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# Characterization of potentially probiotic lactic acid bacteria isolated from olives: Evaluation of short chain fatty acids production and analysis of the extracellular proteome

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## Highlights

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Seventeen olive-isolated probiotics were screened for the production of SCFA. •

Propionic and butyric acids (mg/L) are less abundant than acetic and lactic acids (g/L).

The extracellular proteomes of the two most interesting strains were investigated.

The adhesive ability of *L. plantarum* S11T3E mainly depends on adhesive proteins.

In *L. pentosus* S3T60C adhesion is probably mediated by non proteinaceous molecules.

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## Abstract

Probiotic strains can exert positive effects on human health by various mechanisms, among which the production of short chain fatty acids (SCFA). All the SCFA, mainly acetic, propionic and butyric acid, display beneficial effects on human health; butyric acid is the most interesting for its role in the prevention and treatment of colonic diseases.

In this study the ability of 17 potentially probiotic food-isolated lactic acid bacteria to produce SCFA, directly or indirectly through the production of lactic acid, was investigated. Propionic and butyric acids were quantified by gas chromatography; acetic and lactic acids were quantified by specific enzymatic kits. All the tested strains displayed the ability to produce significant amounts of acetic and lactic acids (in the range of g/L) and just small amounts of propionic and butyric acids (in the range of mg/L).

The extracellular proteomes of two of these strains, *Lactobacillus plantarum* S11T3E and *Lactobacillus pentosus* S3T60C, were evaluated by coupling 2-DE and MALDI TOF-TOF mass spectrometry. This is an interesting approach to investigate a probiotic strain, since secreted

proteins represent the first contact point between bacteria and the host after ingestion. Six and seven proteins, in different isoforms, were identified from *L. pentosus* S3T60C and *L. plantarum* S11T3E, respectively. All of them have a predicted extracellular location, indicating the effectiveness of the used protocol. *L. plantarum* S11T3E secretes several proteins with adhesive function, suggesting that in this strain the ability to adhere to gut mucosa depends on this kind of molecules. In *L. pentosus* S3T60C just one adhesive protein is secreted suggesting that other families of molecules play a role in its adhesive ability.

## Keywords

Probiotics; Short chain fatty acids; Adhesion potential; Extracellular proteins

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## 1. Introduction

Probiotics are defined as living non-pathogenic microorganisms which have a demonstrated beneficial effect on the host when ingested in adequate amounts, ranging in the concentration between  $10^6$  and  $10^9$  CFU/mL ([FAO/WHO, 2002](#)). Even if probiotics may exert positive effects by various and inter-related mechanisms, it is recognized that their activity on human health mainly depends on the control of host immune system, of the balance between saccharolytic and proteolytic species in the gut microbiota and of the production of both antimicrobial compounds and other bio-active metabolites ([Oelschlaeger, 2010](#) and [Pessione, 2012](#)).

Among these bio-active compounds, short chain fatty acids (SCFA), mainly acetic, propionic and butyric acid, exert positive effects on human health, at different levels ([Russel, Hoyles, Flint, & Dumas, 2013](#)). Acetic acid is involved in controlling inflammation and counteracting pathogen invasion ([Fukuda et al., 2011](#) and [Maslowski et al., 2009](#)); it is the most abundant SCFA in plasma where it acts as energetic substrate ([Hara, Haga, Aoyama, & Kiriya, 1999](#)). Both propionic and butyric acid have the ability to influence satiety, to protect against diet-induced obesity and to improve insulin sensitivity ([Arora et al., 2011](#) and [Lin et al., 2012](#)). Furthermore, butyric acid possesses also a key-role in the prevention and treatment of colon cancer by promoting cell differentiation, cell-cycle arrest and apoptosis of altered colonocytes mainly through its ability to alter gene transcription by inhibiting histone deacetylase activity ([Fung, Cosgrove, Lockett, Head, & Topping, 2012](#)).

SCFA are common end-products of the microbial fermentation of dietary carbohydrates, resistant starches and dietary fibers ([Topping & Clifton, 2001](#)). Their production is affected by several factors, including type and number of microorganisms present in the colon, substrate sources and gut transit time ([Wong, de Souza, Kendall, Emam, & Jenkins, 2006](#)). Probiotic lactic acid bacteria (LAB) may indirectly enhance SCFA production by the endogenous colonic microbiota. Actually, lactic acid, the main end-product of LAB metabolism, is normally metabolized to acetate or propionate by lactate-utilizing bacteria such as *Clostridium propionicum*, *Propionibacterium* ssp, *Desulfovibrio* ssp, *Veillonella* ssp, and *Selenomonas* ssp ([Kuchta and Abeles, 1985](#) and [Seeliger et al., 2002](#)), and to butyrate by *Megasphaera elsdenii*, some *Clostridium* ssp, *Eubacterium hallii* and *Anaerostipes caccae* ([Belenguer et al., 2006](#)). Besides this indirect mechanism, probiotic LAB can also contribute to SCFA accumulation in the colon by a direct production. Acetic acid is, in fact, normally produced by heterofermentative LAB and facultative homofermenters, but in the literature there are also reports dealing with the possibility of a direct production of propionic and butyric acid by Lactobacilli ([Nazzaro, Fratianni, Nicolaus, Poli, & Orlando, 2012](#)).

In this study 17 lactic acid bacteria, belonging to three different species, isolated from artisanal-produced olives and their brine ([Botta et al., 2014](#) and [Cocolin et al., 2013](#)) were screened, in order to evaluate their ability to produce SCFA, a class of compounds that could confer them an interesting added value as probiotics.

The extracellular proteome of two of these strains, *Lactobacillus plantarum* S11T3E and *Lactobacillus pentosus* S3T60C, were then analyzed by coupling two-dimensional electrophoresis and MALDI-TOF mass spectrometry. *L. plantarum* S11T3E was selected since it displayed the best probiotic potential in previous experiments ([Botta et al., 2014](#)). *L. pentosus* S3T60C was chosen for its ability to reduce *Listeria monocytogenes* invasion in undifferentiated gut model cells ([Botta et al., 2014](#)); furthermore it was one of the best SCFA producers. The analysis of the extracellular proteome is a very interesting tool to explore a probiotic strain since surface-exposed and secreted proteins represent the first point of interaction between the bacterium and the host after food or nutritional supplement ingestion. Furthermore, the existence of an equilibrium between cell-associated and extracellularly secreted proteins was proved in lactic acid bacteria ([Sánchez, Urdaci, & Margolles, 2010](#)) resulting in the presence, in the exoproteome, of proteins conferring adhesive, antigenic or immunomodulating properties to the strains ([Genovese et al., 2013](#) and [Mangiapane et al., 2013](#)). The proteins involved in this phenomenon are mainly moonlighting proteins, which are single proteins displaying different functions according to their location: inside or outside of cells, within different cell types, in different locations within a cell ([Jeffery, 2005](#)). The most known moonlighting proteins described in *Lactobacillus* species are glycolytic enzymes, chaperones and other stress response-involved proteins, transcriptional and translational factors ([Beck et al., 2009](#), [Bergonzelli et al., 2006](#) and [Granato et al., 2004](#)). The analysis of the extracellular proteome of *L. plantarum* S11T3E and *L. pentosus* S3T60C will be therefore helpful to clarify the protein-based interactions between these two potential probiotics and the host, focusing in particular on adhesion mechanism to gut mucosa, a key requirement for exerting beneficial effects since it prolongs the persistence of the probiotic in the intestine.

## 2. Material and methods

### 2.1. Bacterial strains

In this study 17 lactic acid bacteria strains isolated from fermented olives and their brine were considered. They were selected as potential probiotics since preliminary characterization studies confirmed the exclusion of antibiotic resistance and biogenic amine production, the resistance to gastro-intestinal transit, the ability to adhere to Ca-CO<sub>2</sub> cells and immunostimulating properties ([Botta et al., 2014](#) and [Cocolin et al., 2013](#)). Among those, 14 strains were *L. plantarum*, 1 *L. pentosus* and 2 *Leuconostoc mesenteroides*. All the strains were maintained in MRS broth at – 20°C in 0.5 mL aliquots, collected at the beginning of the stationary phase, with 0.5 mL of 40% (v/v) glycerol.

### 2.2. Culture conditions

#### 2.2.1. Short chain fatty acids and lactic acid quantification

All the strains were grown until stationary phase in commercial MRS broth (Difco) for the quantification of propionic, butyric and lactic acid. Since sodium acetate is significantly present (5 g/L) in commercial MRS medium, for acetic acid quantification all the strains were grown in a modified MRS medium (bacteriological peptone 10 g/L; soy peptone 8 g/L; yeast extract 10 g/L;

saccharose 10 g/L; tween 80: 1 mL/L; dipotassium hydrogen phosphate 2 g/L; triammonium citrate 2 g/L; magnesium sulfate 0.2 g/L; manganese sulfate 0.05 g/L).

All the cultures, in both the media, were incubated in 100 mL screw cap bottles without shaking to guarantee a microaerophylic environment; Lactobacilli were grown at 37 °C, while the two *L. mesenteroides* at 30 °C. For all the strains three biological replicates of the cultures were performed. The bacterial growth was monitored by 600 nm optical density (OD<sub>600</sub>) measurement.

### **2.2.2. Extracellular proteomic experiments**

*L. plantarum* S11T3E and *L. pentosus* S3T60C were grown in MRS broth until the middle exponential phase to avoid cell lysis. The cultures were incubated at 37 °C in 1 L screw cap bottles without shaking to guarantee a microaerophylic environment. All the cultures were realized in three biological replicates. The bacterial growth was monitored by 600 nm optical density (OD<sub>600</sub>) measurement. The same culture conditions, with the addition of saccharose (20 g/L) in the medium, were used to evaluate the production of exopolysaccharides.

## **2.3. Quantification of organic acids**

### **2.3.1. Propionic and butyric acid quantification**

Propionic and butyric acids were quantified by gas chromatography using an Agilent 7890A instrument equipped with a flame ionization detector (FID). The column used for the gas chromatography was the “Agilent J & W GC column DB-WAX” (30 m × 0.32 mm × 0.25 µm). Both the injector and the detector were set up at 250 °C. The temperature gradient of the oven was as follows: 5 min at 125°C, an increase of 15°C/min until 180°C, 3 min at 180°C. Two microliter samples were manually injected with a 5 µL gold syringe (Agilent Technologies).

A calibration curve ranging from 1 mg/L to 100 mg/L was performed using propionic and butyric acids with GC grade purity (Sigma Aldrich) as standards and heptanoic acid GC grade purity (Sigma Aldrich) as internal standard. The solutions at the different concentrations were prepared in MRS medium; one milliliter was acidified with 40 µL HCl and, after shaking the reaction vessel, Short Chain Fatty Acids were extracted with 200 µL diethyl ether ([Garcia-Villalba et al., 2012](#)) and loaded in the column. This extraction procedure was then followed by a short (11 min) GC separation coupled to FID detector analysis. For all the tested concentrations three different solutions were prepared and for all of them two organic extraction and two different injections were performed. The peaks of all the obtained chromatograms were integrated with the Agilent ChemStation software.

To evaluate the production of propionic and butyric acids, 1 milliliter aliquots of the stationary phase bacterial cultures were treated: cells were removed by centrifugation (10,000 ×g, 20 min, 4°C) and SCFA were extracted as described for the calibration curve preparation. Three biological replicates were performed for all the strains and for all of them two organic extraction and two different injections were performed. The peaks of all the obtained chromatograms were integrated with the Agilent ChemStation software.

### **2.3.2. Acetic acid quantification**

Acetic acid was quantified by the enzymatic kit K-ACETRM (Megazyme). One milliliter aliquots of the stationary phase bacterial cultures were treated: cells were removed by centrifugation (4000 ×g, 15 min, 4°C) and the supernatants were treated according to the kit instructions. The

assay is based on enzymatic reactions leading to the production of NAD<sup>+</sup> which is stoichiometric to the amount of acetic acid present in the sample. NADH consumption is measured by evaluating the absorbance decrease at 340 nm. For all the strains three biological and two technical replicates were performed.

### 2.3.3. Lactic acid quantification

Lactic acid was quantified by the enzymatic kit K-DLATE (Megazyme) that allows the quantification of both D-lactic acid and L-lactic acid. One milliliter aliquots of the stationary phase bacterial cultures were treated: cells were removed by centrifugation (4000 ×g, 15 min, 4°C) and the supernatants were treated according to the kit instructions. The assay is based on a set of enzymatic reactions that lead to the production of NADH which is stoichiometric to the amount of lactic acid present in the sample. The produced NADH is quantified by measuring the increase in absorbance at 340 nm. For all the strains three biological and two technical replicates were performed.

### 2.3.4. Statistical analysis

For all the experiments the values are reported as mean values ± standard deviation. The production of metabolites by different strains was statistically analyzed by one way ANOVA, followed by Tukey's post hoc test to evaluate differences among strains (SPSS software, version 21). Differences among strains were considered significant for *p*-values < 0.05.

## 2.4. Extracellular proteomic experiments

### 2.4.1. Preparation of extracellular protein extract

*L. plantarum* S11T3E and *L. pentosus* S3T60C were grown in MRS broth at 37 °C and the cultures harvested at middle exponential phase (OD<sub>600</sub> ≈ 6) and treated as previously described ([Pessione et al., 2012](#)). Briefly, cells of three biological replicates were removed by centrifugation (4000 ×g, 20 min, 4°C) and culture supernatants were filtered in steripup 0.22 μm filters (Millipore). 16% (w/v) trichloroacetic acid was added to supernatants and incubated under shaking overnight at 4 °C to allow extracellular proteins precipitation. The samples were then ultracentrifuged (35,000 ×g, 60 min, 4°C). Pellets were then dried, pulverized and re-suspended in the smallest possible volume of Tris–HCl 50 mM pH 7.3. One mL ice-cold acetone was added to 1 milliliter aliquots and incubated over night at –20°C. Precipitated proteins were collected by centrifugation (15,000 ×g, 20 min, 4°C) and washed with ice-cold acetone again (15,000 ×g, 20 min, 4°C). Pellets were dried and re-suspended in a rehydration solution (6.5 M urea, 2.2 M thiourea, 4% w/v CHAPS, 5 mM Tris–HCl, pH 8.8, 0.5% IPG buffer (GE Healthcare), 1.2% (v/v) DeStreak (GE Healthcare)). Proteins were quantified by Bradford assay (BioRad).

### 2.4.2. Two-dimensional electrophoresis

Isoelectrofocusing (IEF) was performed using a 13 cm IPG strips (GE Healthcare) with a linear gradient ranging from 3 to 10: 200 μg of proteins was loaded by in gel rehydration. IEF was performed using IPGphor (GE Healthcare) at 20 °C, with 83,000 Vhrs. After IEF, the strips were incubated at room temperature in 6 M urea, 30% v/v glycerol, 2% w/v SDS, 50 mM Tris–HCl, pH 8.6, supplemented at first with 2% w/v DTT for 15 min and subsequently with 4.5% w/v iodoacetamide for 15 min. They were then loaded at the top of 1.0 mm vertical second dimension gels. SDS-PAGE was performed on 11.5% T and 3.3% C acrylamide (Biorad Acrylamide) homogeneous gels. The running buffer was 2.5 mM Tris, 192 mM glycine, 0.1% SDS. The running

conditions were 11°C, 600 V constant voltage, 20 mA/gel, 60 W for 15 min and 11°C, 600 V constant voltage, 40 mA/gel, 80 W for about 2.5 h. Molecular weight markers were from the Low Mr Electrophoresis Calibration Kit (GE Healthcare). The gels were automatically stained using Processor Plus (Amersham Biosciences) with freshly prepared Neuhoff stain (Colloidal Coomassie Blue) ([Neuhoff, Arold, & Ehrhardt, 1988](#)). They were digitized with the Personal Densitometer SI (Amersham Biosciences) and then stored after dehydration in a GD 2000 Vacuum Gel Dryer System (GE Healthcare).

### 2.4.3. Image analysis and statistical analysis

Image analysis was performed with the Progenesis PG 220 software (Non Linear Dynamics). Spot detection was automatically performed using the 2005 detection software algorithm and manually verified. After the establishment of some user seeds, matching was automatically performed and manually checked.

Two analytical replicates of all the three biological replicates were performed. A spot was considered significant when it was present in both the technical replicates of at least two out of three biological replicates.

### 2.4.4. Protein identification

The protein spots were excised from the dried gels and rehydrated with MilliQ water. They were washed twice with 50% *v/v* ACN in a 25 mM NH<sub>4</sub>CO<sub>3</sub> and once in 100% *v/v* ACN and vacuum-dried. The proteins were in-gel digested with sequencing-grade, modified porcine trypsin (Promega, Madison, WI, USA) and added to a MALDI target plate as described by [Hewitson et al. \(2008\)](#). Positive-ion MALDI mass spectra were obtained using an Applied Biosystems 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA) in reflectron mode. MS spectra were acquired over a mass range of *m/z* 800–4000 and monoisotopic masses were obtained from centroid of raw, unsmoothed data. Finally, the mass spectra were internally calibrated using the tryptic autoprolysis products at *m/z* 842.509 and 2211.104. CID-MS/MS was performed on the 20 strongest peaks with an S/N greater than 40. A source 1 collision energy of 1 kV was used for CID-MS/MS, with air as the collision gas. The precursor mass window was set to a relative resolution of 50, and the metastable suppressor was enabled. Default calibration was used for the MS/MS spectra, which were baseline-subtracted (peak width 50) and smoothed (Savitsky–Golay with three points across a peak and a polynomial order of 4); the peak detection used a minimum S/N of 5, a local noise window of 50 *m/z*, and a minimum peak width of 2.9 bins. S/N 20 and 30 filters were used to generate peak lists from the MS and MS/MS spectra, respectively. The mass spectral data from the protein spots were submitted to a database search using a locally running copy of the MASCOT program (Matrix Science, version 2.1).

Batch-acquired MS/MS data were submitted to an MS/MS ion search through the Applied Biosystem GPS explorer software interface (version 3.6) with MASCOT.

The search parameters allowed a maximum of one missed cleavage, the carbamidomethylation of cysteine, the possible oxidation of methionine, peptide tolerance of 100 ppm and an MS/MS tolerance of 0.1 Da. The spectra were searched against a recent version of the NCBI non-redundant protein database.

The significance threshold for peptide identification was set at  $p < 0.05$ ; protein identification required that each protein contained at least one peptide with an expect *e*-value  $< 0.05$ .

## 2.5. Evaluation of exopolysaccharides production

Fifty milliliter aliquots of stationary phase cultures of both *L. plantarum* S11T3E and *L. pentosus* S3T60C were collected. Biomass was separated by centrifugation (4000 ×g, 20 min, 4°C) and supernatants were collected. A sterile loop was used to test pellet consistence.

## 3. Results and discussion

### 3.1. SCFA determination

In recent years, a growing number of studies have revealed that SCFA exert positive physiological effects, especially in relation to colonic function ([Russel et al., 2013](#)). The absence of SCFA has been associated to inflammatory bowel diseases (colitis, diarrheas) ([Binder, 2010](#)) and they, especially butyrate, seem to play an important role in the protection against colon carcinogenesis ([Fung et al., 2012](#)). Due to the biological relevance of SCFA, there have been several attempts to develop rapid and selective analytical methods capable of identify and quantify SCFAs in complex biological matrices, especially in fecal samples.

In this paper SCFA production by food-isolated potentially probiotic LAB, belonging to different species, was evaluated by coupling gas chromatography and enzymatic analyses. For propionic and butyric acids, the described gas chromatography-based method displayed good recoveries and repeatability, as well as a high sensitivity, making this method suitable for the analyses of samples with low concentrations of these compounds. On the contrary, this method was not suitable for acetic acid determination, since a very low repeatability was observed both in biological and technical replicates (data not shown).

As shown in [Table 1](#), all the tested strains are able to produce small amounts of both propionic and butyric acid, in the concentration range of mg/L, and higher amounts of both acetic and lactic acid, in the concentration range of g/L. The one way ANOVA test revealed that statistically relevant differences were present within the groups constituted by the considered strains for all the analyzed metabolites. The produced propionic acid ranges from  $0.55 \pm 0.13$  mg/L of *L. plantarum* S1T3B to the  $2.34 \pm 0.56$  mg/L of *L. plantarum* O4T10E. As concerns butyric acid it ranges from  $0.61 \pm 0.12$  mg/L of *L. plantarum* S1T3B to  $2.89 \pm 0.79$  mg/L of *L. plantarum* O4T10E. Nazzaro et al. described a 10-fold higher production of butyric acid by *Lactobacillus acidophilus* DSM 20079 grown in the presence of pectin, a prebiotic inducing SCFA production, as sugar substrate ([Nazzaro et al., 2012](#)); the same authors did not observe a detectable production of butyric acid in the presence of glucose as sugar substrate, the same condition analyzed in the present work. This consideration suggests either a higher production of butyric acid by the LAB tested in this work or a higher sensitivity of the proposed gas-chromatography-based method. Interestingly the trends of production of propionic and butyric acids are similar for the tested strains: the highest and the lowest producers of both these SCFA are always the same two *L. plantarum* strains. These statistically significant differences ( $p < 0.05$ ) in metabolite production by two different strains of the same *L. plantarum* species is not totally surprising: Molenaar et al. reported a genomic analyses on 20 *L. plantarum* strains, displaying the presence of several regions of high variability including part of the region codifying for sugar metabolism ([Moleenar et al., 2005](#)). Acetic and lactic acid were produced in higher amounts ranging from  $0.84 \pm 0.08$  g/L of *L. plantarum* O2T60C and  $1.57 \pm 0.11$  g/L of *L. mesenteroides* F050E and from  $9.17 \pm 0.58$  g/L of *L. mesenteroides* F050E and  $19.37 \pm 0.89$  g/L of *L. plantarum* S2T10D, respectively. The two *L. mesenteroides* strains and *L. pentosus* S3T60C produced a statistically relevant ( $p < 0.05$ ) higher amount of acetic acid than all the 13 tested strains of *L. plantarum*.



Table 1.

quantification of the short chain fatty acids produced by the selected potentially probiotic lactic acid bacteria. The values are represented as mean value  $\pm$  standard deviation.

<b>Microbial strain</b>	<b>Propionic acid (mg/L)</b>	<b>Butyric acid (mg/L)</b>	<b>Acetic acid (g/L)</b>	<b>Lactic acid (g/L)</b>	<b>D-Lactic acid (g/L)</b>	<b>L. Lactic acid (g/L)</b>
<i>L. plantarum</i> O2T60C	0.67 $\pm$ 0.22	1.80 $\pm$ 0.82	0.84 $\pm$ 0.08	13.61 $\pm$ 0.13	8.27 $\pm$ 0.04	5.34 $\pm$ 0.09
<i>L. plantarum</i> O2T60D	1.79 $\pm$ 0.61	2.02 $\pm$ 0.97	1.02 $\pm$ 0.01	17.24 $\pm$ 0.41	10.80 $\pm$ 0.27	6.44 $\pm$ 0.14
<i>L. plantarum</i> O11T30D	1.88 $\pm$ 0.41	2.33 $\pm$ 0.31	0.97 $\pm$ 0.04	16.33 $\pm$ 0.18	12.26 $\pm$ 0.07	4.06 $\pm$ 0.11
<i>L. plantarum</i> S4T30C	1.98 $\pm$ 0.34	2.24 $\pm$ 0.20	1.09 $\pm$ 0.04	19.00 $\pm$ 1.17	12.22 $\pm$ 0.11	6.78 $\pm$ 0.06
<i>L. plantarum</i> S2T10D	2.04 $\pm$ 0.74	1.98 $\pm$ 0.77	0.93 $\pm$ 0.04	19.37 $\pm$ 0.89	15.20 $\pm$ 0.51	4.17 $\pm$ 0.38
<i>L. pentosus</i> S3T60C	2.19 $\pm$ 0.51	2.84 $\pm$ 0.83	1.40 $\pm$ 0.02	18.46 $\pm$ 0.54	11.83 $\pm$ 0.38	6.63 $\pm$ 0.16
<i>L. plantarum</i> S11T3E	1.51 $\pm$ 0.39	1.93 $\pm$ 0.56	0.98 $\pm$ 0.06	15.32 $\pm$ 0.07	11.51 $\pm$ 0.04	3.81 $\pm$ 0.03
<i>L. plantarum</i> O4T10E	2.34 $\pm$ 0.56	2.89 $\pm$ 0.79	1.09 $\pm$ 0.04	15.98 $\pm$ 1.52	12.08 $\pm$ 1.01	3.90 $\pm$ 0.51
<i>L. plantarum</i> O3T15B	1.42 $\pm$ 0.23	1.94 $\pm$ 0.67	1.18 $\pm$ 0.05	16.54 $\pm$ 1.59	10.86 $\pm$ 0.98	5.68 $\pm$ 0.61
<i>L. mesenteroides</i> FS50Q	1.27 $\pm$ 0.20	2.24 $\pm$ 0.02	1.55 $\pm$ 0.04	9.54 $\pm$ 0.21	8.91 $\pm$ 0.17	0.62 $\pm$ 0.04
<i>L. mesenteroides</i> FO50E	1.32 $\pm$ 0.13	1.93 $\pm$ 0.52	1.57 $\pm$ 0.11	9.17 $\pm$ 0.58	8.77 $\pm$ 0.41	0.40 $\pm$ 0.17
<i>L. plantarum</i> O1T90B	1.34 $\pm$ 0.24	1.78 $\pm$ 0.40	1.14 $\pm$ 0.03	18.64 $\pm$ 1.06	12.79 $\pm$ 0.38	5.85 $\pm$ 0.68
<i>L. plantarum</i> O1T90C	1.67 $\pm$ 0.08	2.20 $\pm$ 0.18	1.06 $\pm$ 0.01	13.70 $\pm$ 0.03	10.11 $\pm$ 0.02	3.59 $\pm$ 0.01
<i>L. plantarum</i> O1T90E	1.71 $\pm$ 0.32	2.16 $\pm$ 0.57	1.05 $\pm$ 0.01	13.44 $\pm$ 0.70	10.13 $\pm$ 0.59	3.31 $\pm$ 0.11
<i>L. plantarum</i> S1T10A	1.30 $\pm$ 0.26	1.80 $\pm$ 0.47	1.17 $\pm$ 0.06	18.64 $\pm$ 2.36	12.76 $\pm$ 0.96	5.87 $\pm$ 0.40
<i>L. plantarum</i> S1T30B	1.24 $\pm$ 0.42	1.69 $\pm$ 0.53	1.15 $\pm$ 0.05	15.93 $\pm$ 0.06	10.22 $\pm$ 0.04	5.71 $\pm$ 0.02
<i>L. plantarum</i> S1T3B	0.55 $\pm$ 0.13	0.61 $\pm$ 0.12	1.02 $\pm$ 0.06	16.51 $\pm$ 1.48	12.80 $\pm$ 1.12	3.71 $\pm$ 0.36

[Table options](#)

For all the tested strains, lactic acid was the most abundant produced metabolite, as expected: actually this finding is in agreement with LAB metabolism, which normally produces lactic acid as the major end-product of the glycolytic fermentation and as a significant product (about 50%) of the phosphoketolase route. *L. pentosus* S3T60C and three *L. plantarum* strains (S4T30C, S2T10D, O1T90B) produce significantly higher lactic acid amounts ( $p < 0.05$ ) than the other tested strains with a conversion yield of the glucose present in MRS medium higher than 92%. These values are in agreement with the one reported by Orozco et al. for a strain of *L. plantarum* able to convert lactose in lactic acid with a 94% yield ( [Orozco et al., 2014](#)).

Even if propionic and butyric acid, the most interesting SCFA for human health, are produced in low amounts, it must be taken into account that, after ingestion, potentially probiotic lactic acid bacteria become members of a syntrophic chain with other intestinal bacteria, whose final result is the consumption of carbohydrates not suitable for humans. In this kind of ecological niche LAB may not only directly produce propionic and butyric acid, but also supply lactic acid as a carbon substrate for other bacterial species able to produce higher amounts of these two acids ([Bourriad et al., 2005](#) and [Moat and Foster, 2002](#)). For this reason it is important to underline that in the tested condition all the *Lactobacilli* strains revealed to be able to produce high amounts of lactic acid; on the contrary the lactic acid produced by the 2 tested *L. mesenteroides* strains is statistically significantly lower ( $p < 0.05$ ) than the lactic acid produced by all the other tested strains. Curiously, these 2 strains are the only enantioselective lactic acid producers: they are able to produce almost exclusively D-lactic acid, while all the other strains produce a mix of D- and L-isomers. The lower lactic acid production by *L. mesenteroides* depends on its heterofermentative metabolism in which equimolar amounts of lactic acid, acetic acid, ethanol and CO<sub>2</sub> ( [Axelsson, 1998](#)) are produced. This is also confirmed by the fact that the 2 *L. mesenteroides* strains are the highest producers of acetic acid among all the tested strains ( [Table 1](#)). On the contrary, all the tested *Lactobacilli* are facultative heterofermenters: they are commonly glucose homofermenters but they can change their metabolism under altered conditions or when the initial substrate is a pentose sugar ( [London, 1990](#)).

Further characterization experiments were performed on *L. plantarum* S11T3E and *L. pentosus* S3T60C. *L. plantarum* S11T3E was chosen among the available *L. plantarum* strains since it displayed the best probiotic performance, due to its high resistance to simulated gastric digestion and to its ability to increase trans-epithelial resistance of polarized H4-1 cells as well as to reduce *L. monocytogenes* invasion in undifferentiated gut model cells ( [Botta et al., 2014](#)). This latter feature is also displayed by *L. pentosus* S3T60C which is also one of the highest short chain fatty acid producers.

### **3.2. Extracellular proteomic experiments**

For both *L. plantarum* S11T3E and *L. pentosus* S3T60C, extracellular proteins were recovered from middle exponential phase cultures in order to avoid cytosolic contaminations due to cell lysis and 2-DE gels were performed in the *pI* range of 3–10 ( [Fig. 1](#)).

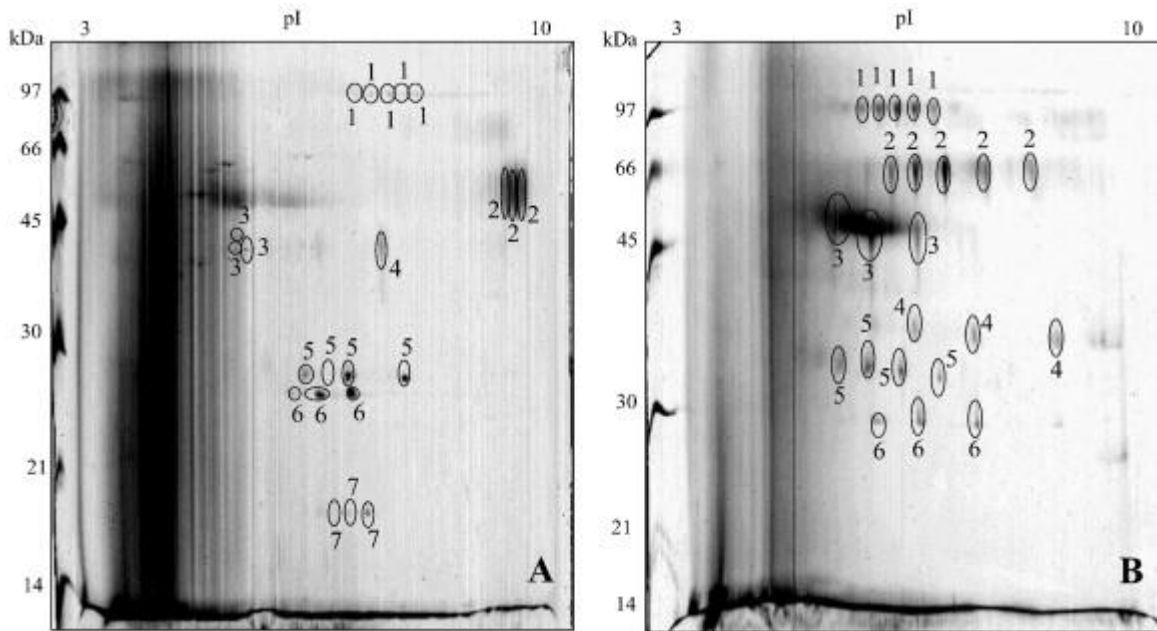


Fig. 1.

2-DE gels of the extracellular proteins recovered from *L. plantarum* S11T3E (A) and *L. pentosus* S3T60C (B). Numbers reported in the part A of the figure refer to the spot number of protein identifications listed in [Table 2](#). Numbers reported in the part B of the figure refer to the spot number of protein identifications listed in [Table 3](#).

#### [Figure options](#)

After image analysis, 22 and 23 spots were present in at least two out of three biological replicates deriving from *L. plantarum* S11T3E and *L. pentosus* S3T60C, respectively. All these spots were analyzed by MALDI TOF-TOF/MS; considering the different isoforms, 7 and 6 proteins were identified for *L. plantarum* S11T3E and *L. pentosus* S3T60C respectively. [Table 2](#) and [Table 3](#) show the proteins identified in this study.

Table 2.

Proteins identified from the maps of extracellular proteins of *Lactobacillus plantarum* S11T3E.

Spot number	Protein name	ID code	pI cal c	Mr Obs (kDa)	Identified peptides	Score	Sequence coverage %
1	Muramidase	C6VIY8	8.99	82066	VTANGQTWLR VTTNGQTWLR YSNLIGVTDYR	145	3%
2	Extracellular protein, Gamma-D-glutamate-meso-diaminopimel	C6VP22	9.37	36837	AGDTVWAYAQK STAYYTSPFAIHM STAYYTSPFAIHM	177	6%

Spot number	Protein name	ID code	pI	Mr Obs (kDa)	Identified peptides	Score	Sequence coverage %
3	ate muropeptidase Glyceraldehyde 3-phosphate dehydrogenase	C6VM91	5.30	3664	IGINGFGR TIVYNVNDDILTADDR	151	7%
4	Extracellular transglycosylase	F9UTQ7	8.93	2629	ESNWNSTVK SYVLSQMQR SYVLSQMQR LANVNLIFIGDK TGVSASTWNTIITR AGDTVSAIAQAHTSVSAIEK VANSYVASR SHWLANGWY	441	24%
5	Extracellular protein, peptidase M23B	U2JL15	8.78	2201	YQLSASYLNGDYSAANQER AGDTVSAIAADHNTTIDAIQQA NHLK	488	29%
6	Extracellular protein	E1TQU5	8.86	2132	GHYILPGQK SGDSVWAIQK SYVLSQMQR SYVLSQMQR FNTTINHVETTNNIK	309	22%
7	Adherence protein, chitin-binding domain	F9UP60	8.89	2224	AGNLSVTWQLTAR	93	6%

[Table options](#)

Table 3.

Proteins identified from the maps of extracellular proteins of *Lactobacillus pentosus* S3T60C.

Spot number	Protein name	ID code	pI	Mr obs (kDa)	Identified peptides	Score	Sequence coverage %
1	Extracellular protein, NlpC/P60 family	I9KZX2	6.64	4895	HGVSVQSIEK AGDSLWALADK ANDTVWVSLAQK SDSNIDLIYVGQNLQISGSK YNTSVHALQQLNLSGNLILV GQK	567	15

Spot number	Protein name	ID code	pI	Mr obs (kDa)	Identified peptides	Score	Sequence coverage %
2	Extracellular protein, gamma-D-glutamate-meso-diaminopimelate muropeptidase	I9KX18	9.39	37405	AGDTVWAYSQK STTYTTPSFAIHMF STTYTTPSFAIHMF	199	6
3	Extracellular transglycosylase	I8R8N8	6.73	26993	SYVLSQMQR SYVLSQMQR TGVSASTWNTIITR AGDTVSEIALAHNTSVSAIEK	338	16
4	Unknown extracellular protein	GOLYK7	9.37	24585	ESGGSYSAR VANSYVASR LANVNLIFIGDK AGDTVSAIAQAHNTSVSAIEK	334	21
5	Extracellular protein, peptidase M23B	G0M3S6	7.90	23031	YGSWANAK ESGGSYSAR VANNYVASR SHWLANNWY AGDTVSQIALDHNTTVDAIQQ ANHLK	499	27
6	Unknown extracellular protein	G0M5T7	7.93	21440	GHYILPGQK SGDSVWAIQK SYVLSQMQR SYVLSQMQR TGVSASTWNTIITR FNTTINNVETTNNIK	446	28

#### [Table options](#)

In both strains all the identified proteins, including those with unknown functions, are classified as extracellular proteins, confirming the effectiveness of the employed protocol to exclude cytosolic contaminations. The only exception is represented by glyceraldehyde 3-P dehydrogenase which is a glycolytic enzyme present in the extracellular proteome of *L. plantarum* S11T3E. The extracellular location of this protein is not surprising: it has been widely described in several lactic acid bacteria as a moonlighting protein that acts as an adhesin when extracellularly-exposed or -released ([Alvarez et al., 2003](#), [Genovese et al., 2013](#), [Mangiapane et al., 2013](#) and [Mangiapane et al., 2014](#)).

The extracellular protein profiles of the two strains share three proteins (gamma-D-glutamate-meso-diaminopimelate muropeptidase; extracellular transglycosylase; extracellular protein peptidase M23B), whereas four proteins are specifically expressed by one of the two bacterial strains: muramidase, glyceraldehyde 3-phosphate dehydrogenase and adherence protein with a chitin-binding domain are present only in *L. plantarum* S11T3E; extracellular protein NlpC/P60 family is present only in *L. pentosus* S3T60C. Furthermore, two spots identified as extracellular proteins,

whose function is yet to be determined, are present in *L. pentosus* S3T60C and one in *L. plantarum* S11T3E.

Most of the identified proteins are involved in the cell wall re-arrangement and in the control of the cell shape during cell division. This result is in agreement with the recovery of the sample during the middle exponential phase, performed to prevent cytosolic contaminations due to cell lysis that generally occurs during the stationary phase. Gamma-D-glutamate-meso-diaminopimelate muropeptidase is expressed by both the analyzed strains; it was firstly identified in *Bacillus subtilis* 168 by Margot et al., which proved its involvement in cell-shape control ( [Margot, Pagni, & Karamata, 1999](#)). This enzyme specifically cleaves the muro-peptide bridge between D-glutamate and meso-diaminopimelate allowing continuous re-arrangement of the peptidoglycan layer during the cell growth ( [Margot et al., 1999](#)). Also muramidase (spot 1 in *L. plantarum* S11T3E maps) and NlpC/P60 family protein (spot 1 in *L. pentosus* S3T60C) are proteins devoted to cell wall renewal; in particular NlpC/P60 belongs to a family of hydrolases with various roles in the dynamics of the bacterial cell wall ( [Bäuerl et al., 2010](#)). As previously referred, in both strains some spots were identified as extracellular proteins with a still unknown function; the presumed secreted location for these proteins is due to the presence in their sequences of at least one LysM domain. Considering that this domain is mainly implicated in the binding to peptidoglycan ( [Buist, Steen, Kok, & Kuipers, 2008](#)) it is reasonable to hypothesize their role in peptidoglycan anchoring and/or turnover.

Both strains express an extracellular transglycosylase, a protein involved in the synthesis of the extracellularly produced exopolysaccharides (EPS) such as dextran, levan and mutan ( [Sutherland, 1982](#)). This enzyme catalyzes the transfer of a monosaccharidic unit from a disaccharidic sugar to a polysaccharide chain. In recent years, the production of EPS by lactic acid bacteria has been extensively studied ( [Patel et al., 2012](#) and [Welman and Maddox, 2003](#)). EPS are commonly described as lightly bound to the bacterial surface and often secreted into their surrounding as a sticky slime. The ability of the two tested strains to produce EPS is confirmed by the sticky and ropy consistence of the pellets, displayed in [Fig. 2](#). *L. pentosus* S3T60C produces a higher EPS amount than *L. plantarum* S11T3E consistently with the higher expression of the extracellular transglycosylase (Spot 3 part B and spot 4 part A of [Fig. 1](#)). EPS are related to biofilm formation, cation sequestration and cellular recognition and play a key role in the protection of microorganisms against adverse conditions such as desiccation and osmotic stress ( [Pessione, 2012](#)). These compounds possess several positive features since they enhance the probiotic potential of the producing strain increasing its resistance to environmental stresses and its ability to adhere, as described by Myszka et al. ( [Myszka & Czaczyk, 2009](#)). In *Lactobacillus delbrueckii* subsp. *bulgaricus* it has been demonstrated that EPS confer better ability of adhesion to CaCo2 cells and enhanced survival in the adverse gastro-intestinal conditions supporting a better competition with autochthonous bacteria ( [Darilmaz, Ashm, Suludere, & Akca, 2011](#)). EPS also contribute to human health as prebiotics or thanks to their antitumor, antiulcer, immunomodulating and cholesterol-lowering activities ( [de Vuyst & Degeest, 1999](#)).



Fig. 2.

Exopolysaccharides production by *L. plantarum* S11T3E (A) and *L. pentosus* S3T60C (B).

#### [Figure options](#)

Both *L. plantarum* S11T3E and *L. pentosus* S3T60C exhibit in their secretome the presence of a protein containing a typical domain of the peptidase M23 family. This domain is present in several zinc metallo-proteases with a wide range of specificity, including endopeptidases involved in the cleavage of peptidoglycan ([Ichimura et al., 2002](#) and [Thumm and Gotz, 1997](#)). The best characterized among these lytic proteins is lysostaphin produced by *Staphylococcus* species ([Naverre & Schneewind, 1999](#)). In the literature there are reports dealing with the identification in a strain of *Treponema denticola* of a group of fibronectin-binding proteins characterized by the presence in their sequence of M23 peptidase domains ([Bamford, Francescutti, Cameron, Jenkinson, & Dymock, 2010](#)). The fibronectin-binding ability of this protein family for sure constitutes a problem in pathogenic strains since it can improve adhesion to the extracellular matrix and hence their virulence potential; on the contrary, in non-pathogenic bacteria, this feature can increase the probiotic potential by enhancing adhesion.

In the exoproteome of *L. plantarum* S11T3E the expression of an adherence protein containing a chitin-binding domain was also observed. Boekhorst et al. ([Boekhorst, Wels, Kleerebezem, & Siezen, 2006](#)) described the predicted secretome of *L. plantarum* WCFS1 by bioinformatic tools in order to shed light on the interaction between this species and the environment. Among the predicted secreted proteins involved in the adhesive potential of the strain they highlighted the presence of one protein containing a domain for chitin binding that is probably the same protein expressed by *L. plantarum* S11T3E. Furthermore, Sanchez et al. proved that, in *L. plantarum*, chitin-binding proteins can bind not only to N-acetylglucosamine present in the chitin exoskeleton of fungi but also to a wide variety of polymers including mucins, demonstrating that they also play a role in adhesion to human epithelial cells ([Sanchez, Gonzalez-Tejedo, Ruas-Madiedo, Urdaci, & Margolles, 2011](#)).

## 4. Conclusions

All the olive-isolated probiotic strains revealed to be able to produce significant amounts of lactic and acetic acid and just small quantities of both propionic and butyric acid. The proteomic studies suggested that the adhesion abilities of the two tested probiotic strains seem to depend on different mechanisms: *L. plantarum* S11T3E secretes several proteins involved in the adhesion processes (adherence protein with chitin-binding domain, glyceraldehyde 3-P dehydrogenase, M23 family peptidase) indicating a key role of these molecules in the phenomenon. Among these proteins *L. pentosus* S3T60C just secretes the M23 family peptidase suggesting the involvement of other biological molecules in its adhesive ability. Exopolysaccharides could be responsible for this property: actually the expression of an extracellular transglycosylase and the sticky and ropy consistence of the pellets suggest their production by both the tested strains.

In conclusion, these two strains, selected either for their ability to produce bio-active molecules such as SCFA (*L. pentosus* S3T60C) or for the already described probiotic properties (*L. plantarum* S11T3E), confirmed to be good candidates to be described and used as probiotics, also thanks to the expressed potential to adhere to gut mucosa. This paper, once more, proves that different approaches, such as proteomics and the study of some metabolites, are more and more useful to describe and characterize a probiotic. Furthermore, these results underline the importance of screening new food matrix (especially artisanal-made fermented food) in order to find more performing strains not yet available in the market.

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