1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Effect of Inulin-type Fructans and Galactooligosaccharides on Cultures of *Lactobacillus* Strains Isolated in Algeria from Camel's Milk and Human Colostrum

Running title: *Lactobacillus* Strains Growing with Fructans and Galactooligosaccharides

Authors: Ibtissem Derriche[2], Alicja M. Nogacka[1,3], Nuria Salazar[1,3], Patricia Ruas-Madiedo[1], M. Gueimonde[1,3], Farid Bensalah[2] *, Clara G. de los Reyes-Gavilán[1,3] *

Affiliations:

[1] Department of Microbiology and Biochemistry of Dairy Products, Dairy Research Institute of Asturias, Spanish Research Council (IPLA-CSIC), 33300 Villaviciosa, Asturias, Spain

[2] Department of Biology, Laboratory of Microbial Genetics, Ahmed Ben Bella ORAN 1 University. Es-Senia 31000 Oran / Algeria

[3] Diet, Microbiota and Health Group, Instituto of Health Research of the Principality of Asturias (ISPA)

For Peer Review

*Corresponding authors:

E-mail: bensalahfarid@yahoo.fr

E-mail : greyes_gavilan@ipla.csic.es,

FUNDING

The present work was financed by the following projects: AGL2013-43770-R, and PCIN-2015-233 (Project *EarlyMicroHealth* from the EU Joint Program Initiative HDHL) financed by Spanish Ministry of Economy and Competitiveness (MINECO) and European Union FEDER funds, by project RTI2018-098288-B-I00, funded by Spanish Ministry of Science, Innovation and University, Agencia Estatal de Investigación and FEDER funds (MCIU/AEI/FEDER, EU), and by grant GRUPIN14-043 funded by Plan Regional de Investigación del Principado de Asturias, Spain. I. Derriche benefited during her stay at IPLA-CSIC of a short stay grant financed by the Ministry of Higher Education and Research in Algeria. Alicja M Nogacka was the recipient of a predoctoral contract granted by MINECO and Nuria Salazar is currently awarded by a postdoctoral contract from the Fundación para la Investigación Biosanitaria de Asturias (FINBA, Asturias, Spain).

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

SUMMARY

Bacteria from the genus *Lactobacillus* are responsible for spontaneous food fermentations. Some species, as *Lactobacillus casei* and *Lactobacillus brevis*, have the "Qualified Presumption of Safety" status recognized by the European Food Safety Authority. Several of their strains are used as probiotics in foods, sometimes included in synbiotic combinations together with prebiotics. New microbial strains isolated from different sources represent an opportunity to use them for the production of traditional food products.

The capacity of three selected strains (one isolated from Camel's milk and identified by partial 16S rRNA gene sequencing as *L. brevis*, and two isolated from human colostrum and identified as *L. paracasei/casei* and *L. brevis*, respectively) were assessed *in vitro* for the ability to survive in gastrointestinal conditions (low pH and high bile salts concentrations). We also tested the capacity of growth and the production of organic acids and volatile compounds by HPLC and gas chromatography, respectively, when these bacteria were incubated anaerobically in the presence of inulin, fructooligosaccharides (FOS), or galactooligosaccharides (GOS) as the main carbon sources. The strains were able to survive in simulated gastrointestinal conditions and to grow in inulin, FOS, and GOS. However, they displayed different profiles of organic acids and volatile compounds, mainly depending on the microbial species and the prebiotic used. The influence that the combined use of strains and different prebiotics could exert on the organic acids and volatiles formed in food and in the gut, should be assessed for each synbiotic combination and food product.

For Peer Review

Keywords: *Lactobacillus*, inulin, fructooligosaccharides, galactooligosaccharides, organic acids, volatile compounds

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

INTRODUCTION

Bacteria from the genus *Lactobacillus* are ubiquitous in plants, animals, and humans. Some species are responsible for spontaneous food fermentations and are included as components of food starter cultures. Probiotics are defined as "live microorganisms that when administered in adequate amounts confer a health benefit to the host" (Hill et al., 2014). Due to their long history of safe use, several species of the genus *Lactobacillus* have the "Qualified Presumption of Safety" (QPS) status recognized by the European Food Safety Authority (EFSA, 2017) and some of their strains are currently available as commercial probiotics. In order to exert health effects, once ingested probiotics must reach alive the colon (so, they must be able to survive to the acidic conditions of the stomach, high concentrations of bile salts in the intestine, and digestive enzymes) and persist, at least transitorily, in this environment.

Most lactic acid bacteria (LAB) isolated from healthy humans and animals seem to exhibit nutritional and health benefits. New isolated LAB from different sources, as milk from humans and other livestock mammals, are therefore gaining interest in recent years to be used as both starters and/or probiotics, for food production (Fguiri et al., 2016; Liu et al., 2020).

The last consensus definition of the term Prebiotics indicates that this is "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al., 2017). Inulin, fructooligosaccharides (FOS), and galactooligosaccharides (GOS) are well-known prebiotic carbohydrates, commercially available. Mixtures of probiotics and prebiotics, known as synbiotics, are often used in functional foods to exploit their synergistic action as well as the ability of some prebiotics to improve the texture of food products and to favour the viability of probiotics in foods and in the gut (De Souza et al., 2011; Marion et al., 2015; Pandey et al., 2015). In spite of currently available studies, there is still scarce information on the influence that different types of prebiotics could exert on the bacterial growth and metabolites produced by LAB, and how the presence of these substrates could modify the growth and metabolic activity of these bacteria in foods and at the intestinal level. Therefore, prior to include combinations of new QPS bacteria and prebiotics in food preparations it is necessary to assess *in vitro* the influence that the addition of different prebiotics may exert on the growth and generation of metabolic compounds by the targeted bacteria. It should be also considered that the addition of probiotics and prebiotics to fermented products could potentially modify their organoleptic properties and in last term the consumer's acceptance.

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

In this context, the aim of the present work was to assess the capacity of three bacterial strains isolated in Algeria from camel's milk and human colostrum to overcome *in vitro* gastrointestinal conditions, and to test the growth and capacity of these strains to produce organic acids and volatile compounds in the presence of inulin-type fructans and GOS. The strains used here were previously isolated by spread-plating on MRS-agar with coagulated skimmed milk by incubation with human colostrum as well as with serial dilutions of raw camel's milk. Ten catalase negative, gram positive and aerotolerant lactobacilli were previously isolated from human colostrum and three other from camel's milk. Among them, those displaying better performance for growth in MRSc broth and that were confirmed as *Lactobacillus* species by partial 16S rRNA gene sequencing, were chosen for the present study. These preliminary assays open the possibility to consider these bacteria in further studies for the manufacture of functional fermented foods.

.MATERIAL AND METHODS

Growth conditions and identification of strains

Three microbial strains, previously isolated from human colostrum and camel's milk, were included in this study (Table 1).The strains were routinely grown in MRS broth (Biokar, Beaubois, France) supplemented with 0.25% L-cysteine (Sigma Chemical Co, St. Louis, MO, USA) (MRSc) and incubated in anaerobic conditions under a 10% H_2, 10% CO_2 and 80% N_2 atmosphere in a MACS MG-500 anaerobic chamber (Don Whitley Scientific, West Yorkshire, UK) at 37°C. Overnight cultures were employed to prepare bacterial inocula to be used in the experiments.

The identity of strains was obtained by partial sequence analysis of the 16S rRNA gene. Briefly, the DNA was extracted from 1 mL of pure cultures by using the GenElute™ Bacterial Genomic DNA Kit (Sigma), following the manufacturer's instructions. The 16S rDNA was amplified using the universal primers plb 16 and mlb 16 (Kullen et al., 2000; Rios-Covian et al., 2013). PCRs were performed in a total volume of 25 µl containing 2 µl of genomic DNA as a template. The reaction mixture was composed of 10x buffer (EurX, Gdansk, Poland), dNTP 0.2 mM (Amersham) and using 0.2 µM of each primer. The PCR conditions used were 5 min at 95°C, 30 cycles of 45 s at 94°C, 60 s at 55°C and 35 s at 72°C, followed by 10 min at 72°C. Amplification was carried out in a SimplioAmp Thermal Cycler (Applied Biosystems). Amplicons (approximately 500 bp length) were purified using the GenElute PCR Clean-Up kit (SIMGA, USA) and were sequenced at Macrogen Inc. (Madrid, Spain) in an automated sequencer ABI3730XL

For Peer Review

(Applied Biosystems, Foster City, CA, USA). The sequences obtained were compared to those held in GenBank using BLAST algorithm at NCBI.

Exposure of the strains to simulated conditions of the gastrointestinal tract

The tolerance to simulated gastric and bile juices was preliminarily assessed. Bacterial cultures were exposed to simulated gastric and bile juices and their survival was determined essentially as described by Arboleya *et al*. (2011). Briefly, 5 mL of bacterial cultures grown overnight were harvested, washed twice with phosphate-buffered saline (PBS) and resuspended in 500 µL of the same solution. 100 µL of bacteria suspensions were added to 900 µL of simulated gastric juice (125 mM NaCl, 7 mM KCl, 45 mM $NaHCO_3$, and 3 g/L pepsin [Sigma Chemical Co.], adjusted to pH 2.5 with HCl) or bile juice (45 mM NaCl, 100 U/mL pancreatin extract [Sigma Chemical Co.], and 3 g/L Oxgall [Sigma Chemical Co.], adjusted to pH 8.0 with NaOH). Suspensions were incubated in anaerobiosis for 90 and 180 min with gastric and bile juices, respectively. Bacterial counts at time 0 and after incubation were obtained, and results were expressed as log units (log UFC/mL) of bacterial suspensions after incubation with respect to time 0.

Commercial prebiotics and carbon sources

Three prebiotics were used as carbohydrate substrates for fermentation in cultures of the strains. Short-chain FOS Actilight 950P was purchased from Beghin Meiji (Marckolsheim, France); it had a 95% purity, with polymerization degree (DP) 2-5 residues, and contained 5% residual glucose, fructose and sucrose. Inulin, extracted from Dahlia tubers (DP ~36 units), was purchased from Sigma-Aldrich (Spain). The GOS Bimuno Daily was provided by Clasado Ltd (Shinfield, Reading, UK) with 79.70% purity (w/v). Glucose (Fluka Analytical, Spain) was used as non-prebiotic universal carbon source and PBS solution as a negative control for fermentation. Sterilization of all substrates added to the culture medium was carried out by filtration through a 0.45 pore size membrane, except for inulin that was autoclaved.

Growth of **L. paracasei/casei** *and* **L. brevis** *strains in MRSc with prebiotics as carbon source*

Growth of *L. paracasei/casei* and *L. brevis* strains was evaluated in MRSc formulated without glucose and supplemented with 0.3% (w/v) of each of the carbohydrates tested (Inulin, FOS Actilight, GOS Bimuno, and glucose) as the sole carbon source added. Culture media were inoculated at 1% (v/v) with overnight cultures

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

of the corresponding strains. Cultures were incubated for 24 h in anaerobiosis at 37ºC. Samples were taken at 0, 5 and 24 h and growth was determined by measuring the optical density at 600 nm (OD_{600}) in an Ultrospect 10 Cell Density Meter (Amersham Biosciences). The OD_{600} values obtained for each culture were fitted to a modified-Gompertz equation (1), a sigmoidal function describing kinetics of bacterial growth (Tjørve et al., 2017; Ware et al., 2017):

$$y = A \cdot \text{exponential}\{-\text{exponential}\,[\mu \cdot e / (\lambda - t) + 1]\} \quad \textbf{(1)}$$

where y is the estimated growth (OD_{600}) at a given time (t), “A” represents the upper asymptote, “μ” is the rate of growth (OD_{600}/h), and “λ” is time lag before exponential phase.

Determination of pH in cultures was carried out by direct measurement with a pH meter Basic 20+ (Crison Instruments SA, Barcelona, Spain). Experiments were made in triplicate.

Analysis of organic acids by HPLC

One mL of each culture collected at 0 and 24 h of incubation was centrifuged (16000 x g for 10 min) and filtered through 0.45 μm-pore-size filters to obtain supernatants which were immediately frozen at -20ºC until analysis.

The quantification of organic acids was carried out in duplicate using an HPLC chromatographic system composed of an Alliance 2690 module injector, and a PDA 996 photodiode array detector, and the Empower software (Waters, Milford, MA, USA). Chromatographic conditions were those indicated previously by Salazar *et al.* (Salazar et al., 2009). Briefly, sample separation was carried out in an ICSep ICE-ION-300 ion-exchange column (Transgenomic, San José, CA) using 8.5 mN H_2SO_4 as the mobile phase at 65ºC, with a flow rate of 0.4 mL/min. Detection was carried out at 210 nm. Organic acids were quantified through regression equations calculated by using different concentration of the corresponding standards. Results were expressed as increments in the concentration of each compound (mM) after 24 h of incubation with respect to time 0.

Analysis of volatile compounds

Volatile compounds produced by *Lactobacillus* strains during incubation were determined by means of headspace (HS) Gas Chromatography (GC). The culture

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

medium with each of the different carbon sources and strains, prepared and inoculated as indicated just above, was placed into separate glass tubes sealed with rubber and metallic caps and incubated in the same conditions as indicated before. After 24 h of incubation, samples in glass tubes were stored at -20ºC until analyses, which were carried out in a 6890N Agilent gas chromatograph coupled with a G1888 series HS automatic injector and with a 5975B inert mass spectrometry (MS) detector (Agilent Technologies Inc., Palo Alto, CA). Data were collected and analysed with ChemStation software (Agilent). Sample preparation and chromatographic separation were performed as described previously (2011). Volatile compounds were identified by comparing their mass spectra with those held in the Wiley 138 library (Agilent) and by comparing their retention times.

The relative chromatographic peak area of each compound at 24 h of incubation was calculated by subtracting to the peak area at this time, the corresponding area for the same compound in the negative control tube. Acetic acid, already quantified by HPLC, was not considered for further calculations. From the remaining volatiles, those displaying relative peak areas above 5% of the sum of relative peak areas of all volatile compounds in at least two samples were considered as the major volatiles produced. The relative abundance of each of these volatiles was then calculated by referring the relative peak area of each of them, to the sum of relative peak areas of all major volatile compounds considered, taking this sum as the 100%.

For Peer Review

Statistical analyses

Statistical analysis of results was performed using the SPSS v.24 software (SPSS Inc., Chicago, USA). Fitting to normal distribution of organic acids data was analysed by means of a Shapiro-Wilk test. The increases of acetic, lactic and formic acid after 24 h of incubation with respect to time 0 in the different cultures were compared by ANOVA and post-hoc Bonferroni's test considering either the strain or the carbohydrate substrate as factors.

RESULTS AND DISCUSSION

Milk and colostrum from mammals, animals and especially humans, constitute a source of LAB that could encounter application in the manufacture of dairy products and functional foods. Microorganisms isolated in the neonatal period (both in animals and humans) could display differential properties related with health-promotion and

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

maturation of the immune system in the offspring, that are not sufficiently explored yet (Arboleya et al., 2011; Jost et al., 2014; Nogacka et al., 2019).

One of the strains was identified as *L. paracasei/casei* (named as *L. paracasei/casei* 6 and assigned the internal culture collection code IPLA 40001) and the other two as *L. brevis* (*L. brevis* 15 [*L. brevis* IPLA 40002]; *L. brevis* 23 [*L. brevis* IPLA 400003]) (Table 1). A 100% identity was obtained by BLAST comparison of their sequences with those held in GenBank database. Members of species *L. paracasei/casei* and *L. brevis* have been previously reported as to be present in human milk and colostrum, and camel's milk (Jimenez et al., 2009; Ozgun et al., 2011; Fguiri et al., 2016; Togo et al., 2019), among other sources. Members of both species have the QPS status recognized by EFSA (2017) and some strains are being used as starters and/or probiotics in fermented dairy products (Fernandez et al., 2015) and other foods. This supports the interest of carrying out a preliminary characterization of our strains in order to assess their suitability to be used in fermented dairy products and in synbiotic combinations.

Table 1

Effect of the simulated conditions of the gastrointestinal tract on* L. paracasei/casei *and* L. brevis *strains

Survival to digestion is an important property so that microorganisms ingested with food could exert health-promoting effects. Therefore, *in vitro* survival in simulated gastric and bile juices were independently assessed as indicators of the survival potential to the gastrointestinal transit of these strains.

The studied strains displayed optimal survival when incubated in simulated gastric and bile juices. Count decreases of the three strains in gastric juice were comprised between 0.28 and 0.83 log units (Figure 1). However, after exposure to simulated bile juice, *L. paracasei/casei* 6 showed a more pronounced decrease (4.71 log units) than *L. brevis* 15 and *L. brevis* 23 (count decreases of 1.8 and 0.02 log units, respectively). These results indicate that after ingestion, the three strains could have the capacity to reach the colon alive. Relating to this, it has been previously reported that some *L. paracasei/casei* and *L. brevis* strains can survive in *in vitro* and *in vivo* gastrointestinal conditions and the oral administration of some particular strains produced beneficial effects in humans (Nishida et al., 2008; Suzuki et al., 2013).

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure 1

Growth of* L. paracasei/casei *and* L. brevis *strains in inulin-type fructans and GOS

Synbiotics combining probiotics and prebiotics could exert synergic benefits for human health. However, prior to design specific synbiotic combinations it is advisable to assess how the presence of different prebiotics could affect microbial viability and growth of probiotics.

In the present work *L. paracasei/casei* and *L. brevis* strains were grown in anaerobiosis in order to mimic environmental conditions in the gut, as well as the oxygen deficient environment occurring in the manufacture of some fermented foods. As shown in Table 1, cultures of the three strains increased their OD_{600}, although at a variable extent, when incubated in MRSc plus glucose, FOS, GOS or inulin, corroborating their ability to grow in the prebiotics tested and suggesting the potential of these substrates to be fermented by our strains. Cultures of *L. paracasei/casei* experienced a higher increase of their OD_{600} at 24 h of incubation than those of *L. brevis* in all substrates assayed. This could be partly attributed to the fact that the absence of oxygen affects microorganisms differently. In this regard, it has been reported that while the growth rate and biomass production by *L. casei* is not significantly influenced by the presence/absence of oxygen (Ricciardi et al., 2019), anaerobic conditions could impair the growth of *L. brevis* (Vazquez et al., 2005). For this last species, the OD_{600} of the strain *L. brevis* 23 in glucose was considerably lower than that of *L. brevis* 15, indicating metabolic differences between these two strains. In accordance with growth results, the decrease of pH values after 24 h of incubation was more pronounced in cultures of *L. paracasei/casei* than in cultures of *L. brevis* strains with any of the carbohydrates tested (glucose, inulin-type fructans, or GOS), evidencing a higher acidifying capacity of *L. paracasei/casei*. Comparing substrates for each bacteria, *L. paracasei/casei* cultures showed a tendency towards lower pH in MRSc medium with glucose, inulin or FOS than with GOS. In contrast, the pH of *L. brevis* 23 cultures was lower when incubated in glucose than in GOS, FOS or inulin and was lower as well than the values obtained for *L. brevis* 15 grown in the presence of any carbohydrate. These results are in good agreement with the substrate preference for growth of each bacteria.

Modelling growth kinetics of bacteria through the Gompertz's function (Fig. 2; Table 2) evidenced important differences among them as depending on the phase of growth and carbohydrate substrate used. In the first hours of incubation, the model indicates a faster growth of *L. brevis* in inulin, FOS and GOS than in glucose whereas

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

the growth of *L. paracasei/casei* seems to be more efficient in glucose than in prebiotics (inulin, FOS and GOS). In contrast, at the stationary phase the model predicts the highest OD_{600} for cultures of *L. paracasei/casei* in inulin-type fructans (inulin and FOS) whereas in cultures of *L. brevis,* glucose would give rise to higher OD_{600} than any prebiotic. Predicting behaviour of these strains in different prebiotics could have application when choosing the most suitable strains and prebiotic substrates to be included in foods, as depending on the manufacture conditions, the specific intestinal site of action and potential health application.

Fig 2

Table 2

Organic acids profile of* L. paracasei/casei *and* L. brevis *strains growing in inulin-type fructans and GOS

For Peer Review

Lactic acid was the major compound synthesized by the three strains. In spite of this, the ability to produce each organic acid and the relative proportions among them in cultures greatly varied as depending on the bacterial species and the carbohydrate (Fig. 3). At this point, it is important to take into account that *L. brevis*, a strict heterofermentative LAB, is able to produce lactic, acetic and formic acids, as well as ethanol and carbon dioxide in all conditions. However, in the species *L. paracasei/casei*, a facultative heterofermentative LAB, the switch from homolactic (lactic acid as the major end product of hexose fermentation) to heterolactic fermentation (production of ethanol and acetic and formic acids in addition to lactic acid) is favoured by the scarcity of easily fermentable carbon sources in conditions of restricted access to oxygen, as is the case of the experimental design in the present work.

Glucose was the substrate that gave rise to more lactic acid production by all the three strains. Comparing bacteria, *L. paracasei/casei* 6 synthesized significantly more lactic acid than strains from the species *L. brevis* in all substrates assayed, excepting GOS. In cultures with this prebiotic *L. paracasei/casei* 6 and *L. brevis* 23 produced similar levels of lactic acid after 24 h of incubation (Fig. 3a). Regarding comparison among prebiotics for each strain, *L. paracasei/casei* produced much more lactic acid in inulin and FOS than in GOS ($p < 0.05$) whereas the contrary was true for both *L. brevis* strains that produced more lactic acid in GOS than in inulin-type fructans (inulin and FOS) ($p < 0.05$).

Fig 3

Conversely to that occurring with lactic acid, the production of acetic acid was significantly higher in prebiotics than in glucose, for all the three strains ($p < 0.05$). Moreover, both *L. brevis* strains produced more acetic acid than *L. paracasei/casei* in the presence of inulin-type fructans, although differences did not reach statistical significance for the comparison among strains in FOS (Fig. 3c). A similar pattern to acetic acid, although with lower final concentrations, was obtained for formic acid, that was produced at higher levels in cultures with prebiotics than with glucose ($p<0.05$), growth in FOS and GOS leading to significantly higher production by *L. brevis* strains than by *L. paracasei/casei* (Fig. 3b).

As a consequence of the aforementioned variations, acetic to lactic acid molar ratios were significantly higher in cultures of both *L. brevis* strains than in cultures of *L. paracasei/casei* (Fig. 3d). In fermentations carried out by *L. paracasei/casei,* the higher acetic to lactic acids ratio was obtained with GOS, followed by inulin/FOS whereas, in contrast, for *L. brevis* strains the highest ratios were obtained with inulin/FOS, followed by GOS. For all the three strains, the lowest acetic to lactic acids ratios corresponded to cultures with glucose as the carbon source.

Together, these results evidenced clearly different production patterns of organic acids as depending on the species and the substrate used for fermentations. As a whole, differences in the production of acetic and formic acids were mainly influenced by the substrate, being higher in prebiotics than in glucose, whereas variations in the concentrations of lactic acid were related with both the substrate and the bacterial species. It is worth considering that organic acids could notably influence the quality of fermented foods, in a different way as depending on the product and the perception of quality by the consumer (Choi et al., 2019). Thus, for example, the better quality of sourdough bread, determined by acidity and rising, was provided by a *L. paracasei* strain (Mantzourani et al., 2019) and correlated with lower spoilage. Relating dairy products, high acetic acid concentration could be considered a negative property in yogurt (Adhikari et al., 2000) but it could be found as a desirable aroma compound in kefir and some types of cheeses (Ranadheera et al., 2019). Therefore, in the selection of the different combinations of lactic acid bacteria strains and prebiotics the optimal organoleptic properties intended for each specific product should be taken into account.

Volatile compounds produced by* L. paracasei/casei *and L. brevis grown in inulin-type fructans and GOS

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Volatile compounds whose chromatographic peak relative areas after 24 h of incubation, excluding acetic acid (which was only considered for quantification by HPLC in the present work), were above 5% of the sum of relative peak areas of all volatile compounds in at least two samples, were considered as the major volatiles formed by *L. paracasei/casei* and *L. brevis* strains. This included ethanol, the aroma compounds acetaldehyde, 2-propanol, 1-butanol, and 1-nonanone, and the sulphurous compound carbon disulphide. The relative area of each of these six compounds was then recalculated and referred to the sum of the relative area of the six of them, considering this as the 100% (Fig. 4).

Fig 4

Proceeding in this way, we found that ethanol constituted more than 60% of volatile compounds formed during incubation of the three strains in all conditions tested, the other compounds representing each of them less than 17%. 1-butanol (relative abundance 3-17.5% in samples) was produced by the three strains in all conditions. Acetaldehyde (relative abundance 12-16% in samples) was only produced by *L. paracasei/casei* in inulin-type fructans (inulin and FOS) but not in glucose or GOS. However, 2-propanol (relative abundance 6-11%) was produced by *L. brevis* in all conditions tested, but not by *L. paracasei/casei*. 2-nonanone was found at low relative abundance (1.5-5.3%) in cultures of *L. brevis* in the four substrates assayed, with the exception of *L. brevis* 23 grown in glucose where this compound was not detected. Ethanol, acetaldehyde, 2-propanol, 1-butanol and 2-nonanone are considered in the literature as important key-flavour compounds contributing to aroma in different fermented dairy foods (Gaspardo et al., 2009; Ranadheera et al., 2019).

For Peer Review

Some volatile sulphur compounds, as carbon disulphide (a compound providing a "cooked" flavour) are basic components of food flavour at low concentrations but they can also cause undesirable organoleptic properties, as depending on the concentration and the type of product (Vazquez-Landaverde et al., 2006). Thus, sulphur compounds are undesirable in UHT milk and yogurt but are considered as desirable attributes in some particular types of cheeses (Al-Attabi et al., 2009). Carbon disulphide was produced by *L. brevis* in all substrates whereas *L. paracasei/casei* produced it only in glucose, which should be taken into account in the manufacture of sweetened fermented dairy products or other fermented foods.

Results obtained in this study reveal important differences between the species *L. paracasei/casei* and *L. brevis* on the profile of volatiles formed, with the presence of

prebiotics being also an important factor influencing the volatile compounds synthesized by each bacteria during fermentation.

CONCLUSIONS

The strains *L. paracasei/casei* 6, *L. brevis* 23 and *L. brevis* 15, the two first isolated from human colostrum and the third one from camel's milk, showed capacity to survive to the simulated *in vitro* conditions of the human gastrointestinal tract. All of them were able to grow in the prebiotics inulin, FOS, and GOS. During fermentation, different profiles of organic acids and volatile compounds were formed, mainly depending on the prebiotic used as carbon source and the microbial species considered. Therefore, the three strains are good candidates to evaluate their use combined with different prebiotics in the manufacture of fermented dairy products or other fermented foods. The influence that the combined use of these strains and prebiotics could exert on the organoleptic properties should be assessed for each specific product.

For Peer Review

CONFLICT OF INTERESTS

Authors disclose any conflict of interest

DATA AVAILABILITY

Data used to support the findings of this study are included within the article. Raw data are available from the corresponding authors upon reasonable request.

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

REFERENCES

Adhikari K, Mustapha A, Grun IU and Fernando L. (2000). Viability of microencapsulated bifidobacteria in set yogurt during refrigerated storage. *Journal of Dairy Science* 83: 1946-1951.

Al-Attabi Z, D'Arcy BR and Deeth HC. Volatile sulphur compounds in UHT milk. (2009). *Critical Reviews in Food Science and Nutrition* 49: 28-47.

Arboleya S, Ruas-Madiedo P, Margolles A, Solís G, Salminen S, de los Reyes-Gavilán CG, et al. (2011). Characterization and *in vitro* properties of potentially probiotic *Bifidobacterium* strains isolated from breast-milk. *International Journal of Food Microbiology* 149: 28-36.

Choi YJ, Yong S, Lee MJ, Park SJ, Yun YR, Park SH, et al. (2019). Changes in volatile and non-volatile compounds of model kimchi through fermentation by lactic acid bacteria. *LWT-Food Science and Technology* 105: 118-126.

EFSA Scientific Topic. Qualified presumption of safety (QPS). (2017). Available from: https://www.efsa.europa.eu/en/topics/topic/qps.Htm

Fernandez M, Hudson JA, Korpela R and de los Reyes-Gavilan CG. (2015). Impact on human health of microorganisms present in fermented dairy products: an overview. *Biomed Research :* Article ID 412714.

Fguiri I, Ziadi M, Atigui M, Ayeb N, Arroum S, Assadi M, et al. (2016). Isolation and characterisation of lactic acid bacteria strains from raw camel milk for potential use in the production of fermented Tunisian dairy products. *International Journal of Dairy Technology* 69: 103-113.

Gaspardo B, Procida G, Volaric S, Sgorlon S and Stefanon B. (2009). Determination of volatile fractions in raw milk and ripened cheese by means of GC-MS. Results of a survey performed in the marginal area between Italy and Slovenia. *Italian Journal of Animal Science* 8: 377-390.

Gibson G, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salminen SJ, et al. (2017). The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nature Reviews Gastroenterology & Hepatology* 14: 491-502.

Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Morelli L, et al. (2014). Expert consensus document: the International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate of the term probiotic. *Nature Reviews Gastroenterology & Hepatology* 11: 506-514.

Jimenez E, Delgado S, Fernandez L, Garcia N, Albújar M, Gómez A, et al. (2009). Assessment of the bacterial diversity of human colostrum and screening of staphylococcal and

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

enterococcal populations for potential virulence factors. *Research in Microbiology* 159: 595-601.

Jost T, Lacroix C, Braegger CP, Rochart F and Chassard C. (2014). Vertical mother-neonate transfer of maternal gut bacteria via breastfeeding. *Environmental Microbiology* 16: 2891-2904.

Kullen MJ, Sanozky-Dawes RB, Crowell and Klaenhammer TR. (2000). Use of the DNA sequence of variable regions of the 16S rRNA gene for rapid and accurate identification of bacteria in the Lactobacillus acidophilus complex. *Journal of Applied Microbiology* 89 : 511-516.

Liu WJ, Chen MX, Duo LN, Wang JC, Guo S, Sun HT, et al. (2020). Characterization and potentially probiotic lactic acid bacteria and bifidobacteria isolated from human colostrum. *Journal of Dairy Science* 103 4013-4025.

Mantzourani I, Plessas S, Odatzidou M, Alexopoulos A, Galanis A, Bezirtzoglou E, et al. (2019). Effect of a novel *Lactobacillus paracasei* starter on sourdough bread quality. *Food Chemistry* 271: 259-265.

Marion P, Costa B, Frasao S, Freitas MQ, Franco RM and Conte-Junior CA. (2015). Cupuassu (*Theobroma grandiflorum*) pulp, probiotic, and prebiotic: influence on color, apparent viscosity, and texture of goat milk yogurts. *Journal of Dairy Science* 98: 5995-6003.

Nishida S, Michinaka A, Nakashima K, Iino H and Fuji T. (2008). Evaluation of the probiotic potential of *Lactobacillus paracasei* KW3110 based on *in vitro* tests and oral administration tests in healthy adults. *Journal of General and Applied Microbiology* 54: 267-276.

Nogacka A, Oddi S, Salazar N, Reinheimer JA, Gueimonde M, Vinderola G, et al. (2019). Intestinal immunomodulation and shifts on the gut microbiota of BALB/c mice promoted by two *Bifidobacterium* and *Lactobacillus* strains isolated from human samples. *Biomed Research International*: article ID 2323540

Ozgun D and Vural HC. (2011). Identification of *Lactobacillus* strains isolated from faecal specimens of babies and human milk colostrum by API 50 CHL system. *Journal of Medical Genetics and Genomics* 3: 46-49. Article 00009778426.

Pandey KR, Naik SR and Vakil BV. (2015). Probiotics, prebiotics and synbiotics- a review. Journal of Food Science and Technology 52: 7577–7587.

De Souza Oliveira RP, Perego P, Nogueira de Oliveira M, and A. Converti. (2011). Effect of inulin as prebiotic and synbiotic interactions between probiotics to improve fermented milk firmness. *Journal of Food Engineering* 107 : 36–40.

Ranadheera CS, Evans CA, Baines SK, Balthazar C, Cruz AG, Esmerino EA, et al. (2019). Probiotics in goat milk products: delivery capacity and ability to improve sensory attributes. *Comprehensive Reviews in Food Science and Food Safety* 18: 867-882.

For Peer Review

Ricciardi A, Zotta T, Ianniello RG, Boscaino F, Matera A and Parente E. (2019). Effect of respiratory growth on the metabolite production and stress robustness of *Lactobacillus casei* N87 cultivated in cheese whey permeate medium. *Frontiers in Microbiology* 10: article 851.

Rios-Covián D, Arboleya S, Hernandez-Barranco, Alvarez-Buylla JR, Ruas-Madiedo P, Gueimonde M, et al. (2013). Interactions between *Bifidobacterium* and *Bacteroides* species in cofermentations are affected by carbon sources, including exopolysaccharides produced by bifidobacteria. *Applied and Environmental Microbiology* 79 : 7518-7524.

Salazar N, Prieto A, Leal JA, Mayo B, Bada-Gancedo JC, de los Reyes-Gavilán CG, et al. (2009). Production of exopolysaccharides by *Lactobacillus* and *Bifidobacterium* strains of human origin, and metabolic activity of the producing bacteria in milk. *Journal of Dairy Science* 92: 4158-4168.

Suzuki N, Michiaki M, Tomoji O, Moroshita S, Koide M, Bae MJ, et al. (2013). Effects of enteric-coated lactoferrin tablets containing *Lactobacillus brevis* subsp. coagulans on fecal properties, defecation frequency and intestinal microbiota of Japanese women with a tendency for constipation: a randomized placebo-controlled crossover study. *Bioscience of Microbiota, Food and Health* 32: 13-21.

Tjørve K andTjørve E. (2017). The use of Gompertz models in growth analyses, and new Gompertz-Model approach: an addition to the unified-Richards family. *PLoS One* 12 (6): e0178691.

Togo A, Dufour JC, Lagier JC, Dubourg G, Raoult D and Million M. (2019). Repertoire of human breast and milk microbiota: a systematic review. *Future Microbiology* 2019; 14: 623-641.

Vazquez JA, Miron J, Gonzalez MP and Murado MA. (2005). Effects of aeration on growth and on production of bacteriocins and other metabolites in cultures of eight strains of lactic acid bacteria. *Applied Biochemistry and Biotechnology* 127: 111-124.

Vazquez-Landaverde PA, Torres JA and Qian MC. (2006). Quantification of trace volatile sulfur compounds in milk by solid-phase microextraction and gas chromatography-pulsed flame photometric detection. *Journal of Dairy Science* 89: 2919-2927.

Ware A and Power N. (2017). Modelling methane production kinetics of complex poultry slaughterhouse wastes using sigmoidal growth functions. *Renewable Energy* 104: 50-59.

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Table 1. Origin, identification, growth, and decrease of pH values after incubation of the strains in the presence of glucose and prebiotic substrates (inulin, FOS and GOS).

Strain (code)	Origin	Species identification by partial 16S rRNA gene sequencing	Carbohydrate source	Growth (Increase OD_{600})	pH change
6	Human colostrum	*L. paracasei/casei*	Glucose	1.74 ± 0.14	-1.07 ± 0.00
15	Camel's milk	*L. brevis*		1.40 ± 0.10	-1.08 ± 0.01
23	Human colostrum	*L. brevis*		0.63 ± 0.13	-0.21 ± 0.01
6	Human colostrum	*L. paracasei/casei*	Inulin (Dahlia extract)	2.62 ± 0.06	-1.15 ± 0.01
15	Camel's milk	*L. brevis*		0.33 ± 0.03	-0.30 ± 0.00
23	Human colostrum	*L. brevis*		0.23 ± 0.01	0.01 ± 0.00
6	Human colostrum	*L. paracasei/casei*	FOS (Actilight)	2.44 ± 0.33	-1.13 ± 0.01
15	Camel's milk	*L. brevis*		0.16 ± 0.03	-0.29 ± 0.02
23	Human colostrum	*L. brevis*		0.21 ± 0.13	0.02 ± 0.00
6	Human colostrum	*L. paracasei/casei*	GOS (Bimuno)	1.77 ± 0.02	-0.68 ± 0.00
15	Camel's milk	*L. brevis*		0.25 ± 0.13	-0.31 ± 0.01
23	Human colostrum	*L. brevis*		0.49 ± 0.01	-0.14 ± 0.00

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Table 2. Kinetic parameters of *L. paracasei/casei* and *L. brevis* strains grown with glucose, inulin, FOS or GOS, determined by the Gompertz-modified model of growth. R^2, determination coefficient

Strain	carbohydrate substrate	A (OD_{600nm})	μ (OD/h)	λ (h)	R^2
L. paracasei/casei 6	Glucose	1.782	0.225	0.881	0.993
L. brevis 15		1.428	0.130	2.426	0.999
L. brevis 23		0.681	0.079	-0.128	0.984
L. paracasei/casei 6	Inulin	3.534	0.148	4.148	1.000
L. brevis 15		0.340	0.102	0.320	0.980
L. brevis 23		0.340	0.576	-0.076	0.993
L. paracasei/casei 6	FOS	2.624	0.172	2.259	0.620
L. brevis 15		0.278	0.518	0.040	0.797
L. brevis 23		0.332	0.457	-0.097	0.809
L. paracasei/casei 6	GOS	1.922	0.123	1.851	0.856
L. brevis 15		0.280	0.552	0.038	0.702
L. brevis 23		0.540	0.102	-0.259	0.975

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

FIGURE CAPTIONS

Fig 1. Survival of the strains after incubation during 90 min in simulated gastric juice at pH 2.5, and during 180 min in simulated bile juice added with 3 g/L Ox Gall pH 8.0 (log units: log UFC/mL).

Fig 2. Growth curves of *L. paracasei/casei* 6, *L. brevis* 15 and *L. brevis* 23 obtained by fitting OD_{600nm} values at different times of incubation in MRSc supplemented with glucose, inulin, FOS or GOS as carbon sources, using the Gompertz's equation.

Fig 3. Increments after 24 h of incubation with respect to time 0 of concentrations (mM) of lactic acid (a), formic acid (b) and acetic acid (c) and the ratio of acetic to lactic acids (d) in cultures of *L. paracasei/ casei* 6, *L. brevis* 15 and *L. brevis* 23 strains growing in MRSc with glucose, inulin, FOS or GOS as the sole carbon sources added. Vertical lines in the bars represent standard deviation. For the same substrate, strains do not sharing the same letter produced significantly different amounts of the corresponding organic acid, $p < 0.05$.

Fig 4. Relative abundance of major volatile compounds (excluding acetic acid) detected by HS/GC//MS after 24 h of incubation of *L. paracasei/casei* 6, *L. brevis* 15 and *L. brevis* 23 growing in MRSC with glucose, inulin, FOS or GOS as the sole carbon sources added.

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41

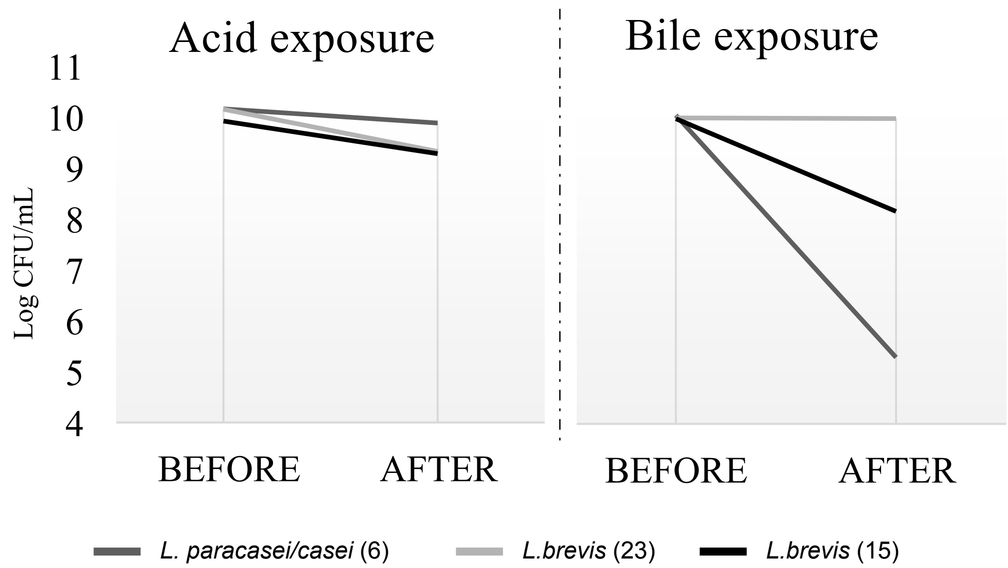


Fig 1. Survival of the strains after incubation during 90 min in simulated gastric juice at pH 2.5, and during 180 min in simulated bile juice added with 3 g/L Ox Gall pH 8.0 (log units: log UFC/mL).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41

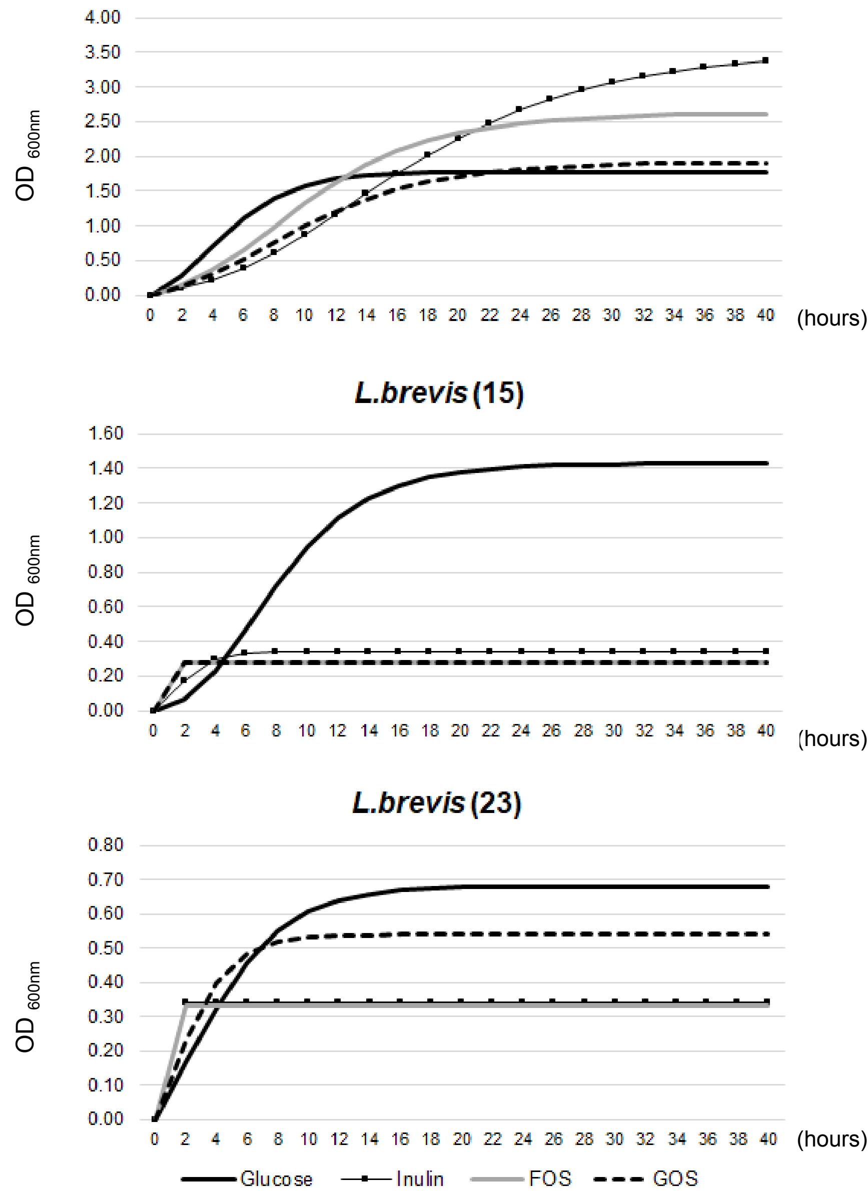


Fig 2. Growth curves of *L. paracasei/casei* 6, *L. brevis* 15 and *L. brevis* 23 obtained by fitting OD_{600nm} values at different times of

incubation in MRSc supplemented with glucose, inulin, FOS or GOS as carbon sources, using the Gompertz's model.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41

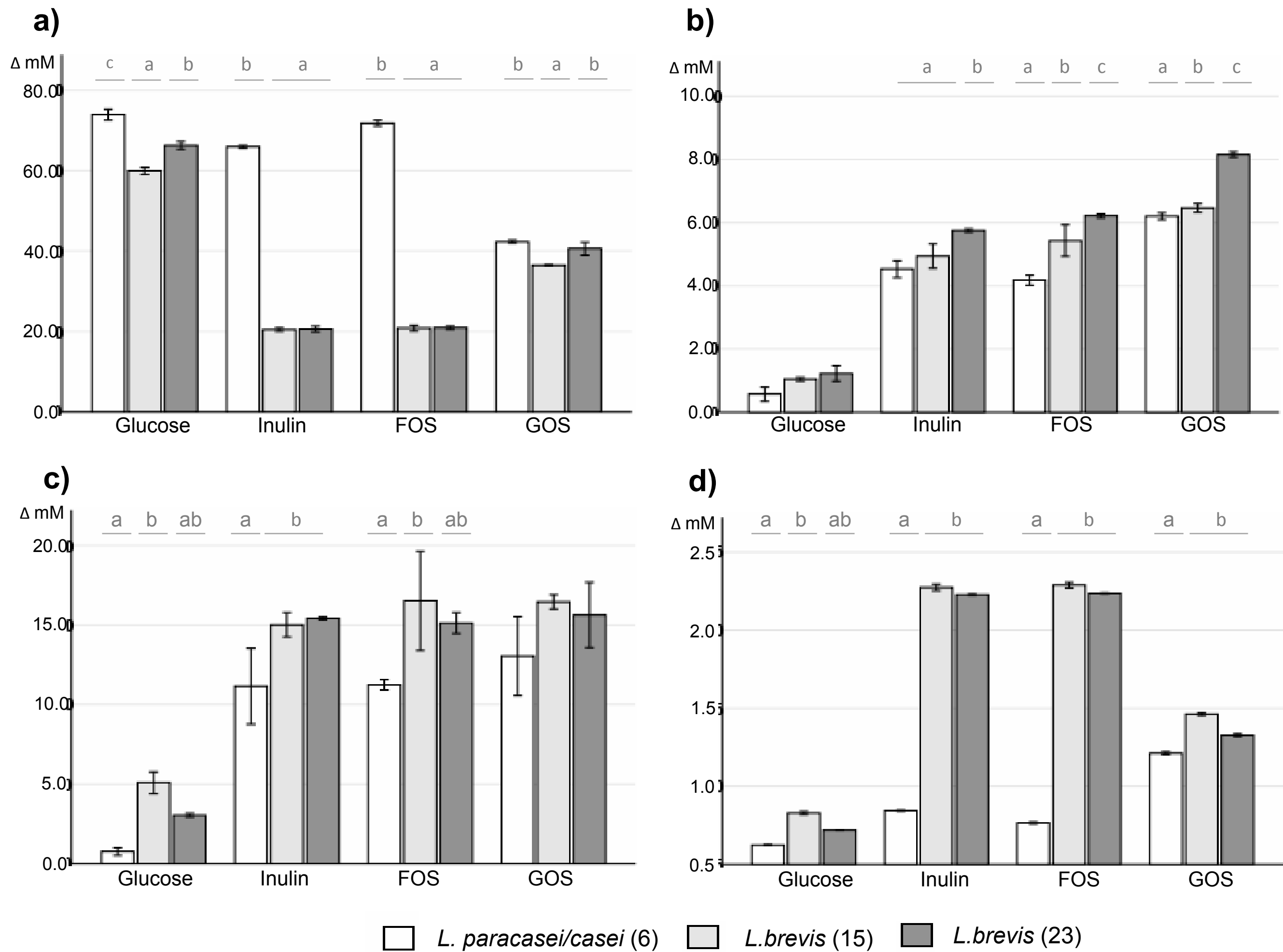
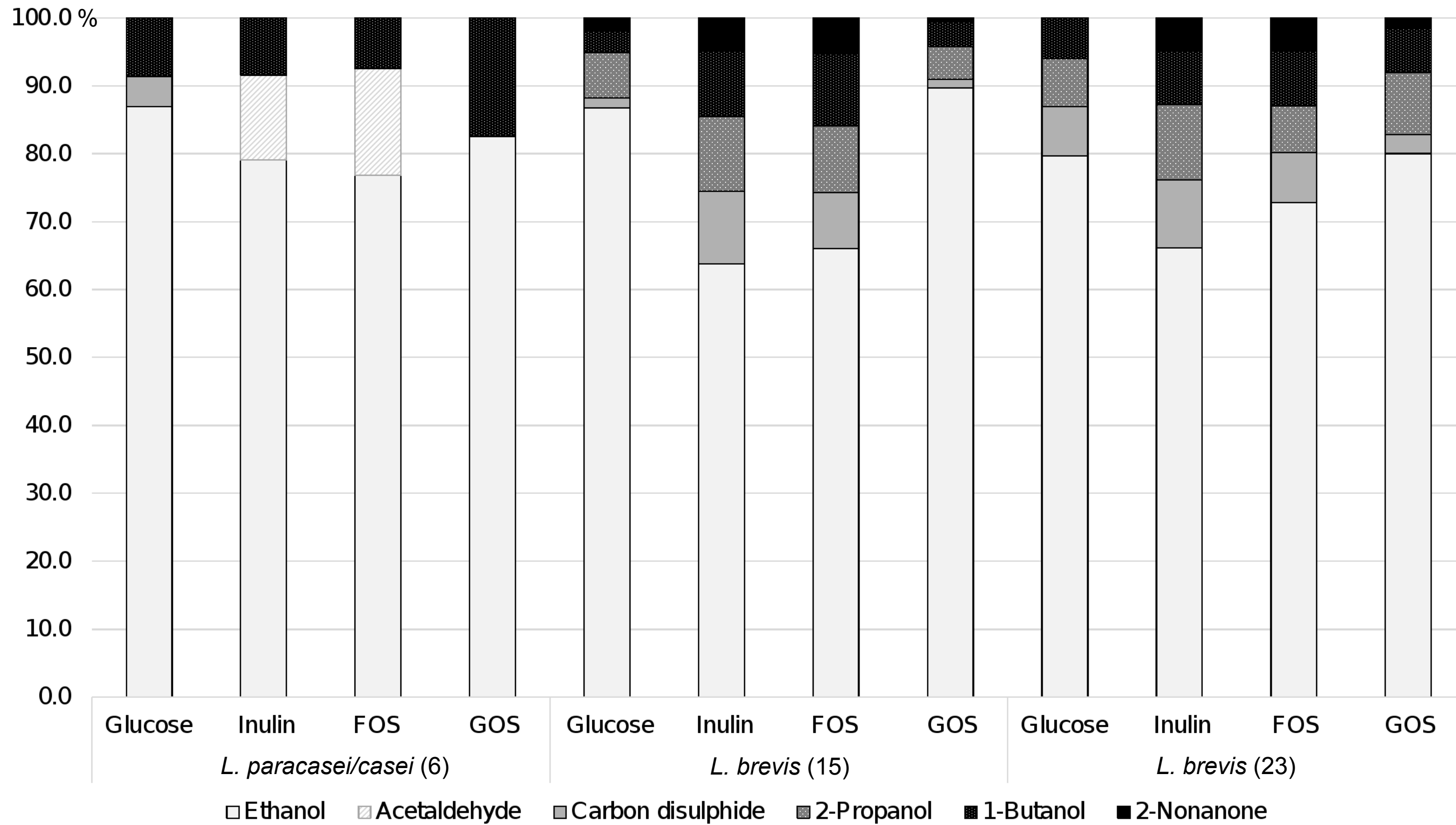


Fig 3. Increments after 24 h of incubation with respect to time 0 of the molar concentrations of lactic acid (a), formic acid (b) and acetic acid (c) and the ratio of acetic to lactic acids (d) in cultures of *L.paracasei/casei* 6, *L. brevis* 15 and *L. brevis* 23 strains growing in MRSc with glucose, inulin, FOS or GOS as the sole carbon sources added. Vertical lines in the bars represent standard deviation. For the same substrate, strains do not sharing the same letter produced significantly different amounts of the corresponding organic acid, $p < 0.05$.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30



31 **Fig 4.** Relative abundance of major volatile compounds (excluding acetic acid) detected by HS/GC//MS after 24 h of incubation of *L. paracasei/casei* 6, *L.*
32
33 *brevis* 15 and *L. brevis* 23 growing in MRSC with glucose, inulin, FOS or GOS as the sole carbon sources added.
34
35
36
37
38
39
40
41