with 4 M sodium acetate and 5 mL of solution containing α-amylase (420 U), papain (12 U), acid phosphatase (22 U) and 0.1% of glutathione (all from Sigma Aldrich) were added. The enzymatic hydrolysis was facilitated by placing samples in an ultrasonic bath for 1 h. Afterward each sample was diluted to 50

mL with 0.01 M HCl. The riboflavin quantification was carried out by HPLC analysis (Agilent-1100 Series, Palo Alto, CA, USA). All samples were filtered through 0.45 µm and then 0.20 µm filters. Chromatography was performed using Zorbax Eclipse Plus C 18 (4.6 x 150 mm, 5 µm i.d.) analytical column with a Zorbax ODS pre-column (4,6 x 12,5 mm, 5 µm i.d.) (Agilent Technologies), and methanol:water (35:65 v/v) mixture as mobile phase. Detection of the eluate was performed by fluorescence at an excitation wavelength of 440 nm and an emission wavelength of 520 nm.

L. plantarum and *L. fermentum* CECT 8447 (Russo *et al*., 2014) parental, non-riboflavin over-producing strains, were used as controls to determine the amount of riboflavin produced by *L. plantarum* CETC 8328 and *L. fermentum* CECT 8448 strains.

Tolerance of L. plantarum CETC 8328 and L. fermentum CECT 8448 to a simulated gastrointestinal system

Bacterial cells were resuspended in three different carrier matrices: i) saline solution (NaCl 8.5 g L^{-1}), ii) reconstituted milk powder in sterilized water (150 g L^{-1}) (Humana 1, GmbH, Herford, Germany), iii) commercial white yogurt used without dilutions. Both reconstituted milk and yogurt were subjected to thermal treatment (72 °C for 20 min) and the absence of any bacterial contamination was checked by plating on MRS agar plates before the gastrointestinal assay. The simulated gastrointestinal transit was adapted from a system previously described (Bove et al. 2013). Briefly, bacterial suspension was subjected to an oral stress step incubating for 5 min with 150 mg L^{-1} lysozyme (Sigma-Aldrich) and adjusting to pH 6.0. Subsequently, pepsin (3g L^{-1}) (Sigma-Aldrich) was added and the pH value was progressively reduced in order to mimic the gastric compartment. Intestinal stress was performed by adjusting the pH value to 6.5 and by addition of bile salts (3g L^{-1}) and pancreatin (1g L^{-1}) (all from Sigma-Aldrich). In relation to the previously designed gastro-intestinal system (Bove et al. 2013), in the present study, only the oral

compartment, gastric steps corresponding to pH values of 3.0 and 2.0 and subsequent intestinal transits (small and large intestine sectors) were used for the evaluation of bacterial survival. Dilutions from control and treated samples of each strain were plated on MRS agar, CFU were counted and percent survival was determined with respect to unstressed control.

In vitro adhesion assay

Adhesion assays were performed on Caco-2 cell monolayers according to Russo et al. (2012a). Bacterial cells were resuspended in absolute DMEM and incubated with Caco-2 cells for 1h, at 37°C, with 5% CO_2 (ratio 1000:1, bacteria to Caco-2 cells). The percentage of adhesion by lactobacilli was determined by plating appropriate dilutions of the bacterial suspensions from control and test wells on MRS agar and subsequent CFU counting.

The ability of the analysed lactobacilli to inhibit the adhesion of *E. coli* O157:H7 to Caco-2 cells was also investigated. *E. coli* cells from mid-exponential phase cultures (OD_{600nm} 1, corresponding to $8x10^8$ CFU/ml) were added to Caco-2 cells in a ratio of 1000:1, likewise the lactobacilli. Three different types of experiments were performed: *i*) competitive adhesion, i.e. simultaneously incubating Caco-2 cells with *E. coli* and each of the *Lactobacillus* strains for 1 h; *ii*) inhibition of adhesion, i.e. pre-incubating Caco-2 cells with the specific lactobacillus strain for 1 h, then adding *E. coli* and further incubating for 1 h; *iii*) displacement, i.e. pre-incubating Caco-2 cells with *E. coli* for 1 h, then adding lactobacilli and further incubating for 1 h (Koo et al. 2012; Candela et al. 2008; Gagnon et al. 2004). The inhibition of pathogen adhesion was determined by a quantitative PCR-based method, according to Candela et al. (2005). Results were expressed as a relative adhesion level with respect to the adhesion observed when *E. coli* O157:H7 was tested alone (control sample). Adhesion rate of *E. coli* was determined by quantitative real time PCR (qPCR) analysis on samples obtained from test and control wells.

Antimicrobial activity assay

To examine the antimicrobial activity of the lactobacilli strains, an agar spot test was used, as described by Gaudana et al. (2010). Briefly, 5 µl of each of the over-night cultures of the lactobacilli were spotted on MRS agar and incubated for 24 h at 37°C to allow growth. *Listeria monocytogenes* CECT 4032, *Salmonella enteritidis* CECT 409, and *Escherichia coli* O157:H7 CECT 4267 were grown overnight in TSB and 150 µl of each pathogenic bacterial strain were mixed with 15 ml of TSB soft agar (containing 0.6% agar, w/v) and poured over MRS agar plates containing the developed colonies of the lactobacilli. Plates were incubated for 24 h and the radii of the inhibition zones were measured.

RNA extraction, cDNA synthesis and qPCR analysis

A qPCR strategy was also employed to determine the percentage of *E. coli* O157:H7 adhesion on Caco-2 cells. To quantify adherent *E. coli* cells by real time PCR, cell suspensions from adhesion assays were heat treated (10 min, 95 °C) and then chilled on ice. Aliquots (3µl) were then mixed with 1x iTaq supermix (Bio-Rad, Hercules, CA, USA), H flagellar antigen specific gene (*fliC* H7) TaqMan probe (200nM) and primers (500 nM each) (Perelle et al. 2004). Reactions were cycled in an ABI 7300 instrument (Applied Biosystems, Foster City, CA, USA) as follows: initial denaturation at 95 °C for 10 min and 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s and fluorescence acquisition (FAM) at 72°C for 30 s. Each PCR assay included duplicate reactions on DNA samples (i.e. heat-treated cell suspensions), on no template (negative) control and on internal standards, i.e. serial dilutions of *E. coli* suspension (corresponding to a concentration) ranging from 1×10^4 to 1×10^8 CFU/ml, to generate a reference standard curve which was used for quantification.

Hemolytic activity, antibiotic resistance and biofilm formation assays

Lactobacilli were assayed for hemolytic activity by spotting cultures onto Columbia agar plates (Oxoid, Basingstoke, England) containing 5% (w/v) of defibrinated human blood from healthy donors. Plates were incubated at 30°C for 48h (Maragkoudakis et al. 2006) and analyzed for α-, β-, and γ-haemolytic activity. *L. monocytogenes* was used as a positive control. Lactobacilli were also tested for resistance against several antibiotics as described by EFSA (2012). Antibiotics include ampicillin (0.5, 1, 2 and 4 mg L^{-1}), gentamycin (4, 8, 16 and 32 mg L^{-1}), kanamycin (16, 32, 64, and 128 mg L^{-1}), erythromycin (0.25, 0.5, 1, and 2 mg L^{-1}), clindamycin (0.5, 1, 2, and 4 mg L^{-1}), tetracycline (8, 16, 32 and 64 mg L^{-1}), chloramphenicol (2, 4, 8, and 16 mg L^{-1}).

The ability to produce biofilms on glass surface was assayed according to Vergara-Irigaray et al. (2009). Cultures of the lactobacilli strains were inoculated in fresh MRS broth (2% v/v) and incubated for 24 h and 48 h at 37 °C, in an orbital shaker (200 rpm). Biofilm rings were washed twice with distilled water and then stained with crystal violet (5 g L^{-1}, 0.5% w/v). The biofilms were solubilized with acetic acid (30% v/v), and optical density (OD) was measured at 570 nm.

Statistical analysis

Each reported finding represents the mean ±SD of three different experiments. Data were analyzed by Student's t-test using the IBM SPSS Statistics 21.0 software program (IBM, Armonk, NY, USA). $p<0.05$ and $p<0.005$ were considered as statistically significant.

Results

Overproduction of riboflavin by Lactobacillus plantarum CETC 8328

L. plantarum CETC 8328 was selected as a roseoflavin-resistant strain by exposure to gradually increasing concentrations of roseoflavin (Russo et al. 2014). Then, the ability of this strain to over-produce riboflavin, compared to the parental strain, was investigated in CDM medium. The riboflavin production by *L. plantarum* CETC 8328 was 3.33 mg L^{-1}, while no riboflavin was

detected in the medium of the parental strain (**Fig. 1**). To our knowledge, and among the *L. plantarum* strains identified so far, *L. plantarum* CETC 8328 is the highest riboflavin producer currently available (Capozzi et al., 2011).

Furthermore, we investigated the capability of *L. plantarum* CETC 8328 and *L. fermentum* CECT 8448 to synthetize riboflavin in co-culture with intestinal Caco-2 cells as reported in **Fig. 2**. Interestingly, riboflavin production was detected in the medium where *L. plantarum* CETC 8328 and *L. fermentum* CECT 8448 were inoculated. The concentrations of riboflavin were 397±2 and 430±4 μg L^{-1} for *L. plantarum* CETC 8328 and *L. fermentum* CECT 8448 respectively. In contrast, the riboflavin contents of the media inoculated with the *L. plantarum* and *L. fermentum* parental isolates, were 117±14 and 192±25 μg L respectively. Uninoculated DMEM medium contained a concentration of riboflavin of 322.9±24.3 μg/L^{-1}, corresponding to the amount of riboflavin stated by the supplier (Fig. 2). Therefore, our results showed that *L. plantarum* CETC 8328 and *L. fermentum* CECT 8448 have the capability to produce riboflavin, 3.4- and 2.2-fold higher than the non roseoflavin-resistant isolates *L. plantarum* and *L. fermentum* strains.

Survival during the GI transit

The capability of *L. plantarum* CETC 8328 and *L. fermentum* CECT 8448 to tolerate the gastrointestinal (GI) tract conditions was investigated using an *in vitro* model that mimics the human digestive process through the oral cavity, the stomach and intestine. Overall, our observations indicated a variable survival depending on strains, vehicle matrices and GI steps (**Fig 3**). The bacterial viability of all strains, expressed as a percentage of survival with respect to untreated samples, was not influenced by oral stress using the different matrices. In contrast, bacterial survival was significantly reduced under gastric conditions in a pH-dependant manner. In saline solution survival decreased by about 3 and 6 Log units, in the gastric sectors at pH 3.0 and 2.0, respectively, with no significant differences between the tested strains. Whereas, at the same

steps, the reduction of bacterial survival was lower, and approximately of 1-3 Log units and 1-4 Log units when tested in milk and yogurt respectively.

Under intestinal stresses, both small and large intestinal challenges, minor reductions of cell viability were found, in all considered matrices. In saline solution, the percentage of survival after the intestinal stress decreased by 4 to 6 Log units, and there were no major differences between bacterial strains, except for *L. acidophilus* LA5, whose survival was mainly negatively compromised by intestinal conditions compared to other strains. In milk, the percentage of survival was reduced in a range of 3-4 Log units, and no relevant differences were observed between bacterial strains.

Adhesion to Caco-2 cells

The ability of *L. plantarum* CETC 8328 and *L. fermentum* CECT 8448 to adhere to human enterocyte-like cells were assayed, and the results are shown in **Fig. 4**. Both the tested isolates possessed a higher adhesion ability compared to the probiotic control strains (*L. plantarum* WCFS1 and *L. acidophilus* LA5), when the adhesion levels were expressed as a percentage (**Fig 4A**) or as the absolute number of adherent bacteria on Caco-2 cell (**Fig. 4B**). The percentage of adhesion to Caco-2 cells was around 16% and 17% for *L. plantarum* CETC 8328 and *L. fermentum* CECT 8448, respectively, whereas *L. plantarum* WCFS1 and *L. acidophilus* LA5 adhesion level was about 12% and 6%, respectively (Fig 4A). The corresponding values of adherence expressed as the number of adherent bacteria on single Caco-2 cell (cfu/Caco-2) were: 120±17 cfu/Caco-2 for *L. plantarum* CETC 8328; 163±25 cfu/Caco-2 for *L. fermentum* CECT 8448; 94±16 cfu/Caco-2 for *L. plantarum* WCFS1 and 11±7 cfu/Caco-2 for *L. acidophilus* LA5 (Fig. 4B).

Influence of L. plantarum and L. fermentum on the adhesion ability of E. coli O157:H7

303 The ability of *L. plantarum* CETC 8328 and *L. fermentum* CECT 8448 to interfere with the
304 adhesion of *E. coli* O157:H7 was quantified by real time PCR. In the competition assay (between
305 lactobacilli and pathogen), *E. coli* O157:H7 and the lactobacilli strains were co-incubated with
306 Caco-2 cells (**Fig. 5**). As shown in **Fig. 5A**, the adhesion ability of *E. coli* O157:H7 was
307 significantly reduced by 1.4- or 1.6-fold, when co-incubating with *L. plantarum* CETC 8328 or *L.*
308 *fermentum* CECT 8448. *L. plantarum* WCF1 also produced a significant decrease (about 1.6-fold)
309 of pathogen adhesion, whereas *L. acidophilus* LA5 did not display any significant effect on *E. coli*
310 O157:H7 binding to enterocytes. When the lactobacilli strains were pre-incubated with Caco-2 cells
311 for 1 h prior to inoculation with *E. coli* O157:H7, and then incubated for a further 1 h, a significant
312 reduction of pathogen adhesion was observed for all the lactobacilli tested, ranging from 2.2- fold to
313 4.8-fold (**Fig. 5B**). The lowest number of adherent *E. coli* O157:H7 cells was detected when pre-
314 incubation was performed with *L. plantarum* WCFS1, although comparable decreases in pathogen
315 adhesion were observed with all the other tested lactobacilli. When Caco-2 cells were first pre-
316 incubated with *Escherichia coli* O157:H7 and lactobacilli were sequentially added, the adhesion
317 levels of *E. coli* O157:H7 (**Fig. 5C**) was significantly increased (from 2.0- to 5.4-fold) with respect
318 to control conditions (no lactobacilli added).
319
320 *Antimicrobial activity, hemolytic activity and antibiotic resistance*
321 *L. plantarum* CETC 8328 and *L. fermentum* CECT 8448 were investigated for a potential
322 antagonistic activity against the pathogens *L. monocytogenes*, *S. enteritidis*, and *E. coli* O157:H7.
323 This was evaluated by measuring the growth inhibition halos produced on agar plates by the tested
324 lactobacilli strains. As reported in **Table 1**, *L. plantarum* CETC 8328 and *L. fermentum* CECT 8448
325 were able to inhibit *L. monocytogenes*, *S. enteritidis*, and *E. coli* O157:H7.
326 The strains analysed presented γ-hemolytic activity (i.e. no hemolysis) on Columbia blood agar
327 plates, unlike *L. monocytogenes* that was used as positive control and exhibited β-hemolytic activity

(Tab 1). Moreover, *L. plantarum* CETC 8328 and *L. fermentum* CECT 8448 strains were tested for antibiotic resistance against a representative range of clinical antibiotics. However, all the analysed strains were susceptible to the tested antibiotics, with *L. plantarum* CETC 8328 able to growth only in presence of 2 mg L^{-1} of chloramphenicol (data not shown).

Biofilm production

The ability of *L. plantarum* WCFS1, *L. acidophilus* LA5, *L. plantarum* CETC 8328 and *L. fermentum* CECT 8448 strains to form biofilms on a smooth glass surface was evaluated. All the strains analysed were able to adhere to glass surface as measured by absorbance spectrophotometry (**Fig. 6**). The biofilm formation was strain- and time-dependent. *L. plantarum* CETC 8328 was the best adherent strain on abiotic surface (OD_{570} 0.58 and 1.75, respectively after 24 and 48h) followed by *L. plantarum* WCFS1 (OD_{570} 0.16 and 0.89, respectively after 24 and 48h), *L. fermentum* CECT 8448 (OD_{570} 0.10 and 0.19, respectively for 24 and 48h) and *L. acidophilus* LA5 (OD_{570} 0.04 and 0.05, respectively for 24 and 48h).

Discussions

Lactic acid bacteria are a group of microorganisms that are widely used in industry as starter cultures for the production of fermented foods. Besides their fermentative capacities, LAB can improve the safety, shelf life, nutritional value, flavor, and overall quality of the product. In addition, LAB may exert a range of properties beneficial to health, and for this reason are used as probiotic microorganisms in a variety of novel products (van Baarlen *et al*. 2013).

We previously selected and characterized a strain of *L. fermentum* able to overproduce riboflavin in CD media or during bread fermentation (Russo et al. 2014). Additionaly, several *L. plantarum* strains were selected from the same matrix (sourdough) as potential riboflavin producers. In this study, we investigated the riboflavin over-production of *L. plantarum* CETC 8328, one of the

strains selected, and we found that this strain was able, in CD media, to produce riboflavin with a concentration of about 3.3±0.13 mg L^{-1} (Fig. 1).

L. plantarum CETC 8328 and *L. fermentum* CECT 8448 strains were analysed for their probiotic potential. Initially, we observed that *L. plantarum* CETC 8328 and *L. fermentum* CECT 8448 were able to produce higher concentrations of riboflavin compared to their parental isolates (3.4- and 2.2 fold-times, respectively) as also occurred in a co-culture system with intestinal Caco-2 cells. Furthermore, we noted that the concentration of riboflavin in the Caco-2 co-culture systems with both of the parental strains decreased by about 60 and 36% with respect to the internal control (uninoculated DMEM medium), presumably due to the utilization of riboflavin by the LAB strains. However, when *L. plantarum* CETC 8328 and *L. fermentum* CECT 8448 were inoculated in the medium, the riboflavin content increased by about 26 and 36% compared to the internal control (Fig. 2). The uptake of riboflavin from the growth medium occurs apparently via a facilitated diffusion mechanism mediated by protein RibU. Homologues of RibU have been found in several gram-positive bacteria that possess the riboflavin biosynthesis operon. A regulatory region called the RFN element is conserved upstream of the *ribU* genes (Burgess 2006b). The *rib* leader region of *L. plantarum* CETC 8328 and *L. fermentum* CECT 8448 showed point mutations at two neighboring locations of the RFN element (Russo et al. 2014). These mutations could explain the low, or no, riboflavin uptake and the consequent enhancement of the external riboflavin concentration due to the over-production. In the case of the parental *L. plantarum* and *L. fermentum*, a negative feed-back regulation of the riboflavin operon may occur due to the riboflavin available in the medium. Thus, these bacteria decreased the total amount of external riboflavin due to their metabolic requirement. Other authors have also indicated that a strain of *Lactococcus lactis* consumes riboflavin from the growth medium, while a derivative strain with a deletion in the *ribU* region, does not decrease the riboflavin from the medium probably due to an non-functional uptake mechanism (Burgess et al. 2006b).

According to our results, *L. plantarum* CETC 8328 and *L. fermentum* CECT 8448 may be able to produce riboflavin in the human intestinal environment and thus make this vitamin available to the host. Orally-delivered probiotics must be resistant to acidic gastric juices, to bile salts and to the action of digestive enzymes, in order to establish themselves in the lower gut and thus guarantee their beneficial effects. Ingested food is subjected to a range of pH during the gastric transit depending on various factors, including food type and gastric juice secretion (Bezkorovainy et al. 2001). The probiotic potential of a microorganism can be evaluated by *in vitro* models that simulate the physical-chemical conditions encountered in the human GI tract (Bove et al. 2013). The food matrix should protect the delivered microbe from the GI hostile environment, sustaining its growth and activity. Commonly used food vehicles are fermented or fresh milks, although alternative carriers are also being investigated (Pitino et al. 2012). In our case, the survival of *L. plantarum* CETC 8328 and *L. fermentum* CECT 8448 under *in vitro* GI stresses was compared with that of two well-known probiotics, *L. plantarum* WCFS1 and *L. acidophilus* LA5 (Fig. 3).

In order to evaluate the effects of carrier matrix on bacterial survival, *L. plantarum* CETC 8328 and *L. fermentum* CECT 8448 were exposed to simulated GI conditions using different vehicles, including milk and yogurt which are frequently used in the preparation of probiotic foods. The bacterial viability of all strains analysed and in all the tested matrices was significantly influenced by acid conditions (pH 3.0 and pH 2.0). Similar results have been reported (Bove et al. 2013; Fernández de Palencia et al. 2008; Both et al. 2010; Maragkoudakis et al. 2006), confirming that low pH is the strongest challenge for probiotic microorganisms and corroborating the necessity to select probiotic strains that are able to tolerate the highly acidic environments of the stomach. Under intestinal stresses, we generally found lesser reductions of cell viability, apparently due to the lower influence of bile and pancreatin on bacterial survival and to the beneficial effect of the higher pH value. Others studies have also indicated that bacterial viability is less affected by bile salts than by acid stress, suggesting a possible recuperation of viability during the intestinal passage

(Maragkoudakis et al. 2006; Fernandez et al. 2003). In all the trials we observed a major susceptibility of bacterial strains to acid stress at pH 2.0 and a subsequent recovery of the bacterial viability after the intestinal stress step. Such recovery, about 1 or 2 Log units, could not be explained by a normal growth rate, therefore we assume that part of the bacterial population exposed to pH 2.0 was in a viable but non-cultivable (VBNC) state. Under intestinal conditions, the bacterial cells could then turn into a cultivable form (Fernández de Palencia et al. 2008; Succi et al. 2005), thereby accounting for the observed CFU increase. Moreover, Fernández de Palencia et al. (2008) suggested a possible formation of bacterial chains or aggregations during gastric challenge that could lead to a single colony on plates resulting in an underestimation of viable cells. The formation of chains and/or clumping could disappear during the intestinal transit.

A clear protective effect of the food matrix was observed when milk and, even more significantly, yogurt, were used. This was indicated by the higher survival rates observed after the complete gastrointestinal transit, and is probably due to the milk proteins that can shield bacterial cells exposed to acid stresses (Fernández et al. 2008). In milk, following the entire digestive process, *L. plantarum* CETC 8328 and *L. plantarum* WCFS1 showed higher percentages of viability compared to *L. acidophilus* LA5 and *L. fermentum* CECT 8448, although *L. fermentum* CECT 8448 exhibited a good tolerance to a pH value of pH 3.0. Moreover, our results suggest that when *L. plantarum* CETC 8328 is vehicled by yogurt, it has a pronounced ability to tolerate GI stress, comparable to that of *L. acidophilus* LA5, which is known to be acid tolerant and is commonly employed for the production of functional food (Fernández de Palencia et al. 2008).

Overall, the good tolerance to the GI tract conditions observed for the tested strains complies with the prerequisites of probiosis (Morelli et al. 2000; de Vrese and Schrezenmeir 2008). Moreover, the presence of a food matrix, i.e. milk and yogurt, commonly used to inoculate probiotics strains, has been proven to improve bacterial delivery to the distal segments of the intestine, thus enhancing the chance of colonization and related beneficial effects.

The ability of probiotic bacteria to adhere on intestinal cells is a feature that could potentiate the colonization of the intestine and is thus considered a key attribute of probiotic microorganisms (Piątek et al. 2012). We have analyzed all strains for their adhesion ability and we expressed the adhesion levels both as an absolute number of adherent bacteria on Caco-2 cells and as a percentage (Fig. 4). Candela *et al*. (2005) proposed a classification of probiotics as non-adhesive, adhesive and strongly adhesive, corresponding to less than 5, between 5 and 40, and more than 40 adherent CFU per Caco-2 cell respectively. On the basis of our results expressed as percentage of adhesion and following the definitions of Candela *et al* (2005) we classified *L. plantarum* CETC 8328 and *L. fermentum* CECT 8448 as strongly adhesive strains.

Furthermore, the ability of *L. plantarum* CETC 8328 and *L. fermentum* CECT 8448 strains to conteract *E. coli O157:H7* adhesion was analysed using three different approaches: *i*) pathogen-probiotic co-incubation, *ii*) probiotic pre-incubation, and *iii*) pathogen pre-incubation assay (Fig. 5). In the co-incubation trial, probiotic and pathogen were incubated at the same time on Caco-2 cells. *L. plantarum* CETC 8328 and *L. fermentum* CECT 8448 showed a pronounced capability to compete with the pathogen for the adhesion on epithelial intestinal cells that could contribute to inhibit and/or decrease the infection process by the pathogen as already suggested elsewhere (Levine et al. 1987; Weinstein et al. 1998). Similarly, Lee et al. (2003) highlighted that the degree of competition is a strain-dependent characteristic and the ability to conteract the pathogen's adhesion probably depends on the affinity of bacterial adhesins for the stereo-specific receptors on epithelial cells (Lee *et al.*, 2003). Thus, each lactobacillus strain should interfere with the adhesion of a restricted range of pathogens, against which it possesses greater affinity for the same binding sites of the intestinal surface.

In the probiotic pre-incubation assay, we investigated the ability of probiotics to exclude the binding of pathogens, in the case in which the probiotic is already adhered on the intestinal cells. Both probiotic strains reduced significantly the pathogen adhesion. The inhibition profile of the

exclusion assay was similar to that of the competitive assay, except for *L. acidophilus* LA5. Indeed, although the mechanism is not yet elucidated, the exclusion of pathogens from intestinal sites by lactobacilli could be connected to a competition process (Lee et al. 2003). In the case of co-incubation with *L. acidophilus* LA5, the number of adherent *E. coli* O157:H7 CFU was much higher. Contrarily, in the pre-incubation trial, *L. acidophilus* LA5 showed an appreciable capability to exclude the pathogen, and, overall, the pathogen adhesion level was consistently reduced compared to the co-incubation trial. According to our data, lactobacilli have greater capability to counteract pathogen adhesion when they have already established adhesion to the epithelial surface, and this is probably achieved by steric hindrance mechanisms (Koo et al. 2012; Sanchez et al. 2008).

Furthermore, we evaluated the potential inhibitory effect of probiotics on pathogen when the latter is already adherent on the enteric surface. Intresting, our observations suggest that probiotic strains increased the adhesion levels of *E. coli* O157:H7 (Fig. 5). However, these findings may be due to an insufficient incubation time. The displacement of pathogens by lactobacilli could take a longer time, presumably more than 1 hour as already suggested by Lee et al. (2003). Therefore, our results suggest that when probiotic bacteria are already settled on the intestinal epithelial surface (e.g. probiotic pre-incubation trial) or reach the enterocytes at the same time with the enteropathogen (e.g. co-incubation trial), their ability to interfere with pathogen adhesion is clearly displayed. Thus, a regular consumption over time of probiotic bacteria could contribute to the formation of a protective barrier against pathogens. In contrast, the use of probiotics against pathogens such as *E. coli* O157:H7, may give unfavorable results when pathogens are already established on the intestinal cells, probably due to the slow action of the probiotics investigated. Elmer et al. (2001) used the term “living drugs” to define the probiotic strength to act as a preventive drug in the prophylaxis of some gastrointestinal disorders, including microbial-associated diarrhea, in the treatment of vaginal and urinary-tract infections, and, moreover, in the immune disorders, lactose

intolerance, hypercholesterolaemia, food allergy, bladder and colon cancer (Mombelli and Gismondo 2000). The therapeutic use of probiotics, although they act slower than drugs, has been evaluated and the results confirmed the potential attractive of these “Low Cost-Slow Action-High Effect drugs”.

The antimicrobial activity of probiotic microorganisms was evaluated measuring the growth inhibition halos on agar plates when probiotic bacteria were grown with three pathogens. According to a previous classification, lactobacilli can be defined as strains of no, mild, strong and very strong inhibition, when they produce inhibition zones of 1 mm, 2 mm, 2-5 mm and more than 5 mm, respectively (Gaudana et al. 2010). On this basis we would categorize *L. plantarum* CETC 8328 and *L. plantarum* WCFS1 as strong inhibiting strains towards all tested pathogens, whereas *L. fermentum* CECT 8448 was a strong inhibitor strain of the Gram-negatives *S. enteritidis* and *E. coli* O157:H7 and a mild inhibitor of the Gram-positive *L. monocytogenes*. *L. acidophilus* LA5 had the ability to antagonize the growth of only *L. monocytogenes*, as it did not produce any significant inhibition zones against *S. enteritidis* and *E. coli* O157:H7 (Tab. 1). Overall, *L. plantarum* CETC 8328 showed the strongest antagonism capability of all the analyzed *Lactobacillus* strains.

None of the strains analysed presented hemolytic activity and none antibiotic resistance (Tab. 1). As indicated by EFSA (2012), we categorized all bacteria strains as susceptible according to the microbiological cut-off values indications. These results confirm the GRAS status of lactobacilli strains and comply with the key attribute of safety required for probiotics (Salminen et al. 1998).

Biofilm formation is the way by which several bacteria grow and conteract exogenous stresses. The adhesion of bacterial cells to cell tissue or other surfaces occurs by self-synthesized exopolysaccharides that ensure the biofilm stability (Rendueles et al. 2013). In the human oro-gastrointestinal tract, probiotic bacteria, but also pathogens, can be present as single or association cells, i.e. biofilm (Macfarlane and Dillon 2007). The ability of probiotic microorganism to form biofilms could be advantageous, creating a closer cell-to-intestine interaction and hence beneficial

effects to the host, including the reduction of pathogen biofilms (Söderling et al. 2011; Rendueles et al. 2013). Comparing the ability of adhesion to abiotic (glass) and biotic (Caco-2 cells) surfaces, we observed that *L. acidophilus* LA5 showed a low capability to bond to either surface, while *L. plantarum* CETC 8328 was the best adherent bacteria forming biofilm and a very strong adherent probiotic on intestinal cells. Conversely, *L. fermentum* CECT 8448 was the probiotic with the strongest adherent ability on Caco-2 cells, whereas its aptitude to form biofilm on a glass surface was mediocre (Fig. 6).

Humans require a dietary intake of riboflavin, as they are unable to biosynthesize this vitamin. However, a relevant source of vitamin B_2 can arise from the anabolic activity of members of the gut microbiota (Hill et al. 1997). Taking into account recent animal trials, vitamin-producing probiotics, administered orally, could be used as an effective endogenous source of vitamins (Pompei et al. 2007; LeBlanc et al. 2010a). Thus, the identification and development of vitamin-producing probiotics can be regarded as a promising perspective in the therapeutic application of probiotics. Indeed, selected microorganisms could both exert the beneficial effect of probiotics and provide an endogenous source of vitamins, thereby preventing the vitamin deficiencies associated with the onset of various pathologies (Rossi et al. 2011).

In this work, two strains of *Lactobacillus*, previously selected for their ability to overproduce riboflavin (Russo et al. 2014) or analysed in the present work (*L. plantarum* CETC 8328), were investigated in order to assess their potential probiotic activity. Riboflavin overproduction was detected under conditions resembling the intestinal environment (i.e. lactobacilli and Caco-2 cells co-cultures). Survival to an *in vitro* OGI system was satisfactory, comparable to that of reference probiotics and particularly marked when yogurt was used as carrier matrix. Both strains exhibited a strong adherence on human enterocyte-like cells, which suggests the potential for intestinal colonization. Biofilm assays indicated good adhesion ability even on abiotic surfaces. Moreover, the investigated strains were able to consistently hinder the intestinal adhesion of an enteropathogen

and antagonize growth of three potential food borne detrimental microbes. Similarly to the control probiotic strains, the investigated lactobacilli were devoid of β-hemolytic activity and antibiotic resistance.

Based on our findings, the investigated *L. plantarum* CETC 8328 and *L. fermentum* CECT 8448 strains possess the potential ability to survive the OGI transit, reach the intestine in a viable state and there exert various probiotic activities, including the production of vitamin B_2 in the body compartment where it can be adsorbed.

The selection of appropriate lactobacilli strains with the above mentioned properties could be exploited to develop novel functional food which is both vitamin-enriched, thanks to the bacterial metabolism, and, simultaneously, presents probiotic beneficial effects for the host health.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Acknowledgments

G.S. is supported by the Italian Ministry for Development in the framework of the "Industria 2015 Bando Nuove Tecnologie per il Made in Italy - Realizzazione di una innovativa pasta alimentare funzionale arricchita di componenti bioattivi e probiotici", and by MIUR [PON02_00186_2937475] in the framework of the project named "Protocolli innovativi per lo sviluppo di alimenti funzionali" [Pro.Ali.Fun.]". P.L. and G.S. are supported by the Spanish Ministry of Economy and Competitiveness grants AGL2012-40084-C03-01. We would like to thank Prof. M. Kleerebezem for providing the *L. plantarum* WCFS1 strain and Dr. Fergal Rattray [Chr. Hansen, Hörsholm Denmark] for providing *Lactobacillus acidophilus* LA5.

References

Bezkorovainy A (2001) Probiotics: determinants of survival and growth in the gut. Am J Clin Nutr 73:399S-405S.

Both E, György É, Kibédi-Szabó CZ, Tamás É, Ábrahám B, Miklóssy I, Lányi S (2010) Acid and bile tolerance, adhesion to epithelial cells of probiotic microorganisms. UPB Sci Bull Series B 72:37-44.

Bove P, Russo P, Capozzi V, Gallone A, Spano G, Fiocco D (2013) *Lactobacillus plantarum* passage through an oro-gastro-intestinal tract simulator: carrier matrix effect and transcriptional analysis of genes associated to stress and probiosis. Microbiol Res 168:351-359.

Burgess C, O'Connell-Motherway M, Sybesma W, Hugenholtz J, van Sinderen D (2004) Riboflavin production in *Lactococcus lactis*: potential for in situ production of vitamin-enriched foods? Appl Environ Microbiol 70:5769-5777.

Burgess CM, Smid EJ, Rutten G, van Sinderen D (2006a) A general method for selection of riboflavin-overproducing food grade micro-organisms. Microb Cell Fact 5:24-35.

Burgess CM, Slotboom DJ, Geertsma ER, Duurkens RH, Poolman B, van Sinderen D (2006b) The riboflavin transporter RibU in *Lactococcus lactis*: molecular characterization of gene expression and the transport mechanism. J Bacteriol 188:2752-2760.

Candela M, Seibold G, Vitali B, Lachenmaier S, Eikmanns BJ, Brigidi P (2005) Real-time PCR quantification of bacterial adhesion to Caco-2 cells: Competition between bifidobacteria and enteropathogens. Res Microbiol 156:887-895.

Candela M, Perna F, Carnevali P, Vitali B, Ciati R, Gionchetti P, Rizzello F, Campieri M, Brigidi P (2008) Interaction of probiotic *Lactobacillus* and *Bifidobacterium* strains with human intestinal epithelial cells: Adhesion properties, competition against enteropathogens and modulation of IL-8 production. Int J Food Microbiol 125:286-292.

Capozzi V, Russo P, Dueñas MT, López P, Spano G (2012) Lactic acid bacteria producing B-group vitamins: a great potential for functional cereals products. Appl Microbiol Biotechnol 96:1383-1394.

Delcenserie V, Martel D, Lamoureux M, Amiot J, Boutin Y, Roy D (2008) Immunomodulatory effects of probiotics in the intestinal tract. Curr Iss Mol Biol 10:37-54.

de Vrese M, Schrezenmeir J (2008) Probiotics, prebiotics, and synbiotics. Adv Biochem Eng Biotechnol 11:1-66.

Dunne C, O'Mahony L, Murphy L, Thornton G, Morrissey D, O'Halloran S, Feeney M, Flynn S, Fitzgerald G, Daly C, Kiely B, O'Sullivan GC, Shanahan F, Collins JK (2001) *In vitro* selection criteria for probiotic bacteria of human origin: correlation with in vivo findings. Am Soc Clin Nutr 73:386S-92S.

Elmer GW (2001) Probiotics: 'Living Drugs'. Am J Health-Syst Pharm 58 1101-1109.

European Food Safety Authority (EFSA) (2012) Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), EFSA J 10:2740-2749.

FAO/WHO (2001) Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria. Córdoba, Argentina. Food and Agriculture Organization of United Nations and World Health Organization Working Expert Consultation Report.

FAO/WHO (2002) Guidelines for the evaluation of probiotics in food. London, Ontario: Food and Agriculture Organization of United Nations and World Health Organization Working Group Report.

Fernández MF, Boris S, Barbés C (2003) Probiotic properties of human lactobacilli strains to be used in the gastrointestinal tract. J Appl Microbiol 94:449-455.

Fernández de Palencia P, López P, Luis CA, Peláez C, Requena T (2008) Probiotic strains: survival under simulated gastrointestinal conditions, in vitro adhesion to Caco-2 cells and effect on cytokine secretion. Eur Food Res Technol 227:1475-1484.

Gagnon M, Kheadr EE, Le Blay G, Fliss I (2004) In vitro inhibition of *Escherichia coli* O157:H7 by bifidobacterial strains of human origin. Int J Food Microb 92:69-78.

Gaudana SB, Dhanani AS, Bagchi T (2010) Probiotic attributes of *Lactobacillus* strains isolated from food and of human origin. Br J Nutr 103:1620-1628.

Hill MJ (1997) Intestinal flora and endogenous vitamin synthesis. Eur J Cancer Prev 6:43-45.

Holzapel WH, Haberer P, Snel J, Schillinger U, Huis in't Veld JHJ (1998) Overview of gut flora and probiotics. Int J Food Microbiol 41:85-101.

Hooper LV, Midvedt T, Gordon JI (2002) How host microbial interactions shape the nutrient environment of the mammalian intestine. Ann Rev Nutr 22:283-273.

Jakobsen J (2008) Optimisation of the determination of thiamin, 2-(1-hydroxyethyl)thiamin, and riboflavin in food samples by use of HPLC. Food Chem 106:1209-1217.

Kleerebezem M, Boekhorst J, van Kranenburg R, Molenaar D, Kuipers OP, Leer R, Tarchini R, Peters SA, Sandbrink HM, Fiers MWEJ, Stiekema W, Lankhorst RMK, Bron PA, Hoffer SM, Groot MNN, Kerkhoven R, de Vries M, Ursing B, de Vos WM, Siezen RJ (2003) Complete genome sequence of *Lactobacillus plantarum* WCFS1. PNAS 100:1990-1995.

Koo OK, Amalaradjou MAR, Bhunia AK (2012) Recombinant probiotic expressing Listeria adhesion protein attenuates *Listeria monocytogenes* virulence *in vitro*. PLoS ONE 7. DOI: 10.1371/journal.pone.0029277

LeBlanc JG, Sybesma W, Starrenburg M, Sesma F, deVos WM, de Giori GS, Hugenholtz J (2010a) Supplementation with engineered *Lactococcus lactis* improves the folate status in deficient rats. Nutrition 26:835-841.

LeBlanc JG, Taranto MP, Molina V, Sesma F (2010b) B-group vitamins production by probiotic lactic acid bacteria. In: Mozzi F, Raya R, Vignolo G (ed) Biotechnology of lactic acid bacteria: novel applications. Wiley-Blackwell, Ames.

LeBlanc JG, Lainõ JE, Juarez del Valle M, Vannini V, van Sinderen D, Taranto MP, Font de Valdez G, Savoy de Giori G, Sesma F (2011) B-Group vitamin production by lactic acid bacteria-current knowledge and potential applications. J Appl Microbiol 111:1297-1309.

Lee YK, Lim CY, Teng WL, Ouwehand AC, Tuomola EM, Salminen S (2000) Quantitative Approach in the Study of Adhesion of Lactic Acid Bacteria to Intestinal Cells and Their Competition with Enterobacteria. Appl Enviro Microbiol 66:3692-3697.

Levine MM (1987) *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. J Infect Dis 155:377-389.

Maccaferri S, Klinder A, Brigidi P, Cavina P, Costabile A (2012) Potential probiotic *Kluyveromyces marxianus* B0399 modulates the immune response in Caco-2 cells and peripheral blood mononuclear cells and impacts the human gut microbiota in an in vitro colonic model system. Appl Enviro Microbiol 78:956-964.

Macfarlane S, Dillon JF (2007) Microbial biofilms in the human gastrointestinal tract. J Appl Microbiol 102:1187-1196.

Maragkoudakis PA, Zoumpopoulou G, Miaris C, Kalantzopoulos G, Pot B, Tsakalidou E (2006) Probiotic potential of *Lactobacillus* strains isolated from dairy products. Inter Dairy J 16:189-199.

Marteau PR, de Vrese M, Cellier CJ, Schrezenmeir J (2001) Protection from gastrointestinal diseases with the use of probiotics. Am J Clin Nutr 73:430-436.

Mombelli B, Gismondo MR (2000) The use of probiotics in medical practice. Intern J Antimicrob Ag 16:531-536.

Morelli L (2000) In vitro selection of probiotic lactobacilli: A critical appraisal. Curr Iss Intest Microbiol 1:59-67.

Patel RM, Lin PW (2010) Developmental biology of gut-probiotic interaction. Gut Microbes 1:186-195.

Perelle S, Dilasser F, Grout J, Fach P (2004) Detection by 5'-nuclease PCR of Shigatoxin producing *Escherichia coli* O26, O55, O91, O103, O111, O113, O145 and O157:H7, associated with the world's most frequent clinical cases. Mol Cell Prob 18:185-192.

Piątek J, Gibas-Dorna M, Olejnik A, Krauss H, Wierzbicki K, Żukiewicz-Sobczak W, Głowacki M (2012) The viability and intestinal epithelial cell adhesion of probiotic strain combination-in vitro study. Ann Agric Environ Med 19:99-102.

Pitino I, Randazzo CL, Cross KL, Parker ML, Bisignano C, Wickham MS, Mandalari G, Caggia C (2012) Survival of *Lactobacillus rhamnosus* strains inoculated in cheese matrix during simulated human digestion. Food Microbiol 31:57–63.

Pompei A, Cordisco L, Amaretti A, Zanoni S, Matteuzzi D, Rossi M (2007) Folate production by bifidobacteria as a potential probiotic property. App Environ Microbiol 73:179-185.

Rendueles O, Kaplan JB, Ghigol J-M (2013) Antibiofilm polysaccharides. Environ Microbiol 15:334-346.

Riley MA, Gordon DM (1999) The ecological role of bacteriocins in bacterial competition. TIM 7:129-133.

Rossi M, Amaretti A, Raimondi S (2011) Folate Production by Probiotic Bacteria. Nutrients 3:118-134.

Russo P, López P, Capozzi V, Fernández de Palencia P, Dueñas MT, Spano G, Fiocco D (2012a) Beta-Glucans Improve Growth, Viability and Colonization of Probiotic Microorganisms. Intern J Mol Sci 13:6026-6039.

Russo P, Fernández de Palencia P, Romano A, Fernández M, Lucas P, Spano G, López P (2012b) Biogenic amine production by the wine *Lactobacillus brevis* IOEB 9809 in systems that partially mimic the gastrointestinal tract stress. BMC Microbiol 12:247-256.

Russo P, Russo P, Capozzi V, Arena MP, Spadaccino G, Dueñas MT, López P, Fiocco D, Spano G (2014) Riboflavin overproducing strains of *Lactobacillus fermentum* for riboflavin enriched bread. Appl Microbiol Biotechnol doi:10.1007/s00253-013-5484-7.

Sanchez B, Bressollier P, Urdaci MC (2008) Exported proteins in probiotic bacteria: adhesion to intestinal surfaces, host immunomodulation and molecular cross-talking with the host. FEMS Immunl Med Microbiol 54:1-17.

Söderling EM, Marttinen AM, Haukioja AL (2011) Probiotic Lactobacilli interfere with *Streptococcus* mutans biofilm formation *in vitro*. Curr Microbiol 62:618-622.

Succi M, Tremonte P, Reale A, Sorrentino E, Grazia L, Pacifico S, Coppola R (2005) Bile salt and acid tolerance of *Lactobacillus rhamnosus* strains isolated from Parmigiano Reggiano cheese. FEMS Microbiol Lett 244:129-137.

van Baarlen P, Wells JM, Kleerebezem M (2013) Regulation of intestinal homeostasis and immunity with probiotic lactobacilli. Trend Immunol 34:208-15.

van Bokhorst-van de Veen H, van Swam I, Wels M, Bron PA, Kleerebezem M (2012) Congruent strain specific intestinal persistence of *Lactobacillus plantarum* in an intestine-mimicking in vitro system and in human volunteers. PLoS One 7. DOI: 10.1371/journal.pone.0044588.

van Loon AP, Hohmann HP, Bretzel W, Humbelin M, Pfister M (1996) Development of a fermentation process for the manufacture of riboflavin. Chimia 50:410-412.

Vergara-Irigaray M, Valle J, Merino N, Latasa C, García B, Ruiz de Los Mozos I, Solano C, Toledo-Arana A, Penadés JR, Lasa I (2009) Relevant role of fibronectin-binding proteins in *Staphylococcus aureus* biofilm-associated foreign-body infections. Infect Immun 77:3978-91.

Vinderola G, Matar C, Perdigon G (2005) Role of intestinal epithelial cells in immune effects mediated by gram-positive probiotic bacteria: involvementt of toll-like receptors. Clin Diagn Lab Immunol 12:1075-1084.

Weinstein DL, O'Neill BL, Hone DM, Metcalf ES (1998) Differential early interactions between *Salmonella enterica* serovar Typhi and two other pathogenic *Salmonella* serovars with intestinal epithelial cells. Infect Immun 66:2310-2318.

Yu J, Kaper JB (1992) Cloning and characterization of the *eae* gene of enterohaemorrhagic *Escherichia coli* O157:H7. Mol Microbiol 6:411-417.

Tab. 1 Antimicrobial and hemolytic activity exhibited by the examined lactobacilli. According to Gaudana et al. (2010), the lactobacilli strains producing inhibition zones of 1 mm, 2mm, 2-5 mm and more than 5mm were classified as no (+/-), mild (+), strong (++), and very strong (+++) inhibitors. *L. plantarum* WCFS1, *LpWCFS1*; *L. acidophilus* LA5, *La5*.

	L. monocytogenes	*E. coli* O157:H7	*S. enteritidis*	*Hemolytic activity*
LpWCFS1	++	+	+	γ-hemolitic
La5	++	+/-	+/-	γ-hemolitic
L. plantarum CECT 8328	++	++	++	γ-hemolitic
L. fermentum CECT 8448	+	++	++	γ-hemolitic

FIGURE CAPTIONS:

Fig. 1 Riboflavin production of lactobacilli in CDM. *L. plantarum* CETC 8328 displayed the capability to *in vitro* over-produce riboflavin with respect to the non-roseoflavin-resistant isolate *L. plantarum.* Values represent mean ± standard deviation of three different experiments. *L. plantarum*, *Lp*; *L. plantarum* CECT 8328, *Lp8328*. Nd= not detected

Fig. 2 Riboflavin production in co-cultures of lactobacilli and intestinal Caco-2 cells. Both *L. plantarum* CETC 8328 and *L. fermentum* CECT 8448 displayed the capability, *in vitro,* to over-produce riboflavin compared to the parental *L. plantarum* and *L. fermentum* strains, used as control to determine the level of riboflavin overproduction. Values represent mean ± standard deviation of three different experiments. Statistical analyses were carried out by Student's t-test. (*$p<0.05$ and **$p<0.005$). *L. plantarum*, *Lp*; *L. plantarum* CECT 8328, *Lp8328*; *L. fermentum*, *Lfm*; *L. fermentum* CECT 8448, *Lfm8448.*

Fig. 3 Bacterial survival in an *in vitro* model simulating the human gastrointestinal tract, including oral, gastric (pH 2.0 and pH 3.0) and intestinal (small and large intestine) stresses, each determined in three different vehicle matrices (see the experimental procedure for details). Viability was calculated by CFU count analysis and expressed as percent survival relative to untreated control (i.e. bacteria resuspended in the corresponding vehicle matrix at time zero, before simulated digestion). Values represent mean ± standard deviation of three different experiments. Statistical analyses were carried out by Student's t-test and significant differences are relative to saline solution used as negative control carrier matrix (*$p<0.05$ and **$p<0.005$). *L. plantarum* WCFS1, *LpWCFS1*; *L. acidophilus* LA5, *La5*; *L. plantarum* CECT 8328, *Lp8328*; *L. fermentum* CECT 8448, *Lfm8448.*

Fig. 4 Bacterial adhesion to Caco-2 cells. The adhesion ability was expressed both as the percentage of adhesion (a) and as the number of adherent bacteria per Caco-2 cell (b). Values represent mean ± standard deviation of three different experiments. *L. plantarum* WCFS1, *LpWCFS1*; *L. acidophilus* LA5, *La5*; *L. plantarum* CECT 8328, *Lp8328*; *L. fermentum* CECT 8448 *Lfm8448.*

Fig. 5 Influence of lactobacilli on the adhesion of *E. coli* O157:H7 to Caco-2 cells. In the co-incubation trial, probiotics and *E. coli* O157:H7 were co-incubated with Caco-2 cells; in the probiotic pre-incubation trial, probiotics were pre-incubated with Caco-2 cells, then *E. coli* O157:H7 was added; in the pathogen pre-incubation trial *E. coli* O157:H7 was pre-incubated with Caco-2 cells, then probiotics were added. The inhibition of pathogen adhesion was determined by a quantitative PCR-based method, and expressed as a relative level with respect to the adhesion observed when *E. coli* O157:H7 was tested alone (control sample). Values represent mean ± standard deviation of three different experiments. Statistical analyses were carried out by Student's t-test and significant differences are relative to control sample (*$p<0.05$ and **$p<0.005$). *E. coli* O157:H7, *E.* coli; *L. plantarum* WCFS1, *LpWCFS1*; *L. acidophilus* LA5, *La5*; *L. plantarum* CECT 8328, *Lp8328*; *L. fermentum* CECT 8448, *Lfm8448*.

Fig. 6 Ability to produce biofilms on glass surface. Cultures of the tested lactobacilli (/*L. plantarum* WCFS1, *L. acidophilus* LA5, *L. plantarum* CECT 8328, *L. fermentum* CECT 8448) were inoculated in fresh MRS broth and incubated for 24 h and 48 h at 37 °C. **a**) Optical density measured at 570 nm to quantify the biofilm. **b**) Biofilms formation on smooth glass surface. Values represent mean ± standard deviation of three different experiments. *L. plantarum* WCFS1, *LpWCFS1*; *L. acidophilus* LA5, *La5*; *L. plantarum* CECT 8328, *Lp8328*; *L. fermentum* CECT 8448, *Lfm8448*.

Figure

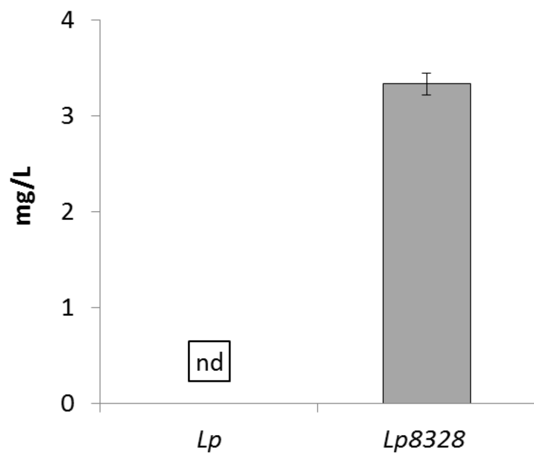


Fig. 1

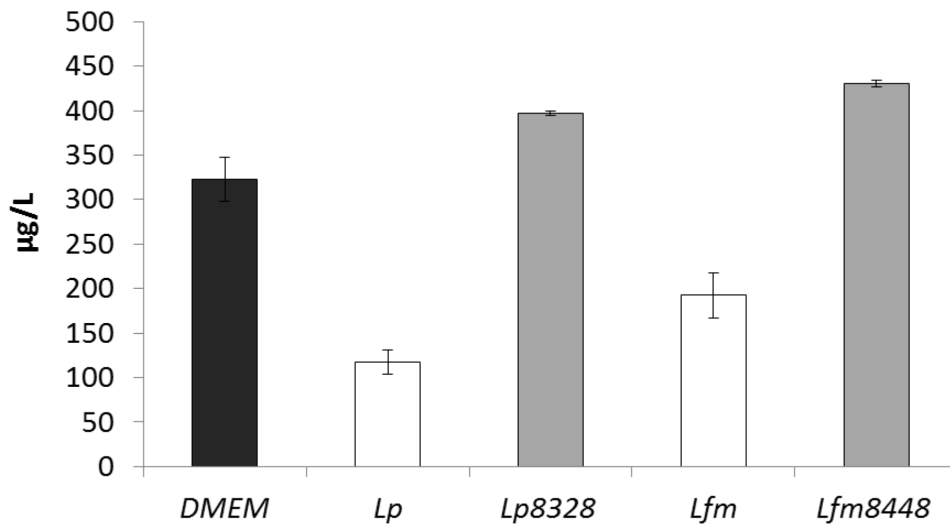


Fig. 2

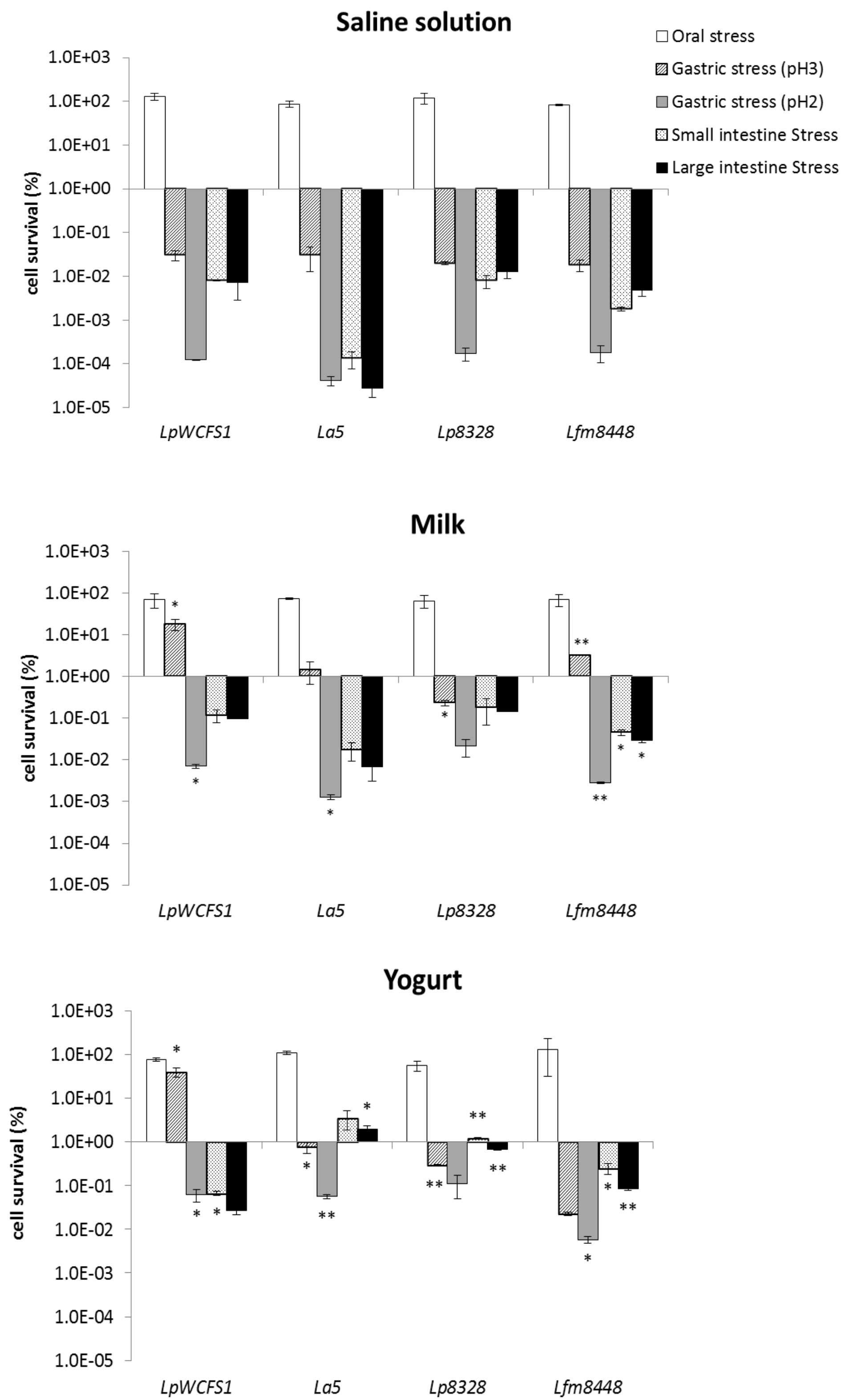


Fig. 3

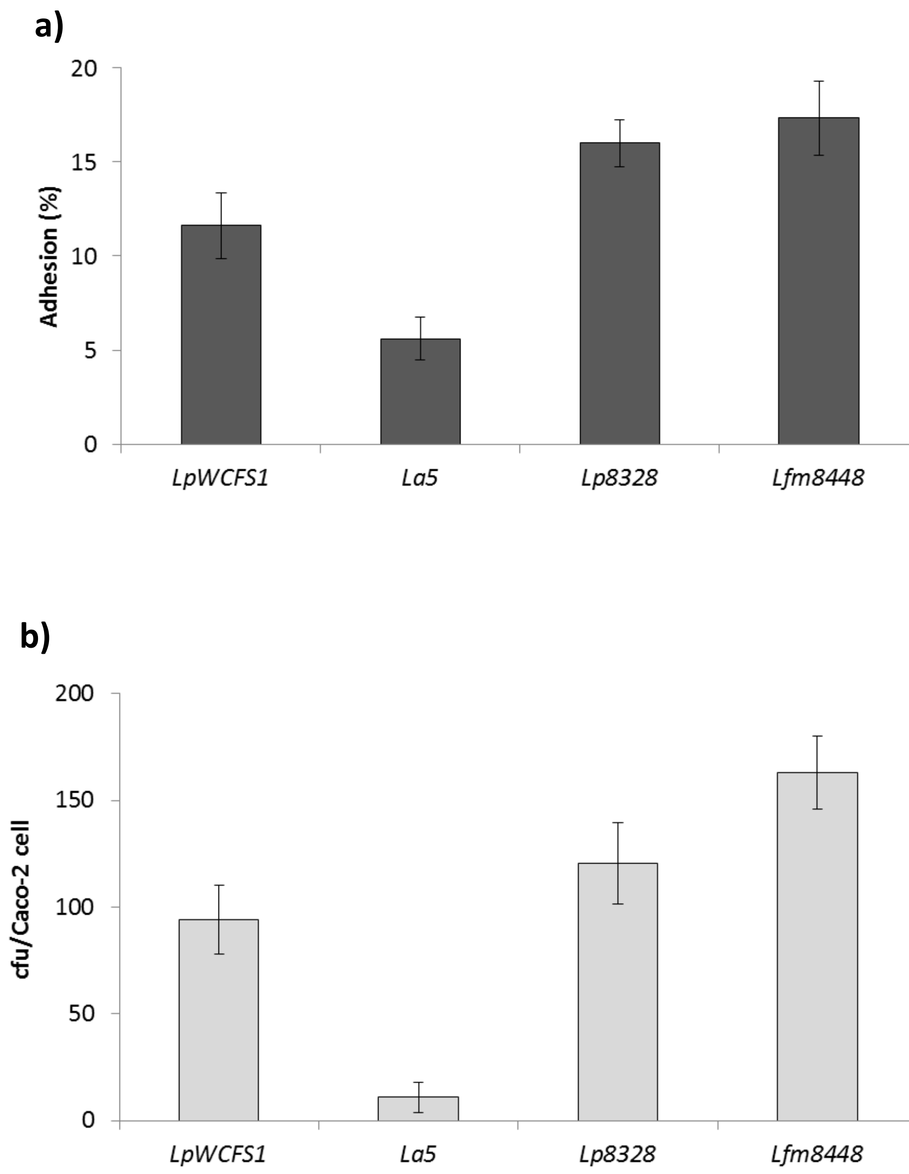
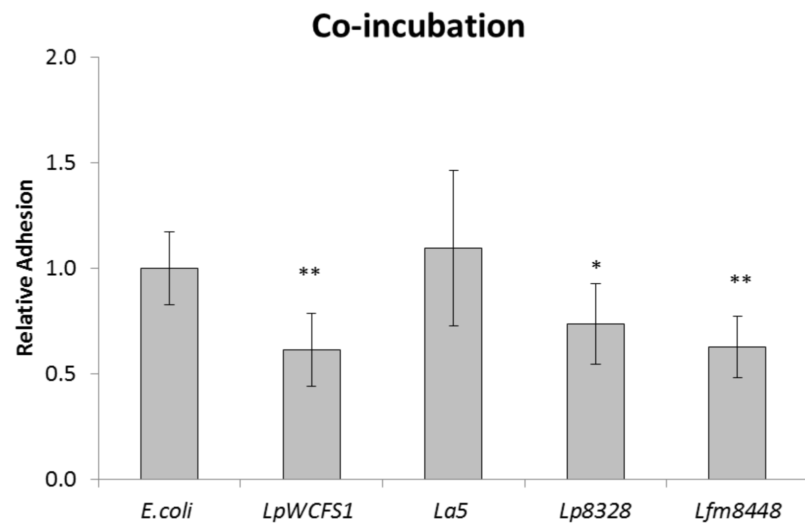
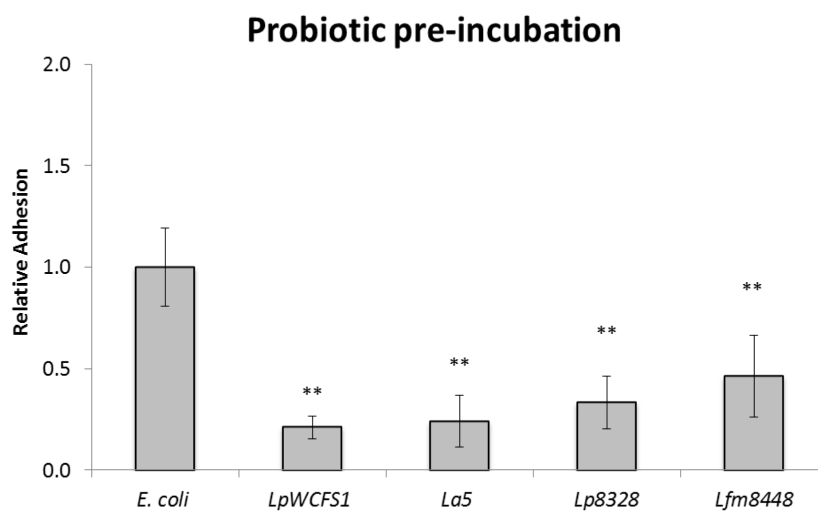


Fig. 4





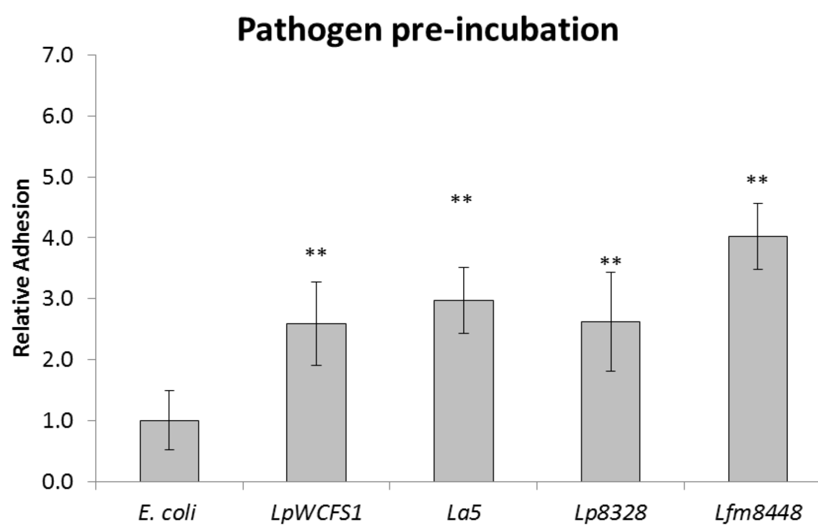
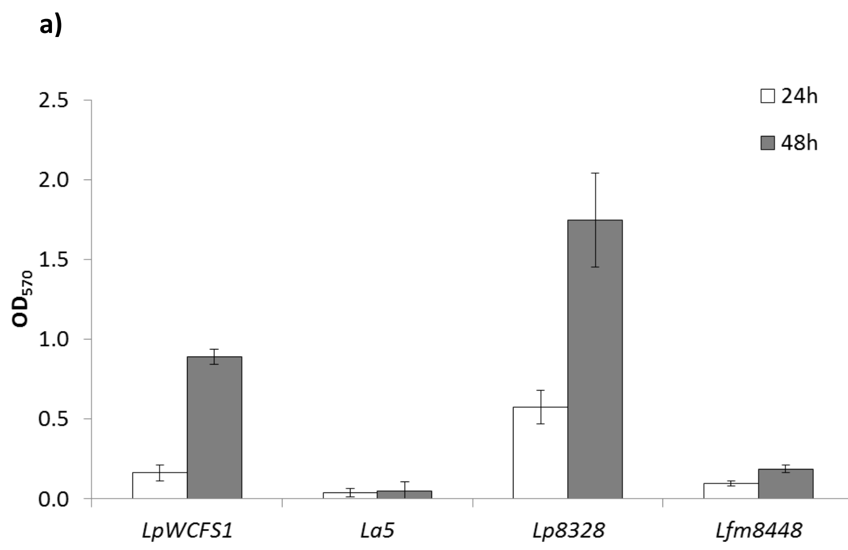


Fig. 5



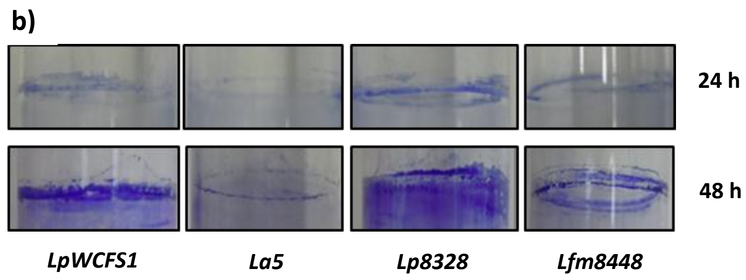


Fig. 6