Production of Exopolysaccharides by *Lactobacillus* and *Bifidobacterium* Strains from Human Origin and Metabolic Activity of the Producing Bacteria in Milk.

N. Salazar*, A. Prieto†, J.A. Leal†, B. Mayo*, J.C. Bada-Gancedo*, C.G. de los Reyes-Gavilán* and P. Ruas-Madiedo*[1].

***** Instituto de Productos Lácteos de Asturias (IPLA-CSIC). Departamento de Microbiología y Bioquímica de Productos Lácteos. Carretera de Infiesto s/n, 33300 Villaviciosa, Asturias Spain

† Centro de Investigaciónes Biológicas (CIB-CSIC). Ramiro de Maeztu 9, 28040 Madrid, Spain

[1]Corresponding author: Instituto de Productos Lácteos de Asturias. Departamento de Microbiología y Bioquímica de Productos Lácteos. Carretera de Infiesto s/n (P.O. box 85), 33300 Villaviciosa (Asturias), Spain

Phone: +34 985892131, Fax: +34 985892233, E-mail: ruas-madiedo@ipla.csic.es

ABSTRACT

This work reports the physico-chemical characterization of 21 exopolysaccharides (EPS) produced by *Lactobacillus* and *Bifidobacterium* strains isolated from the human intestinal microbiota, as well as the growth and metabolic activity of the EPS-producing strains in milk. The strains belong to the species *Lactobacillus casei, Lactobacillus rhamnosus, Lactobacillus plantarum, Lactobacillus vaginalis, Bifidobacterium animalis, Bifidobacterium longum* and *Bifidobacterium pseudocatenulatum*. The molar mass distribution of EPS fractions showed two peaks of different sizes, which is a feature shared with some EPS from bacteria of food origin. In general, we have detected an association between the EPS-size distribution and the EPS-producing species, although due to the low number of human bacterial EPS tested we cannot establish a conclusive correlation. The main monosaccharide components of the EPS under study were glucose, galactose and rhamnose, which are the same than those found in food polymers; however rhamnose and glucose ratios was generally higher than galactose in our human bacterial EPS. All EPS-producing strains were able to grow and acidify milk; most lactobacilli produced lactic acid as the main metabolite. The ratio lactic/acetic acid in bifidobacteria was 0.7, close to the theoretical one, indicating that they did not produce an excessive amount of acetic acid that could adversely affect the sensory properties of fermented milks. With respect to the viscosifying ability, *L. plantarum* H2 and *L. rhamnosus* E41 and E43R were able to increase the viscosity of stirred fermented milks at a similar extent to the EPS-producing *Streptococcus thermophilus* strain used as positive control. Therefore, these human EPS-producing bacteria could be used as adjuncts in mixed cultures for the formulation of functional foods if probiotic characteristics were demonstrated. This is the first article reporting the physico-chemical characteristics of EPS isolated from human intestinal microbiota.

Key words: “exopolysaccharide”, “human origin”, “*Bifidobacterium*”, “*Lactobacillus*”

INTRODUCTION

Recently, the concept of "functional starter" has been introduced referring to "cultures that posses properties which contribute to food safety and / or offer one or more organoleptic, technological, nutritional, or health advantages" (Leroy and De Vuyst, 2004). In this context, some exopolysaccharide (EPS)-producing lactic acid bacteria (LAB) strains could cover both technological and health aspects. Their capability to improve the rheological characteristics of fermented milks has been proven and, more recently, health benefits have been attributed to some of these biopolymers. It is well known that the key parameters determining the viscosity and texture intensifying capability of EPS are mainly their chemical composition, molar mass and the structure of the repeating units (Laws and Marshall, 2001) as well as their interaction with the food protein matrix (Hassan, 2008). However, there are very few articles reporting the relationship between the physico-chemical characteristics of EPS and their putative health promoting properties (Nagaoka et al., 1994; Kitazawa et al., 1998). These studies have been conducted with polymers synthesized by food origin LAB, and as far as we know no data are available about the physico-chemical characteristics of polymers isolated from human origin strains. Indeed, these strains could be good candidates to study the relationship between the EPS production phenotype and its putative involvement in their probiotic properties. In this way, we have demonstrated that human isolated *Lactobacillus* and *Bifidobacterium* strains are able to produce EPS (Ruas-Madiedo et al., 2007).

Nowadays, fresh fermented and unfermented dairy products, such as milk, yogurt, ice cream and desserts are widely employed for the delivery of probiotic strains and the incorporation into cheeses is under development (Boylston et al., 2004; Grattepanche et al., 2008). The viability of probiotics in fermented milks has improved in recent years but this was to the detriment of the variability of strains employed in the formulation of these foods

(Gueimonde et al. 2004). These strains have been selected as they have been shown to survive well in the fermented product but their health promoting properties have not been taken into account. Thus, the exploration of both technological and probiotic properties is crucial for the potential application of new strains in functional food formulations.

In the current work, we aimed to study the growth and metabolic activity of human isolated EPS-producing strains in milk. This characterization could allow us to identify potential probiotic strains with appropriate technological properties for dairy applications. Indeed, in a previous work we have shown that the EPS produced by these *Bifidobacterium* strains had the ability to modulate the intestinal microbiota, promoting a bifidogenic effect comparable to that of the prebiotic inulin (Salazar el al., 2008). Thereby, the physico-chemical characteristics of EPS synthesized by these strains have been analyzed in order to establish a preliminary classification of these biopolymers based on their molar mass and monosaccharide composition. This could help us in a future to correlate the intrinsic characteristics of EPS with their putative health benefits.

MATERIAL AND METHODS

Bacterial Strains and Isolation of EPS Fraction from Culture Media

The human-origin strains employed in this study (Table 1) have been previously screened for EPS production and they were identified by 16S rRNA gene sequencing (Ruas-Madiedo et al., 2007). Strains were grown in MRSC broth [MRS (BioKar Diagnostics, Beauvais, France) supplemented with 0.25% (wt/vol) L-cysteine (Sigma Chemical Co., St. Luis, MO)] and incubated at 37ºC for 24 h under anaerobic conditions as previously described (Salazar et al., 2008). The strain *Streptococcus thermophilus* ST-body-1 (Chr. Hansen, Horsholm, Denmark), currently employed in yoghurt manufacture, was used as an EPS-producing positive control. This control strain was directly inoculated from the lyophilized

powder into M17 broth (Oxoid Ltd., Basingstoke, Hampshire, UK) and grown overnight at 37ºC. This culture was employed to inoculate (2%) fresh M-17 broth which was incubated during 24 h.

The EPS fraction of strains was isolated from the cellular biomass harvested from agar-MRSC plates. In short, strains were grown on the surface of agar-MRSC for 5 days under anaerobic conditions at 37ºC. Cellular biomass was collected with ultrapure water and mixed with 1 vol of 2 *M* NaOH. After gently stirring overnight at room temperature, cells were removed by centrifugation and EPS from the supernatants were precipitated for 48 h at 4ºC using 2 vol of absolute cold ethanol. After centrifugation, the EPS fraction was resuspended in ultrapure water, dialyzed against ultrapure water for 3 days at 4ºC in dialysis tubes (Sigma) of 12 to 14 kDa molecular mass cut off and finally freeze-dried.

Molar Mass Distribution of EPS Fractions

Size exclusion chromatography (SEC) was employed to determine the molar mass (MM) distribution of the EPS fractions. The lyophilized fractions were resuspended (5 mg/mL) in 0.1 *M* $NaNO_3$, kept overnight under gently stirring, and finally centrifuged (10000*g*, 10 min) before analysis. A HPLC chromatographic system composed of an Alliance 2690 module injector, a Photodiode Array PDA 996 detector, a 410 refractive index (RI) detector and the Empower software (Waters, Milford, MA, USA) was used. The separation (50 μL vol injection sample) was carried out in two columns placed in series, TSK-Gel G3000 PW_{XL} + TSK-Gel G5000 PW_{XL} protected with a TSK-Gel guard column (Supelco-Sigma). The mobile phase was 0.1 *M* $NaNO_3$ and the separations took place at 40ºC at a flow rate of 0.45 mL/min. The EPS peaks were detected with the RI detector and the presence of proteins was monitored through the PDA detector set at 220 nm. Standards of dextran (Fluka-Sigma), ranging from $5x10^3$ to $4.9x10^6$ Da, were used for quantification and MM determination. For quantification, the corresponding regression equations were calculated from four different

concentrations of each standard (R^2 ≥0.98) and for the MM determination the regression equation was calculated using the elution time of seven dextran standards of different MM (R^2 =0.99). The peaks obtained after SEC were arbitrarily distributed amongst four molar mass ranges. The percentage of each peak was calculated with respect to the total amount of peaks detected in each EPS fraction (μg peak range x 100)/ (μg total peaks).

Monosaccharide Composition of EPS Fractions

The EPS fractions were hydrolyzed with 0.15, 1.5 or 3 *M* trifluoroacetic acid for 1 h at 121ºC. The products were converted into their corresponding alditol acetates and then identified and quantified by gas-liquid chromatography (GLC), with inositol as the internal standard (IS). The neutral sugars composition was determined in an Autosystem instrument (Perkin Elmer, Norwalk, CT, USA) equipped with a flame ionization detector, using a TR-CN100 capillary column (30 m x 0.25 mm, 0.2 μm film thickness) and a temperature program from 210ºC (1 min) to 240ºC (ramp rate of 15ºC per min, final temperature during 7 min) employing He as gas carrier. Injection was performed in the split mode (split ratio 50:1). The percentage of each monosaccharide was calculated with respect to the total monosaccharide content. For amino-sugars identification, sample components were analyzed by GC-MS in an Agilent 7980A-5975C instrument (Agilent Technologies Inc., Palo Alto, CA, USA), using a HP-5 column (30 m x 0.25 mm, 0.2 μm film thickness) and a temperature program of 170 to 210ºC, 1 min initial hold, 2 ºC per min ramp rate. Peaks were identified on the basis of the sample coincidence with relative retention times of commercial standards and by their mass spectra.

Growth of EPS-producing Strains in Milk

Commercial pasteurized milk (Central Lechera Asturiana, Asturias, Spain) was purchased from the supermarket and was supplemented with 1% Difco™ skimmed milk (Becton Dickinson, MD, USA) and 0.2% yeast extract (Biokar) giving a total solid content of 12.5%. This supplemented milk was pasteurized again at 90ºC during 5 min. MRSC or M17

cultures of each strain were washed twice with sterile PBS buffer pH 7.0 and were separately employed to inoculate (2%) 500 mL of pasteurized milk. The inoculated milks were incubated overnight (17± 1 h) in a water bath at 37ºC and, at the end, a sample was collected in sterile conditions for bacterial counting and pH measurements. Afterwards, the fermented milks were cool-down to approximately 18ºC with running tap water, then they were stirred 20-times up and down with a spoon and stored overnight at 4ºC. Three replicated batches of fermented milks were carried out for each strain.

For bacterial counts, serial dilutions of cultured milks were made in Ringer's solution (Merck, Darmstadt, Germany) and deep-plated on agar-MRSC or agar-M17. The plates were incubated at 37ºC during 3 days under anaerobic conditions for *Lactobacillus* and *Bifidobacterium* and under aerobic conditions for *S. thermophilus*. Counts were expressed as Log cfu/g and the increase of the Log units during milk fermentation was calculated.

Isolation of EPS from Cultured Milk and Apparent Viscosity of Stirred-Fermented Milks

The EPS fraction was isolated from milks cultured with the human origin EPS-producing strains. Briefly, 40 g of cultured milk was mixed with TCA solution (12% final concentration) and strongly stirred for 45 min at room temperature. Precipitated bacteria and proteins were removed by centrifugation (10000*g*, 4ºC, 30 min) and the pH of supernatant was raised to 4.5±0.5. Finally, supernatants were intensively dialyzed and freeze-dried as described in the previous paragraph. Given that the yeast extract added to milk could be a source of polysaccharides (mainly gluco- and galacto-mannans), the same procedure was employed to obtain the precipitated-fraction of uncultured supplemented pasteurized milk. The EPS yield (mg/100 mL of whey) of each strain growing in milk was calculated after

subtracting the precipitated-fraction (15.0±1.5 mg/100 mL of whey) obtained from the uncultured milk.

The apparent viscosity of stirred-fermented milks was measured using the Posthumus funnel (Hellinga et al., 1989). The funnel was filled with approximately 450 g of stirred-fermented milk and the time (s) taken to pass the mark inside the funnel was recorded. The measurements were carried out in a chamber refrigerated at 4ºC.

Lactose Consumption and Organic Acids Production in Fermented Milks

The same HPLC chromatographic system described before was employed to quantify the lactose consumption and organic acid production in fermented milks. Samples for HPLC analysis were prepared as follows: 5 g of cultured milk was mixed with 20 mL of 9 m*N* H_2SO_4, kept at 37ºC with constant shaking for 2 h and, after centrifugation (10000*g*, 20 min), supernatants were filtered through filter paper. The sample separation was carried out in an ICSep ION-300 ion-exchange column (Transgenomic, San Jose, CA, USA) using 8.5 m*N* H_2SO_4 as mobile phase at 65ºC and with a flow rate of 0.4 mL/min. Lactose was detected by using the RI detector and the organic acids by using the PDA detector set at 210 nm. For quantification, the regression equations (R^2 ≥0.99) were calculated using different concentrations of the corresponding standards purchased from Fluka-Sigma. Results were expressed as m*M*.

Production of Volatile Compounds in Fermented Milks

Volatile compounds produced by lactobacilli and bifidobacteria in fermented milks were determined by means of head-space (HS) GC-MS. Samples (10 g) of fermented milk were mixed with cyclohexanone (0.36 mg/mL) as IS and were placed into glass tubes sealed with rubber and metallic caps. They were analyzed in a 6890N Agilent GC coupled with a HS automatic injector G1888 series and with a 5975B inert MS detector (Agilent). Data was collected and analyzed with the ChemStation software. Samples in the HS were kept at 50ºC

during 30 min under stirring and then separated in a HP-Innovax column (60 m x 0.25 mm x 0.25 μm film thickness, Agilent). The temperature of the injector was kept at 220ºC and the split ratio was 20:1. The chromatographic conditions were 35ºC during 5 min, 100ºC (ramp ratio 5ºC per min) final temperature during 10 min, 240ºC (ramp ratio 8ºC per min) final temperature during 5 min, using He as gas carrier. The electron impact energy of the MS detector was set to 70eV and data were collected within the 20 to 250 amu range (at 3.12 scans/s). The volatile compounds were identified by comparing their mass spectra with those held in the Wiley 138 library (Agilent) and by comparing their retention times with those of the corresponding standards (Fluka-Sigma). The peaks were quantified as the relative total ionic count abundance with respect to the IS. The concentration (μg/mL) of each volatile compound was calculated using linear regression equations (R^2 >0.99) of the corresponding standards.

RESULTS AND DISCUSSION

Characterization of EPS Produced by Human Bifidobacterium *and* Lactobacillus *Strains*

A broad MM distribution, ranging from about 10^3 to $5x10^6$ Da, of the EPS fractions isolated from agar-MRSC plates of *Bifidobacterium* and *Lactobacillus* strains from human intestinal origin was detected. In addition, within each EPS-fraction more than one peak varying in size was monitored (Table 1). Then, an arbitrary classification of MM ranges has been established as follows: M1: $>10^6$ Da, M2: 10^5-10^6 Da, M3: 10^4-10^5 Da, and M4: $<10^4$ Da. Most EPS-fractions showed two different MM peaks and all of them included the smaller peak range, in some cases being present at the highest (more than 80%) proportion. The four *Lactobacillus plantarum* as well as *Lactobacillus. casei* BA61 and F72 strains showed a second peak within the M3 range. However, *L. casei* E51 was the only lactobacilli presenting a peak ($2x10^6$ Da) of the highest M1, whereas in the two *Lactobacillus rhamnosus* strains and

the *Lactobacillus vaginalis* strain the M2 peak was detected. This data indicates a different pattern of EPS-MM distribution within each *Lactobacillus* species. Similar behavior was also noted for the *Bifidobacterium* species. Thus, the two *Bifidobacterium animalis* strains have EPS with a second peak in the M3 range; in 3 out of 4 *Bifidobacterium. longum* strains the M2 range was detected, and most *Bifidobcterium pseudocatenulatum* strains presented the M1 highest range. However, the scarce number of strains analyzed for each species do not allow us to establish a definitive direct correlation between EPS-size distribution and the EPS-producing species. As far as we are aware, there is no available data in the literature reporting the MM values of EPS synthesized by lactobacilli of human origin. Mozzi and co-workers (2006) collected data of EPS from several mesophilic and thermophilic *Lactobacillus* strains from food origin and most showed MM values lower than 10^6 Da as it was evidenced in our human origin EPS-strains. But none of the food strains presented simultaneously more than one MM peak. However, the EPS-fraction produced by several strains of *Lactobacillus delbrueckii* subsp. *bulgaricus*, *S. thermophilus* and *Lactococcus lactis* subsp. *cremoris* as well as that synthesized by *Lactobacillus pentosus* LPS26 presented simultaneously two EPS of different MM (Grobben et al., 1997; Degeest and De Vuyst, 1999; Petry et al., 2003; Vaningelgem et al., 2004; Ruas-Madiedo et al., 2005; Sánchez et al., 2006). Thus, it seems that the production of two EPS peaks of different size is a relatively common characteristic among LAB from food and intestinal origin. With respect to the EPS synthesized by *Bifidobacterium*, scarce data is available in the literature regarding their MM distribution (Nagaoka et al., 1995; Roberts, 1995; Hosono et al., 1997). The production of two EPS-fractions of different sizes has been detected as well in *B. longum* and *Bifidobacterium infantis* (Abbad-Andaloussi et al., 1995; Tone-Shimokawa et al., 1996). It has been reported that the variation in the culture conditions (nitrogen and carbon sources, pH, temperature, etc.) could modify the ratio of high to low MM (Degeest and De Vuyst, 1999) or might

induce the production of two EPS of a different chemical / structure composition (Cerning et al., 1994; Petry et al., 2000; Wang & Bi, 2008). It has been also postulated that the occurrence of more than one EPS fraction produced by a single strain could be due to a partial hydrolysis of the highest peak as a consequence of prolonged fermentations (De Vuyst et al., 1998). We can not discard a partial hydrolysis of our EPS after incubation of the producing bacteria in the surface of agar-MRSC for 5 days. In fact, we were able to cultivate in MRSC broth the biomass collected from solid medium after this period. However, part of the microbial population could have become not viable or even dead and thus releasing glycolytic enzymes. In spite of this, a relative long incubation period (5 days) has been used in our case due to the generally slow growth of strains from human origin and the need for obtaining optimal biomass production in order to provide enough EPS for their physico-chemical characterization.

Regarding the chemical composition (Table 1), the monosaccharides galactose and glucose were present in all polymers and rhamnose in half of them. These are also the three major sugar components of EPS isolated from food environments. Mannose, fucose and N-acetyl-glucosamine were detected in minor proportions in 4 out of the 11 *Bifidobacterium* strains and *L. vaginalis* C32. N-acetyl-aminated sugars are also commonly found in EPS from LAB, whereas fucose and mannose have been described in only a few strains (Ruas-Madiedo et al., 2009). The ratio among the major monosaccharides varied depending on the strain and a species-associated pattern has not been evidenced for most of them. In general for all EPS, with the exception of the two *B. animalis* strains, galactose was present at a lower ratio than glucose and rhamnose, when this last monosaccharide was present. On the opposite, in EPS from lactobacilli of food origin galactose is often found at the same or higher proportion than the other monosaccharides (Mozzi et al., 2006; Ruas-Madiedo et al., 2009). In the EPS produced by our *B. animalis* and *B. longum* strains (with the exception of strain E44) from

human origin the monosaccharide rhamnose was present, whereas in *B. pseudocatenulatum* it was absent. Even more, strains *B. longum* H73 and H67 presented a high content of rhamnose as it was also detected for the EPS from most *L. casei* and *rhamnosus* strains. In this respect, it is interesting to note that the polymer composition of 25 EPS produced by LAB strains from food revealed that only 7 (28%) had rhamnose in their composition (Ruas-Madiedo et al., 2009). Whereas, the EPS produced by our strains of human origin presented higher rhamnose occurrence (11 out of 21 strains, 52%) and also higher proportion (ratio above 1). In this respect, the high rhamnose content of EPS from *Bifidobacterium* has been related with their capacity of protection against induced gastric ulcers in rats (Nagaoka et al., 1994). The chemical characterization of the human bacterial EPS carried out in this work confirmed our previous results relating the screening of genes involved in EPS synthesis (Ruas-Madiedo et al., 2007) which pointed out that they were heteropolysaccharides. Finally, it is necessary to be careful when using laboratory media for analyzing EPS produced by intestinal bacteria. In fact, environmental conditions of laboratory and gut are drastically different and it is known that culturing conditions have a major influence on the type of EPS produced. Hence differences in EPS maybe expected between that found in our study and that actually produced in the gut environment by the same strain.

Growth and Metabolic Activity of Human Origin EPS-producing Strains in Milk

The EPS-producing strains isolated from human microbiota were grown in pasteurized milk supplemented with 0.2% of yeast extract which was added for increasing the available nitrogen source in order to allow these strains to grow in milk as single cultures. The pH values and the increase of the Log cfu/g after 17±1h of incubation at 37ºC are depicted in Figure 1. The initial pH of milk was 6.5±0.2 and all lactobacilli were able to decrease the pH below 4.5, except *L. vaginalis* C32 that only reached a value of 5.1. The pH of milks cultured

297 with *Bifidobacterium* strains presented values around 4.5, with the exception of those of *B.*
298 *longum* L55 and E44 that displayed lower values and those made with *B. animalis* C64MRa
299 and E43 which were above 5.0. Regarding the ability to grow in milk, all bacteria increased
300 their counts more than 1.5 Log cfu/g with respect to the initial levels. Among lactobacilli, *L.*
301 *casei* strains reached the highest counts followed by *L. rhamnosus* and *L. plantarum*.
302 Remarkably, several *Bifidobacterium* strains were able to increase their counts more than 2.5
303 Log units. Contrary to that expected, *B. animalis* strains showed the poorest ability to grow in
304 milk which was in accordance with their low capacity to produce organic acids.

305 Figure 2 shows the lactose consumption and organic acids production in milks
306 fermented with the EPS-producing strains from human origin. Milks cultured with *B. animalis*
307 C64MRa and E43 presented the lowest lactose consumption and consequently the lowest
308 lactic and acetic acid production, as compared with the other strains. The initial lactose
309 content in the pasteurized milk was 28.7±2.4 m*M* and the consumption ranged between 8%
310 (for *B. animalis* E43) and 36% (for *L. plantarum* C64MRb) showing a wide variability among
311 strains. The average lactic/acetic acid ratio obtained in these fermented milks was 0.85±0.14
312 for *Bifidobacterium* and 4.51±1.12 for *Lactobacillus.* From a sensory point of view, the
313 production of no excessive high amounts of acetic acid as compared with the lactic acid is a
314 desirable characteristic for preserving the sensory properties of fermented milk. Ethanol was
315 produced in variable amounts depending on the strain (Table 2). Acetaldehyde and diacetyl
316 were also detected in most milks cultured with bifidobacteria. A moderate production of
317 acetic acid and a very low level of ethanol were detected in milks fermented with *L. casei*, *L.*
318 *rhamnosus* and *L. plantarum* groups with most glucose, coming from the hydrolysis of
319 lactose, being converted into lactic acid. The strain *L. vaginalis* C32 had a different behavior,
320 as it was the highest ethanol (277 μg/mL on average) and the lowest lactic acid producer
321 among lactobacilli which is in good accordance with the heterofermentative metabolism of

this species (Embely et al., 1989). The ability of our strains to grow in milk is strain-dependent as it was previously indicated for other probiotic *Bifidobacterium* and *Lactobacillus* strains (Gilliland et al., 2002; Ostlie, et al., 2003). It is known that the milk composition influences the growth capacity of the strains (Kehagias et al., 2008). This could be related to the different amino acid availability among the milk types. Because in our case a fast-use nitrogen source (yeast extract) was available, other factors (such as variation in β-galactosidase activity, carbohydrate and peptide / amino acid transport systems, oxygen / pH tolerance, etc.) behind differences in proteolytic activity could account for the different capacity to grow in milk of our strains. Variations in the levels of metabolites produced during milk fermentation were also detected among our strains as other authors previously indicated (Baron et al., 2000; Ostlie et al., 2003). In this way, it is well known that the metabolic profile of potential probiotic strains growing in milk correlates directly with the sensory characteristics of the product, thereby being an important selection criterion for the formulation of functional dairy foods. Finally, this study was conducted to select strains with potential application for human consumption. However, since the yeast extract can not be included into the formulation of fermented milks, would be the use of starters (*S. thermophilus* and/or *L. delbrueckii* subsp. *bugaricus*) the next step in the assay of mixed fermentations for application in dairy industry.

The apparent (Posthumus) viscosity of milks cultured with some human EPS-producing strains, as well as the amount of EPS-fraction purified from the fermented milks, is presented in Table 3. The amount of the EPS produced in milk by the strains under characterization was low. It is worthy to mention that this value could have been underestimated due to the subtraction of the precipitated fraction from the uncultured milk. However, this was necessary in order to avoid the quantification of possible polysaccharides coming from the small amount of yeast extract added to the milk that could be co-precipitated

347 during the EPS purification process. In general, the viscosity intensifying capability of the
348 human origin strains was very scarce and most strains showed values of apparent viscosity
349 lower than 35 s (data not shown). Only three of our lactobacilli strains conferred to the
350 fermented milks a smooth and creamy consistency comparable to that of the EPS-producing
351 *S. thermophilus* strain used as control (Table 3). These were *L. rhamnosus* E41 and E43R and
352 *L. plantarum* H2. Interestingly, the same strains were also able to increase the apparent
353 viscosity of the stirred fermented milks, although their values were lower than that of *S.*
354 *thermophillus* ST-body-1. Among these three lactobacilli, only *L. plantarum* H2 increased the
355 viscosity of the fermented milk by producing similar low EPS amount to those produed by *S.*
356 *thermophilus*, their yield being lower than that of *L. rhamnosus* E41 and E43R strains
357 although their viscosity intensifying ability was higher. Other EPS intrinsic characteristics
358 beyond the concentration reached in milk, could also account for the increase in viscosity of
359 milks fermented with the human origin EPS-producing strains (Laws and Marshall, 2001).
360 The MM of an EPS strongly influences the viscosity of fermented milks made with EPS-
361 producing strains and, in general, polymers having high MM can produce fermented milks
362 with higher viscosity (Ruas-Madiedo et al., 2009). However, in the present study we can not
363 correlate the MM values obtained in MRSC medium with the viscosity of the fermented milk
364 because, as previously stated, MM and chemical composition of EPS could greatly vary
365 depending on the culture conditions. Finally, factors such as interactions between the EPS and
366 the milk protein network (Hassan et al., 2008) or the acidification rate of the strains in milk
367 (Lucey and Singh, 1998), among others, could affect the viscosity of the stirred fermented
368 milk. However, since our human origin strains E41, E43R and H2 and the control strain
369 reached similar final pH after the same incubation period (3.82±0.08, 3.76±0.02, 3.88±0.09
370 and 3.97±0.06, respectively) differences in acidification could have not account for variation
371 in the viscosity of the fermented milks.

CONCLUSION

Our EPS-producing strains from human origin presented higher rhamnose content than strains of food origin previously studied by other authors. In a future this characteristic could be correlated with a putative biological effect at intestinal mucosa level. Nowadays, it is not possible to reproduce in the laboratory the gut conditions in order to be able to study the EPS production in this environment. Thereby, the use of laboratory media is a valuable technique to undertake the preliminary characterization of polymers from new origin. However, given the influence of the culturing conditions on both MM and chemical composition of EPS, the characterization of EPS produced in the gut environment remains as a challenge for the future.

For dairy industry applications, it is important to know the growth capability and the metabolic activity of these microorganisms in milk. Our study indicates that three EPS-producing lactobacilli, *L. rhamnosus* E41, *L. rhamnosus* E43R, and mainly *L. plantarum* H2, could have good technological properties. They all grew and acidified milk and, additionally, they were able to increase the viscosity and to confer a desirable texture to the fermented product. Most bifidobacteria were able to grow and acidify milk without producing an excessive amount of acetic acid which would not be a desirable sensory characteristic in fermented milks. Therefore, both lactobacilli and bifidobacteria could be used as adjuncts in mixed cultures for the formulation of functional foods if they would present probiotic characteristics.

ACKNOWLEDGEMENTS

This work was financed by FEDER funds (European Union) and the Spanish Plan Nacional de I+D+i through projects AGL2006-03336 and AGL2007-62736. N. Salazar was

funded by pre-doctoral FPI-fellowship from the Spanish Ministry of Education and Science. Isabel Cuesta (IPLA-CSIC) and Ana Hernández-Barranco are acknowledged for their excellent technical assistance in the HPLC and GC-MS analysis. The authors are grateful to Christian-Hanssen ((Hørsholm, Denmark) for kindly supply the strain *S. thermophilus* ST-body-1 for research purposes.

REFERENCES

1. Abbad-Andaloussi, S., H. Talbaoui, R. Marczak, and R. Bonaly. 1995. Isolation and characterization of exocellular polysaccharide produced by *Bifidobacterium longum*. Appl. Microbiol. Biotechnol. 43:995-1000.

2. Baron, M., D. Roy, and J.C. Vuillemard. 2000. Biochemical characteristics of fermented milk produced by mixed-cultures of lactic starters and bifidobacteria. Lait 80:465-478.

3. Boylston, T.D., C.G. Vinderola, H.B. Ghoddusi, and J.A. Reinheimer. 2004. Incorporation of bifidobacteria into cheeses: challenges and rewards. Int. Dairy J. 14:375-387.

4. Cerning, J., C.M.G.C. Renard, J.F. Thibault, C. Bouillanne, M. Landon, M. Desmazeaud, and L. Topisirovic. 1994. Carbon source requirements for exopolysaccharide production by *Lactobacillus casei* CG11 and partial structure analysis of the polymer. Appl. Environ. Microbiol. 60:3914-3919.

5. Degeest, B., and L. De Vuyst. 1999. Indication that the nitrogen source influences both amount and size of exopolysaccharides produced by *Streptococcus thermophilus* LY03 and modelling of the bacterial growth and exopolysaccharide production in a complex medium. Appl. Environ. Microbiol. 65:2863-2870.

6. De Vuyst, L., F. Vanderveken, S. van de Ven, and B. Degeest. 1998. Production by and isolation of exopolysaccharides from *Streptococcus thermophilus* grown in a milk

421 medium and evidence of their growth-associated biosynthesis. J. Appl. Microbiol. 84:
422 1059-1068.
423 **7.** Embely, T.M., N. Faquir, W. Bossart, and M.D. Collins. 1989. *Lactobacillus vaginalis* sp.
424 nov. from the human vagina. Int. J. Syst. Bacteriol. 39:368-370.
425 **8.** Gilliland, S.E., S.S. Reully, G.B. Kim, and H.S. Kim. 2002. Viability during storage of
426 selected probiotic lactobacilli and bifidobacteria in a yogurt-like product. J. Food Sci.
427 67:3091-3095.
428 **9.** Grattepanche, F., S. Miescher-Schwenninger, L. Meile, and C. Lacroix. 2008. Recent
429 developments in cheese cultures with protective and probiotic functionalities. Dairy Sci.
430 Technol. 88:421-444.
431 **10.** Grobben, G.J., W.H.M. van Casteren, H.A. Schols, A. Oosterveld, G. Sala, M.R. Smith, J.
432 Sikkema, and J.A.M. de Bont. 1997. Analysis of the exopolysaccharides produced by
433 *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB2772 grown in continuous culture on
434 glucose or fructose. Appl. Microbiol. Biotechnol. 48:516-521.
435 **11.** Gueimonde, M., S. Delgado, B. Mayo, P. Ruas-Madiedo, A. Margolles, and C.G. de los
436 Reyes-Gavilán. 2004. Viability and diversity of probiotic *Lactobacillus* and
437 *Bifidobacterium* populations included in commercial fermented milks. Food Res. Int.
438 37:839-850.
439 **12.** Hassan, A.N. 2008. *ADSA Foundation Scholar Award*: Possibilities and challenges of
440 exopolysaccharide-producing lactic cultures in dairy foods. J. Dairy Sci. 91:1282-1298.
441 **13.** Hellinga, C., D.J. Somesen, and J.P.J.M. Koenraads. 1989. Viscosity of stirred yoghurt:
442 modern techniques useful in analysing and improving routine measurements. Neth. Milk
443 Dairy J. 40:217-240.
444 **14.** Hosono, A., J. Lee, A. Ametani, M. Natsume, M. Hirayama, T. Adachi, and S.
445 Kaminogawa. 1997. Characterization of a water-soluble polysaccharide fraction with

446 immunopotentiating activity from *Bifidobacterium adolescentis* M101-4. Biosci.
447 Biotechnol. Biochem. 61:312-316.
448 **15.** Kehagias, C., J. Csapó, S. Konteles, E. Kolokitha, S. Koulouris, and Z. Csapó-Kiss. 2008.
449 Support of growth and formation of D-amino acids by *Bifidobacterium longum* in cows´,
450 ewes´, goats´ milk and modified whey powder products. Int. Dairy J. 18:396-402.
451 **16.** Kitazawa, H., T. Harata, J. Uemura, T. Saito, T. Kanebo, and T. Ithoh. 1998. Phosphate
452 group requirement for mitogenic activation of lymphocytes by an extracellular
453 phosphopolysaccharide from *Lactobacillus delbrueckii* ssp. *bulgaricus.* Int. J. Food
454 Microbiol. 40:169-175.
455 **17.** Laws, A.P., and V.M. Marshall. 2001. The relevance of exopolysaccharides to the
456 rheological properties in milk fermented with ropy strains of lactic acid bacteria. Int.
457 Dairy J. 11:709-721.
458 **18.** Leroy, F., and L. De Vuyst. 2004. Lactic acid bacteria as functional starter cultures for the
459 food fermentation industry. Trends Food Sci. Technol. 15:67-78.
460 **19.** Lucey, J.A., and H. Singh. 1998. Formation and physical properties of acid milk gels: a
461 review. Food Res. Int. 30:529-542.
462 **20.** Mozzi, F., F. Vaningelgem, E.M. Hébert, R. van der Meulen, M.R. Foulquié-Moreno, G.
463 Font de Valdez, and L. De Vuyst. 2006. Diversity of heteropolysaccharide-producing
464 lactic acid bacterium strains and their biopolymers. Appl. Environ. Microbiol. 72:4431-
465 4435.
466 **21.** Nagaoka, M., S. Hashimoto, T. Watanabe, T. Yokokura, and Y. Mori. 1994. Anti-ulcer
467 effects of lactic acid bacteria and their cell wall polysaccharides. Biol. Pharm. Bull.
468 17:1012-1017.

22. Nagaoka, M., H.Shibata, I. Kimura, S. Hashimoto, K. Kimura, H. Sawada, and T. Yokokura. 1995. Structural studies on a cell wall polysaccharide from *Bifidobacterium longum* YIT4028. Carbohydr. Res. 274: 245-249.

23. Ostlie, H.M., M.H. Helland, and J.A. Narvhus. 2003. Growth and metabolism of selected strains of probiotic bacteria in milk. Int. J. Food Microbiol. 87:17-27.

24. Petry, S., S. Furlan, M.A. Crepeau, J. Cerning, and M. Desmazeaud. 2000. Factors affecting the exocellular polysaccharide production by *Lactobacillus delbrueckii* subsp. *bulgaricus* grown in a chemically defined medium. Appl. Environ. Microbiol. 66:3427-3431.

25. Petry, S., S. Furlan, E. Waghorne, L. Saulnier, J. Cerning, and E. Maguin. 2003. Comparison of the thickening properties of four *Lactobacillus delbrueckii* subsp. *bulgaricus* strains and physicochemical characterization of their exopolysaccharides. FEMS Microbiol. Lett. 221:285-291.

26. Roberts, C.M., W.F. Fett, S.F. Osman, C. Wijey, J.V. O'Connor, and D.G. Hoover. 1995. Exopolysaccharides production by *Bifidobacterium longum* BB-79. J. Appl. Bacteriol. 78:463-468.

27. Ruas-Madiedo, P., A. Alting, and P. Zoon. 2005. Effect of exopolysaccharides and proteolytic activity of *Lactococcus lactis* subsp. *cremoris* strains on the viscosity and structure of fermented milks. Int. Dairy J. 15:155-164.

28. Ruas-Madiedo, P., J.A. Moreno, N. Salazar, S. Delgado, B. Mayo, A. Margolles, and C.G. de los Reyes-Gavilán. 2007. Screening of exopolysaccharide-producing *Lactobacillus* and *Bifidobacterium* strains isolated from the human intestinal microbiota. Appl. Environ. Microbiol. 73:4385-4388.

29. Ruas-Madiedo, P., N. Salazar, and C.G. de los Reyes-Gavilán. 2009. Biosynthesis and chemical composition of exopolysaccharides produced y lactic acid bacteria. Pages (in

494 press, ISBN 978-1-904455-45-5) in Bacterial polysaccharides: current innovations and
495 future. M. Ullrich, ed. Horizon Scientific Press, Norwich, UK.
496 **30.** Salazar, N., M. Gueimonde, A.M. Hernández-Barranco, P. Ruas-Madiedo, and C.G. de
497 los Reyes-Gavilán. 2008. Exopolysaccharides produced by intestinal *Bifidobacterium*
498 strains act as fermentable substrates for human intestinal bacteria. Appl. Environ.
499 Microbiol. 74:4737-4745.
500 **31.** Sánchez, I., B. Martínez, R. Guillén, R. Jiménez-Díaz, and A. Rodríguez. 2006. Culture
501 conditions determine the balance between two different exopolysaccharides produced by
502 *Lactobacillus pentosus* LPS26. Appl. Environ. Microbiol. 72:7495-7502.
503 **32.** Tone-Shimokawa, Y., T. Toida, and T. Kawashima. 1996. Isolation and structural analysis
504 of polysaccharide containing galactofuranose from the cell walls of *Bifidobacterium*
505 *infantis.* J. Bacteriol. 178:317-320.
506 **33.** Vaningelgem, F., M. Zamfir, F. Mozzi, T. Adriany, M. Vancanneyt, J. Swings, and L. De
507 Vuyst. 2004. Biodiversity of exopolysaccharides produced by *Streptococcus thermophilus*
508 strains is reflected in their production and their molecular functional characteristics. Appl.
509 Environ. Microbiol. 70:900-912.
510 **34.** Wang, M., and J. Bi. 2008. Modification of characteristics of kefiran by changing the
511 carbon source of *Lactobacillus kefiranofaciens.* J. Sci. Food Agric. 88:763-769.

512 **Table 1**: *Bifidobacterium* and *Lactobacillus* strains of human origin and physico-chemical characteristics of their EPS fractions isolated from the
513 cell biomass harvested from the surface of agar-MRSC plates.

Species	Strain	EPS-fractions							
		Molar mass (Da) distribution (%)[1]				Monosaccharide Ratio[2]			Presence[3]
		$> 10^6$	10^5-10^6	10^4-10^5	$< 10^4$	Ram	Gal	Glc	Other
B. animalis	C64MRa	-	-	9	91	1	1.5	2.5	-
	E43	-	-	12.3	87.7	1	2	2.5	Man
B. longum	H73	-	70.7	-	29.3	2	1	2.5	Fuc
	L55	-	45.9	-	54.1	1	1	4	-
	H67	-	52.6	-	47.4	4	1	4	Fuc
	E44	11.1	-	-	88.9		1	1	-
B. pseudocatenulatum	A102	40.6	-	22.7	36.7		1	2	-
	C52	10.4	-	-	89.6		1	1	Ram
	E515	47.1	-	-	52.9		1	2	-
	E63	38.0	-	-	61.9		1	1	-
	H34	-	56.5	-	43.5		1	1.5	Fuc, NAGlc
L. casei	BA61	-	-	55.4	44.6	2	1	4	-
	E51	16.1	-	-	83.9		1	2	-
	F72	-	-	58.9	41.1	2	1	2.4	-
L. rhamnosus	E41	-	76.9	-	23.1	3	1	3	-
	E43R	-	55.9	-	44.1	4	1	2	-
L. plantarum	C64MRb	-	-	18.4	81.6		1	9	-
	E112	-	-	7.5	92.5		1	5	-
	G62	-	-	11.3	88.7		1	5	-
	H2	-	-	32.3	67.7		1	3	Ram
L. vaginalis	C32	-	68.6	-	31.4		1	2	Man, NAGlc

514 [1]Percentage calculated with respect to the total amount (μg) of peaks measured by GPC.
515 [2]Ram: rhamnose, Gal: galactose, Glc: glucose.
516 [3]Content lower than 10% of the total monosaccharides. Man: mannose, Fuc: fucose, NAGlc: N-acetyl-glucosamine.

517 **Table 2**: Volatile compounds produced in milks fermented with EPS-producing
518 *Bifidobacterium* and *Lactobacillus* strains from human origin.

		Mean ± SD (μg /mL)		
Species	**Strain**	Acetaldehyde [1]	Diacetyl	Ethanol
Uncultured milk	-	0.27±0.06	0.19±0.01	2.17±0.04
B. animalis	C64MRa	21.86±1.24	0.99±0.16	47.91±5.81
	E43	ND	2.00±0.58	167.25±46.39
B. longum	H73	6.20±0.83	3.34±0.57	40.50±3.76
	L55	10.04±1.54	1.75±0.20	43.00±3.61
	H67	14.70±0.72	4.35±0.33	51.01±1.28
	E44	7.67±0.93	1.22±0.01	37.43±4.05
B. pseudocatenulatum	A102	16.43±0.28	3.32±1.41	35.80±2.51
	C52	12.35±5.78	2.41±0.21	31.75±0.88
	E515	14.30±0.55	3.72±1.69	30.48±5.7
	E63	ND	3.45±0.18	25.80±5.72
	H34	11.48±5.74	5.96±3.42	30.75±12.15
L. casei	BA61	ND	0.08±0.01	18.37±0.43
	E51	ND	1.35±0.30	35.32±0.70
	F72	ND	0.19±0.12	9.45±1.45
L. rhamnosus	E41	ND	0.32±0.04	13.53±0.87
	E43R	ND	0.25±0.05	12.64±0.46
L. plantarum	C64MRb	ND	0.31±0.10	4.09±1.53
	E112	ND	0.54±0.34	11.59±0.19
	G62	1.62±1.02	0.44±0.18	3.28±0.39
	H2	ND	0.43±0.22	13.79±0.35
L. vaginalis	C32	ND	1.92±1.32	277.09±125.73
S. thermophilus [2]	St-body-1	9.38±0.56	0.75±0.11	4.92±0.79

519 [1]ND: level under detection limit
520 [2]The strain *Streptococcus thermophilus* ST-body-1 was used as EPS-producing strain
521 control.
522

Table 3: EPS production and apparent (Posthumus) viscosity of stirred milks fermented with some EPS-producing *Lactobacillus* strains from human origin.

		Mean ± SD	
Species	**Strain**	**Viscosity** (s)	**EPS-fraction** [1] (mg/100 mL whey)
L. rhamnosus	E41	63.3±3.7	29.0
	E43R	69.0±7.9	51.4±11.4
L. plantarum	H2	72.0±24.0	18.9
S. thermophilus [2]	St-body-1	143.3±20.8	15.5±0.2

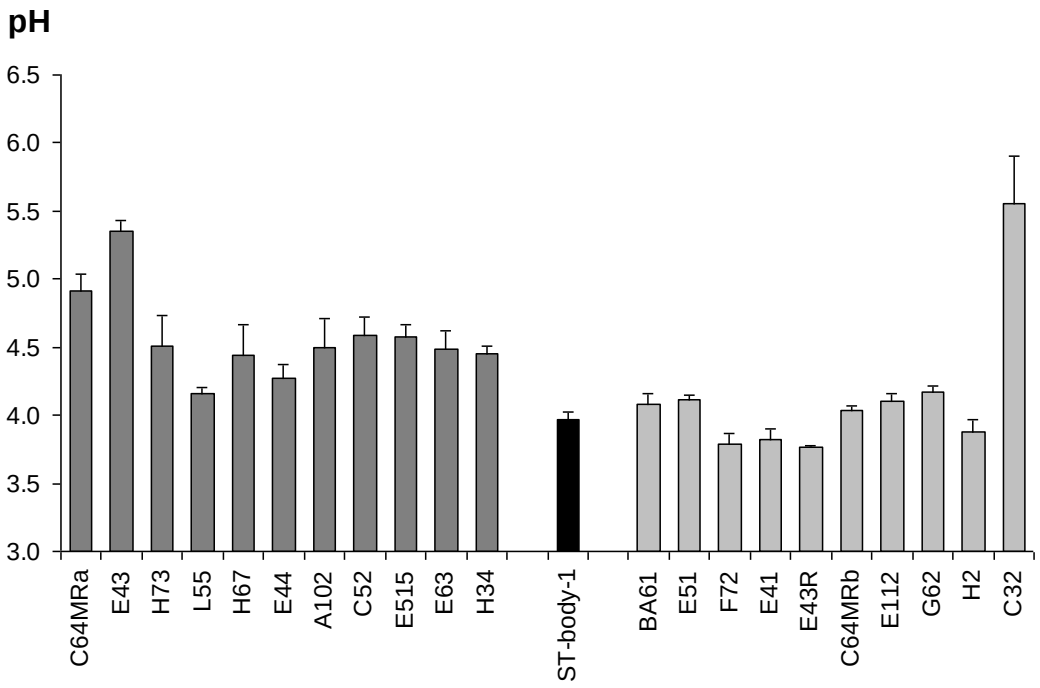
[1]The values were calculated after subtracting the precipitated-fraction isolated from non-fermented milk.

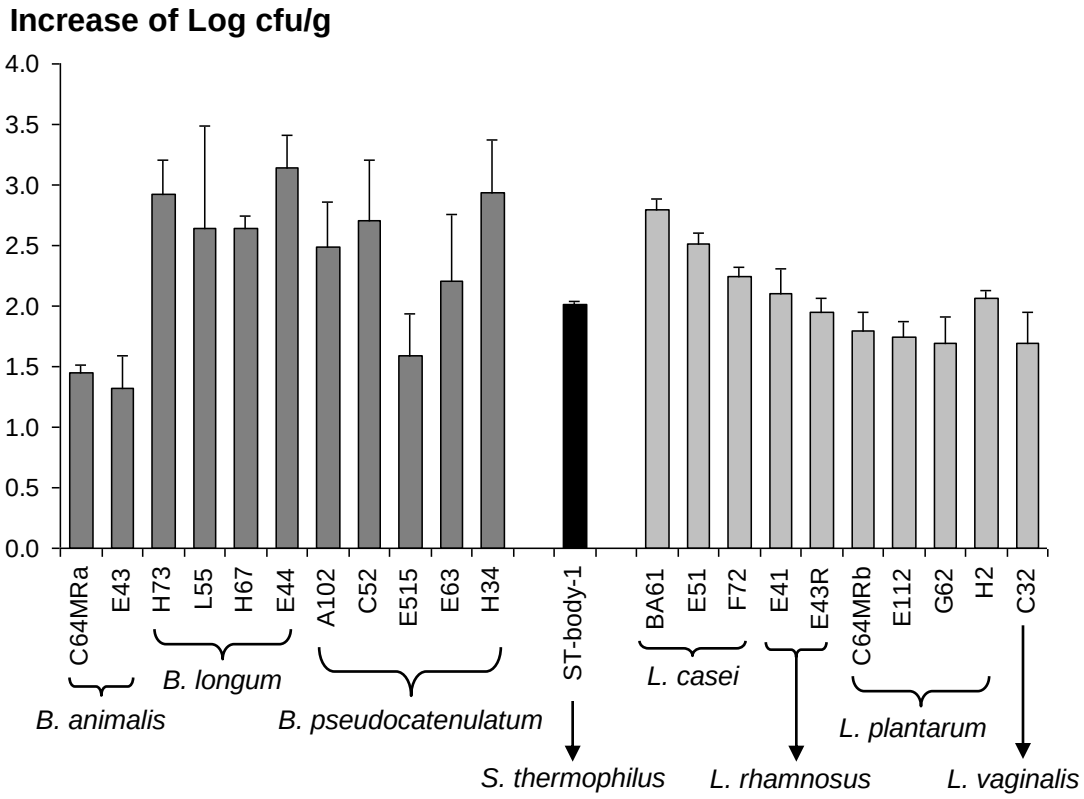
[2] *Streptococcus thermophilus* ST-body-1 was used as EPS-producing strain control with high viscosity intensifying capability.

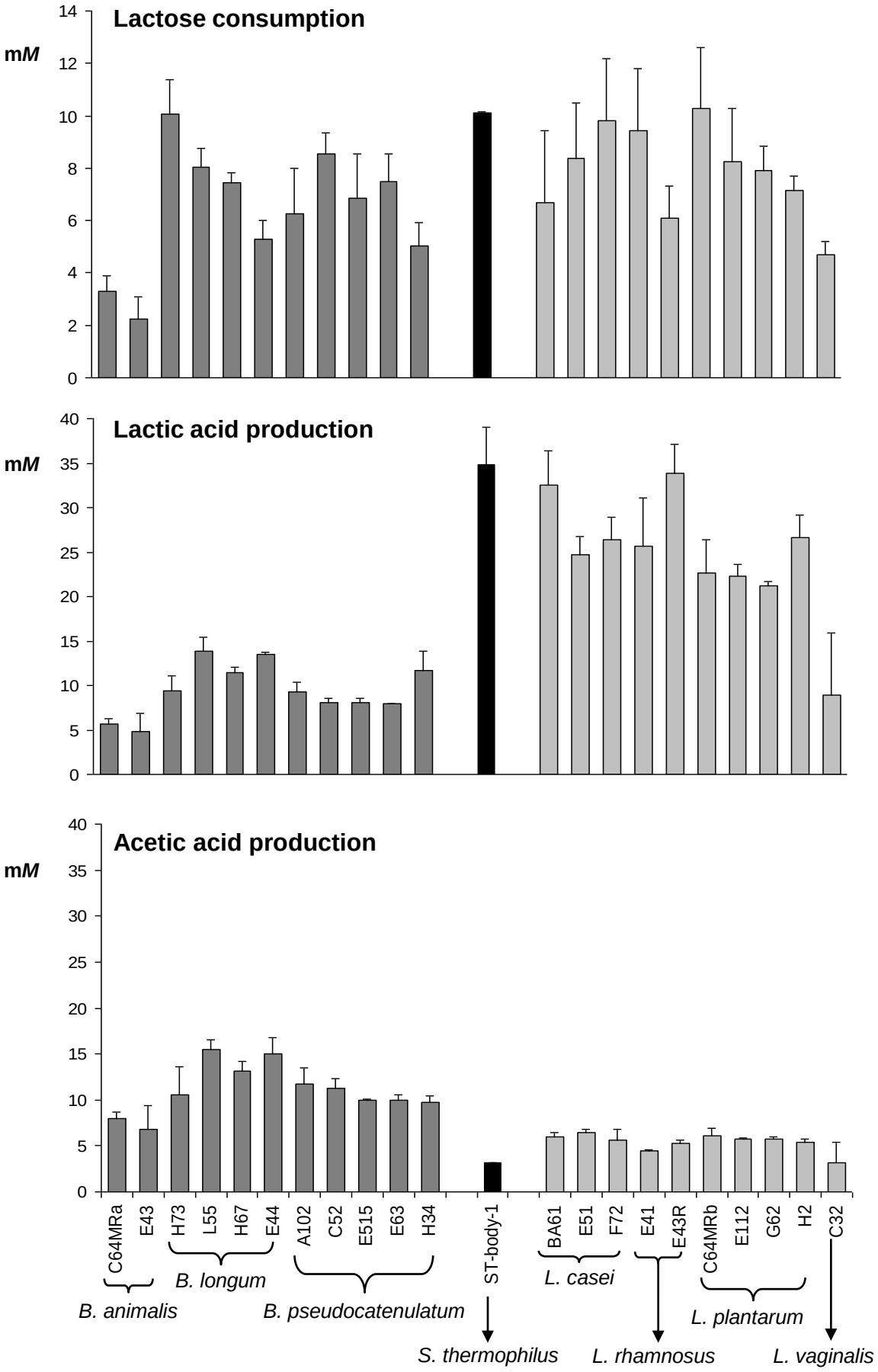
FIGURE LEGENDS

Figure 1. pH values of milks fermented with EPS-producing *Bifidobacterium* and *Lactobacillus* strains from human origin and increase of the Log cfu/g after incubation at 37ºC during 17±1 h. The strain *Streptococcus thermophilus* ST-body-1, employed in yoghurt manufacture, was used as an EPS-producing strain control.

Figure 2. Mean values (m*M*) of lactose consumption and production of lactic acid and acetic acid in milks fermented with EPS-producing *Bifidobacterium* and *Lactobacillus* strains from human origin at 37ºC during 17±1 h. The strain *Streptococcus thermophilus* ST-body-1, employed in yoghurt manufacture, was used as an EPS-producing strain control.


pH
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
C64MRa
E43
H73
L55
H67
E44
A102
C52
E515
E63
H34
ST-body-1
BA61
E51
F72
E41
E43R
C64MRb
E112
G62
H2
C32


Increase of Log cfu/g
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
C64MRa
E43
H73
L55
H67
E44
A102
C52
E515
E63
H34
ST-body-1
BA61
E51
F72
E41
E43R
C64MRb
E112
G62
H2
C32
B. animalis
B. longum
B. pseudocatenulatum
S. thermophilus
L. casei
L. rhamnosus
L. plantarum
L. vaginalis


Lactose consumption
mM
14
12
10
8
6
4
2
0
Lactic acid production
mM
40
35
30
25
20
15
10
5
0
Acetic acid production
mM
40
35
30
25
20
15
10
5
0
C64MRa
E43
H73
L55
H67
E44
A102
C52
E515
E63
H34
ST-body-1
BA61
E51
F72
E41
E43R
C64MRb
E112
G62
H2
C32
B. animalis
B. longum
B. pseudocatenulatum
L. casei
L. plantarum
S. thermophilus
L. rhamnosus
L. vaginalis