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Limosilactobacillus fermentum from buffalo milk is suitable for potential biotechnological process development and inhibits *Helicobacter pylori* in a gastric epithelial cell model

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ABSTRACT

Probiotics are living microorganisms that give beneficial health effects while consumed, and each strain possesses diverse and unique properties and also different technological characteristics that affect its ability to be produced at large scale. *Limosilactobacillus fermentum* is a widely studied member of probiotics, however, few data are available on the development of fermentation and downstream processes for the production of viable biomasses for potential industrial applications.

In the present study a novel *L. fermentum* strain was isolated from buffalo milk and used as test example for biotechnological process development. The strain was able to produce up to 10^9 CFU/mL on a (glucose based) semi-defined medium deprived of animal-derived raw materials up to the pilot scale (150 L), demonstrating improved results compared to commonly used, although industrially not suitable, media rich of casein and beef extract. The study of strain behavior in batch experiments indicated that the highest concentration of viable cells was reached after only 8 h of growth, greatly shortening the process. Moreover, initial concentrations of glucose in the medium above 30 g/L, if not supported by higher nitrogen concentrations, reduced the yield of biomass and increased production of heterolactic fermentation by-products. Biomass concentration via microfiltration on hollow fibers, and subsequent spray-drying allowed to recover about 5.7×10^{10}CFU/g_{powder} of viable cells, indicating strain resistance to harsh processing conditions.

Overall, these data demonstrate the possibility to obtain and maintain adequate levels of viable *L. fermentum* cells by using a simple approach that is potentially suitable for industrial development. Moreover, since often exopolysaccharides produced by lactobacilli contribute to the strain's functionality, a partial characterization of the EPS produced by the newly identified *L. fermentum* strain was carried out.

Finally, the effect of *L. fermentum* versus *H. pylori* in a gastric epithelial cell model was evaluated demonstrating its ability to stimulate the response of the immune system and displace the infective agent.

1. Introduction

Probiotics are live microorganisms that are intended to provide health benefits to their host [16], and every strain possesses different and often unique properties that justify the search for new probiotics. Lactic acid bacteria (LAB) are a group of probiotics with specific nutritional requirements (e.g. vitamins, amino acids etc.) and low redox potential for growth. They are often used as starter cultures for food fermentation due to the production of lactic acid (LA), bacteriocins (small proteins with antibacterial activity) and exopolysaccharides

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(EPS) as all these metabolites can compete with pathogenic microorganisms and prevent food decomposition [3]. *Limosilactobacillus fermentum*, an heterofermentative member of the LAB family (previously addressed as *Lactobacillus fermentum* and recently reclassified as *Limosilactobacillus* [62]), can be found mainly in vegetable and cereal-based fermented foods [17, 49]. Furthermore, it was also identified in the oral cavity [40], in human breast milk and in the vagina [27, 38]. Recently, *L. fermentum* was found in the fecal microbiota of Koreans from a village that is famous for the longevity of its inhabitants[46].

This microorganism was studied for several interesting applications and uses in recent years [43]. Different strains showed anti-infectious and immunomodulatory properties [61], cholesterol-reduction [53] on both mice [45] and human volunteers (strain ME3) [34], prevention of lactational mastitis in women during breastfeeding [31] and wound healing induction in animal models [5]. Moreover, it was also evaluated as antimicrobial agent against infections caused by *Candida* spp. [12, 18], *Salmonella typhimurium* [55] and *Staphylococcus aureus* [47]. Therefore, the industrial production of *L. fermentum*-based probiotic products is of great interest. However, very few scientific publications are available on the development of fermentation processes for the large-scale production [2, 19]. *L. fermentum* was demonstrated to grow on renewable feedstocks such as starch [11], whey [4], cereal-based substrates [13] and even anaerobic digester sludge [32]. Nevertheless, most studies and patents still use De Man, Rogosa and Sharpe (MRS) medium for bioreactor experiments [28, 33, 52] even if this medium is not well suited for commercial food or pharmaceutical production processes due to presence of animal derived components (beef extract and peptone). In fact, although several medicinal commonly used products are of animal origin, or rely on raw materials of animal origin, research is moving towards the identification of animal substitutes and the elimination of animal (and human) derived contaminants. Indeed, not only issues related to moral or religious commitments but also those regarding the risks due to the presence of prionic or viral particles, address biopharmaceutical product manufacturing to the avoidance of animal-derived supplements (https://www.ivtnetwork.com/article/animal-derived-ingredients-fda-and-regulations).

The aim of this study was the identification of a semi-defined medium deprived of ingredients of animal origin that could support growth of the newly identified *L. fermentum* strain isolated from buffalo milk, and to demonstrate scalability up to the pre-pilot scale. A simple and short fermentation process followed by biomass concentration and spray drying were also evaluated to implement a complete and industrially applicable biotechnological approach providing highly viable *L. fermentum* cells.

Since the EPS have been demonstrated to participate to beneficial effects of *Lactobacilli* on the gastric mucosa [26], a partial characterization of the EPS produced by the newly identified *L. fermentum* strain was carried out.

Moreover, due to the high prevalence rates of *H. pylori* infections in the adult population in industrialized countries, as well as in developing ones [29], and considering the ability of certain *L. fermentum* strains to improve immune response and resistance against infections caused by this pathogen [26], the ability of the newly isolated strain (produced with the developed process) to reduce the inflammation in adenocarcinoma gastric cells infected with *H. pylori*, was investigated in different experimental conditions.

2. Materials and methods

2.1. Bacterial strain and media

L. fermentum was isolated from buffalo milk and identified by molecular analysis and amplification of specific sequences [51] and stored at −80 °C in MRS broth. Twenty% v/v glycerol stock suspensions prepared with exponentially growing cells were stored at − 80 °C.

Before use for cell culture experiments, the strain was grown in MRS anaerobically at 37 °C for 24–48 h, centrifuged at 4.000 × g for 10 min, at 4 °C, washed twice with saline, resuspended at the concentration of 0.5 OD_{600} in DMEM without antibiotics and added to cultures.

Helicobacter pylori ATCC® 43,629™ was cultivated on Trypticase Soy Agar (Oxoid; Unipath, Basingstoke, UK). All medium components and salts were supplied by Sigma-Aldrich (St. Louis, MO, USA). Yeast extract was furnished by Organotechnie (La Corneuve, France), while sulphuric acid was purchased by Biochem s.r.l. (Turin, Italy). The different semi-defined media used for growth experiments are listed in table 1. Glucose to a final concentration of about 30 g/L, $CaCl_2$ and Na_2S were filter sterilised and added to all semi-defined media after autoclaving.

2.2. Bottle experiments

All experiments were performed in 100 mL screw-cap bottles with a working volume of 90 mL. Bottles were incubated at 37 °C and 150 rpm in a rotary shaker incubator (model Minitron, Infors, Bottmingen, Switzerland) for up to 24 h. Bottle inhibition tests were conducted on medium 2 supplemented with 20 g/L of glucose as carbon source. After sterilization, L-Lactic acid was added to reach a final concentration of 20, 40, 60, 80 and 100 g/L in the medium; before strain inoculation media were buffered with NaOH 20 M to a pH of 6.4. Samples were withdrawn every hour for 7 times to analyze optical density (600 nm) carbon source consumption, and acid and ethanol production. All bottle experiments were performed at least in duplicate.

Viability was evaluated by serially diluting the samples and plating on MRS-agar medium. Plates were incubated at 37 °C for 36 h before counting viable cells. Each sample was analysed in triplicate.

Viability data on the different semi-defined media and on medium 2 supplemented with different carbon sources were analysed by one-way Anova and following post-hoc Tukey comparisons.

2.3. Bioreactor experiments

Medium 2 was used for all bioreactor processes. Fermentation experiments were performed in a Biostat CT plus (Sartorius Stedim, Gottingen, Germany) bioreactor with a working volume of about 2.2 L. Temperature was controlled at 37 °C, pH at 6.1 and agitation was fixed at 150 rpm. Glucose at a concentration of about 10 to 50 g/L was used as carbon source. Before each experiment, a concentrated stock solution was inoculated in 0.45 L of medium 2 at 37 °C and 150 rpm and grown for 8 h. The pre-culture was then transferred to the bioreactor with a peristaltic pump (model 313 U, Watson-Marlow, England) to reach up to 10% (v/v) of the working volume inside the fermenter. The airflow was kept constant at 0.44 vvm and stirring was set to 150 rpm.Experiments lasted up to 24 h. The batch process was scaled on a Biostat D100 (Sartorius Stedim, Gottingen, Germany) with a working volume of 60 L. Process parameters were those previously described. The scale-up strategy was based on maintaining constant the power input and tip speed used on the 3 L scale. In particular, the equations used were:

$$P_0(W) = N_p \rho N_i N^3 D_I^5 \quad (1)$$

$$V_t = \pi \cdot D_I \cdot \frac{rps}{100} \quad (2)$$

Where $P_0(W)$ is the ungassed power; N_p is the ungassed power number of the impeller (rushtone in this case); ρ is the broth density; N is the number of impellers and D_I is the diameter of the impellers. Samples were withdrawn during the experiments to analyze optical density (600 nm), cell viability, carbon source consumption, and acid and ethanol production. Viability was evaluated as previously described.

2.4. HPLC quantification of sugars, organic acids, and ethanol

Samples of about 10 mL were withdrawn during the experiments. The broth was centrifuged at 10,000 × g to separate the biomass and

Table 1
Composition of the semi-defined media used in 100 mL bottle experiments. a: Casein peptone; b: Bactocasitone; c: Soya peptone. d: Beef extract.

Component (g/L)	MRS	Medium 1	Medium 2	Medium 3	Medium 4	Medium 5
Yeast extract	5	5	10	10	2.5	5
Peptone	b*10 d*10	b* 10	c* 10	a* 7	a* 5 c* 5	c* 10
K_2HPO_4	2	2	/	2	/	3
$MgSO_4$ * 7 H_2O	0.1	0.1	0.25	0.2	0.1	/
$MnSO_4$ * H_2O	0.05	0.05	0.05	0.05	/	/
Yeast nitrogen base	/	5	/	/	5	/
$Na_3C_6H_5O_7$	/	5	2	/	/	/
Tween80 (mL/L)	1	1	0.453	/	/	/
$C_6H_8O_7 \bullet 2NH_3$	2	2	/	/	/	/
L-ascorbic acid	/	/	0.5	/	/	/
Sodium acetate	5	/	/	/	/	/
Di-sodium glycerophosphate	/	/	/	/	19	/
Ammonium sulfate	/	/	/	/		2
$CaCl_2$ * H_2O	/	/	/	/	/	0.2
$MgCl_2$ * 6 H_2O	/	/	/	/	/	0.2
NaCl	/	/	0.2	/	/	2
Na_2S *$_9H_2O$	/	/	/	/	/	0.001

recover the supernatant. One mL was then UF/DF on 3 kDa centrifugal filter devices (Centricon, Amicon, Sigma-Aldrich) at 10,000 × g and the permeate was analysed for the determination of the concentrations of carbon source, acids and ethanol by HPLC (UHPLC Dionex Ultimate 3000; Thermofisher) on a Alltech IOA-2000 column (500 mm × 6.5 mm ID) as previously reported [15].

2.5. Downstream process

The broth collected at the end of the batch process performed on the 150 L bioreactor was treated on a 0.22 μm hollow fiber module. Eleven liters of culture were filtered on 1.65 m^2 (with 1250 PES fibers, 1 mm thick) to separate biomass and supernatant recirculating for about 1 h. The concentrated biomass was spray-dried, as described in the following paragraph. The permeate was ultrafiltrated on 10 kDa cut-off membranes with a filtering area of 0.1 m^2 (Sartorius Stedim, Gottingen, Germany). Tangential flow filtration was performed on a Sartoflow alpha (Sartorius Stedim, Gottingen, Germany) system connected with a thermostatic bath that kept a constant temperature of about 20–25 °C. After the concentration step, 3 vol of diafiltration (VDF) with milliQ water were performed to remove low molecular weight molecules still attached to the membrane, and salts. The retentate was then precipitated with 2 vol of 1:1 ethanol/acetone solution and dried in vacuum oven at 40 °C over-night. The obtained powder was then resuspended in milliQ water and treated with 2% p/v activated charcoal. Precipitation was repeated as described above to obtain a powder for EPSs characterization.

2.6. Phenol sulfuric acid assay for exopolysaccharides quantification

Quantification of exopolysaccharides was performed by phenol sulfuric acid test [21], a colorimetric assay used for the determination of total carbohydrates in a sample. Pentoses through hydrolysis are dehydrated to furfural, and hexoses to hydroxymethylfurfural producing a yellow-gold color in presence of phenol. The calibration curve was obtained with standard solutions of D (+) glucose at concentrations ranging from 0.01 to 0.1 mg/mL. Briefly, 200 μL of standards were placed in a reaction tube with 200 μL of aqueous solution of phenol 5% w/v. Then, 1 mL of concentrated sulfuric acid (98% w/w) was added, and the reaction tube was quickly closed and after vigorous stirring, the reaction was carried out for 30 min at 30 °C. Sample absorbance was read at 490 nm using distilled H_2O as blank.

2.7. Spray dryer

After microfiltration the concentrated biomass (about 1.7 L) was diafiltered with 2 vol (3.4 L) of sterile phosphate buffer solution (PBS) and after addition of trehalose and sucrose it was spray-dried on a Mobile minor™ (Gea Process Engineering, Düsseldorf, Germany). Based on the theoretical wet to dry biomass ratio, a 1:1 ratio between dry biomass and total sugars was maintained. The sample was fed to the spray dryer at 2.4 L/min, with a peristaltic pump (model 730 U, Watson-Marlow, England). The inlet temperature was set at 165 °C, outlet temperature was 85 °C, while atomizer pressure was set to 1 bar.

2.8. Cell cultures

Human gastric adenocarcinoma cell-line AGS cells ATCC® CRL-1739™ (American Type Culture Collection, Rockville, MD, USA), were routinely cultured in Ham's F-12 K medium (Gibco, Waltham, Massachusetts,USA) supplemented with 1% (v/v) Penstrep, 1% (v/v) glutamine and 10% (v/v) fetal calf serum (Gibco) at 37 °C and 5% CO_2.

2.9. Cell infection with H. pylori and/or L. fermentum

The ability of *L. fermentum* to reduce the inflammation in AGS cells after infection with *H. pylori*, was investigated in three different experimental types: (i) Competitive assay, in which AGS cells (10^5) were incubated simultaneously for with *L. fermentum* (10^8 CFU/mL) and *H. pylori* (10^8 CFU/mL) for 2 h; (ii) Inhibition assay, in which AGS cells (10^5 cells) were preincubated with *L. fermentum* (10^8 CFU/mL) for 1.5 h and then *H. pylori* (10^8 CFU/mL) was added and incubated for 2 h; (iii) Displacement assay in which AGS cells were pre-incubated with *H. pylori* (10^8 CFU/mL) for 2 h and then *L. fermentum* (10^8 CFU/mL) was added and further incubated for 1.5 h.

2.10. Evaluation of proinflammatory genes expression

At the end of cell culture experiments, to evaluate the expression of pro- and anti-inflammatory cytokines, the cells were washed three times with sterile PBS, and the total RNA was extracted using High Pure RNA Isolation Kit (Roche Diagnostics, Monza, Italy). Two hundred nanograms of total cellular RNA were reverse-transcribed (Expand Reverse Transcriptase, Roche Diagnostics) into complementary DNA (cDNA) using random hexamer primers (Random hexamers, Roche Diagnostics) at 42 °C for 45 min, according to the manufacturer's instructions. Real time PCR for IL-6, IL-8, TNF-α, IL-1α, IL-1β, TGF-β and HBD-2 was carried out with the LC Fast Start DNA Master SYBR Green kit using 2 μL of cDNA, corresponding to 10 ng of total RNA in a 20 mL final volume, 3 mM $MgCl_2$ and 0.5 mM sense and antisense primers (Table 2). After amplification, melting curve analysis was performed by heating to 95 °C for 15 s with a temperature transition rate of 20 °C/s, cooling to 60 °C for

Table 2
Primer sequences and amplification programs used for gene expression studies.

Gene	Primers sequence	Conditions	Product size (bp)
IL-6	5′-ATGAACTCCTTCTCCACAAGCGC-3′ 5′-GAAGAGCCCTCAGGCTGGACTG-3′	5′'at 95 °C, 13″ at 56 °C, 25′'at 72 °C for 40 cycles	628
IL-8	5′-ATGACTTCCAAGCTGGCCGTG-3′ 5′- TGAATTCTCAGCCCTCTTCAAAAACTTCTC- 3′	5′'at 94 °C, 6″ at 55 °C, 12′'at 72 °C for 40 cycles	297
IL-1β	5′-GCATCCAGCTACGAATCTCC-3′ 5′-CCACATTCAGCACAGGACTC-3′	5′'at 95 °C, 14″ at 58 °C, 28′'at 72 °C for 40 cycles	708
TGF-β	5′-CCGACTACTACGCCAAGGAGGTCAC-3′ 5′-AGGCCGGTTCATGCCATGAATGGTG-3′	5′'at 94 °C, 9″ at 60 °C, 18′'at 72 °C for 40 cycles	439
IL-1α	5′- CATGTCAAATTTCACTGCTTCATCC-3′ 5′- GTCTCTGAATCAGAAATCCTTCTATC −3′	5″at 95 °C, 8″at 55 °C, 17″at 72 °C for 45 cycles	421
HBD-2	5′- GGATCCATGGGTATAGGCGATCCTGTTA-3′ 5′-AAGCTTCTCTGATGAGGGAGCCCTTTCT- 3′	5″at 94 °C, 6″at 63 °C, 10″at 72 °C for 50 cycles	198
TNF-α	5′- CAGAGGGAAGAGTTCCCCAG −3′ 5′- CCTTGGTCTGGTAGGAGACG −3′	5′'at 95 °C, 6″ at 57 °C, 13′'at 72 °C for 40 cycles	324

15 s with a temperature transition rate of 20 °C/s, and then heating the sample at 0.1 °C/s to 95 °C. The results were then analysed using LightCycler software (Roche Diagnostics). The standard curve of each primer pair was established with serial dilutions of cDNA. All PCR reactions were run in triplicate. The specificity of the amplification products was verified by electrophoresis on a 2% (w/v) agarose gel and visualization by ethidium bromide staining. Significant differences among groups were assessed by two-way ANOVA using GraphPad Prism 6.0, and the comparison between the means was calculated by t-student test. The data are expressed as means ± standard deviation (SD) of three independent experiments.

2.11. ELISA assay

Supernatants of AGS cells infected as described aboveat the end of the experiment were harvested and the presence of cytokines IL-6, IL-8, IL-1α and HBD-2 was analysed by enzyme-linked immunosorbent assay (ELISA; ThermoFischer Scientific Inc., Waltham, Massachusetts,USA; Phoenix Pharmaceuticals, Burlingame, USA). Statistical analyses were performed as described in the previous paragraph.

2.12. Scanning electron microscope analysis

AGS cells (10^5) were infected with *H. pylori* or *L. fermentum* alone, both at 10^8 CFU/mL for 2 h and after incubation fixed in 4% v/v paraformaldehyde in PBS. Samples were then dehydrated by washing in increasing ethanol concentrations (30% to 95% for 10 min, and 100% 3 times for 15 min). Immediately afterwards the samples were further dried in the EMITECH K850 critical point dryer, then stutter coated in Denton Vacuum DESKV sputter coater with platinum – palladium target, at 77 mAmps for 120 s. The samples thus prepared were observed using a Fe-SEM Supra 40 field-emission scanning electron microscope, Zeiss, Germany (EHT = 5.00 kV, WD = 22 mm, Inlens detector).

2.13. Monosaccharide analysis and permethylation of EPS

Monosaccharide compositional analysis (acetylated methyl glycosides) was performed as reported elsewhere [3]. Monosaccharide derivatives were recognized based on their GC–MS spectrum fragmentation pattern and by comparison of their retention time with that of authentic standards.

A sample (1 mg) of the EPS was permethylated using methyl iodide and sodium hydroxide [14] as already reported [25]. After hydrolysis with 2 M TFA (1 h, 120 °C), the partially methylated monosaccharides were reduced with $NaBD_4$. After the usual work-up, the sample was submitted to acetylation with acetic anhydride and pyridine 1:1 (30 min, 100 °C), and the mixture of partially methylated alditol acetates was analysed by GC–MS.

2.14. NMR spectroscopy

^{1}H and 2D NMR spectra of the EPS from *L. fermentum* were recorded in deuterated water (D_2O) using a Bruker 600 MHz instrument equipped with a cryoprobe at 298 K.

3. Results

3.1. Medium optimization and acid inhibition test

Initial bottle experiments were performed with 90 mL of each medium to evaluate viability and optical density. Results are showed in Fig. 1.

Different media were tested to identify those that better supported biomass production. Various nitrogen sources and their combinations were tested together with different C:N ratios. Glucose at a fixed concentration of about 30 g/L was chosen for these trials. Viability data were analysed by one-way Anova with post-hoc Tukey comparisons showing significantly higher concentrations of viable cells on medium 2 ($p<0.001$) compared to results obtained on the control medium (MRS). A maximum viability of about $9.3 \pm 1.3 \times 10^8$ CFU/mL was reached after 16 h of growth, that corresponded to about 1.35±0.01 g/L of dry cell biomass and an optical density of about 4.5 ± 0.1 OD_{600} (Fig. 1a). This medium was then chosen for further experiments, and it was supplemented with different carbon sources at the same initial concentration (Fig 1b). *L. fermentum* reached the same final OD on media containing maltose and glucose however, the latter showed a 38% higher number of viable cells after 16 h of growth and was therefore selected for bioreactor experiments. Finally, bottle experiments on the same medium supplemented with increasing concentrations of lactic acid, were also performed. Growth was followed during the first 7 h and the specific growth rate (μ) was calculated as linear regression of OD measurements.

Luong-model [39] exponential inhibition Eq. (3) showed the best fit to experimental data:

$$\mu = \mu max\left(1 - \frac{P}{Pmax}\right)^{NP} \quad (3)$$

where μ_{max} is the maximum specific growth rate calculated in the absence of lactic acid, P is the concentration of product (lactic acid), P_{max} is the critical product concentration (when $P = P_{max}$, $\mu = 0$) and n_p is the inhibition constant (for non-competitive inhibition $np > 0$). We found a P_{max} of 100 g/L and a μ_{max} equal to 0.59 h^{-1} determined experimentally (Fig. 2). A regression coefficient (R^2) equal to 0.93 was found analysing data.

3.2. Fermentation experiments

All fermentations were performed on medium 2 under controlled conditions of temperature, pH and aeration rate. Batch processes were initially performed with and without air sparging (0.44 vvm), and slightly better results were obtained in the presence of air (data not shown), therefore this condition was used in all further experiments.

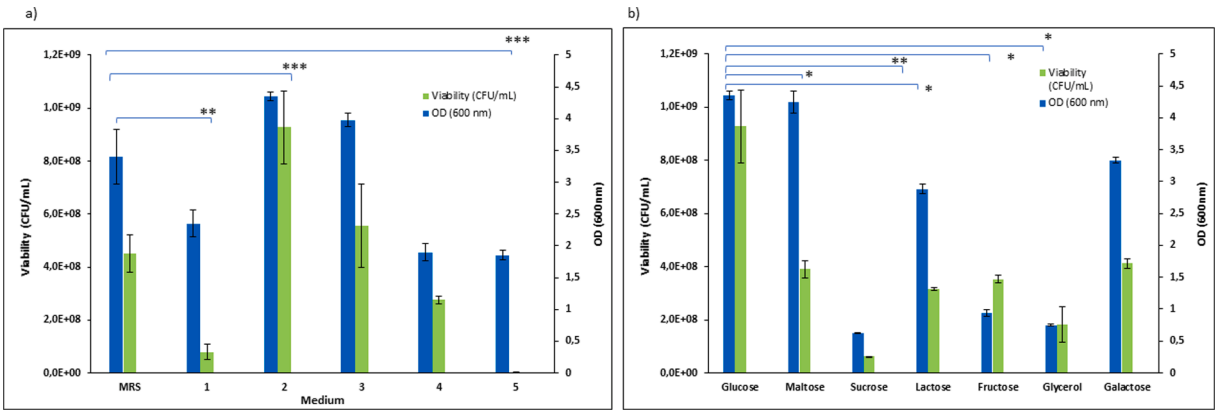

Fig. 1. Small scale experiments performed in 100 mL bottles at 37 °C and 150 rpm. a) Optical density and concentration of viable cells obtained on different semi-defined media compared to MRS; b) Optical density and concentration of viable cells obtained on medium 2 with different carbon sources. Viability data were analysed by Anova and post hoc Tukey comparison; data significance was indicated as *p<0.05, **p<0.01. ***p<0.001.

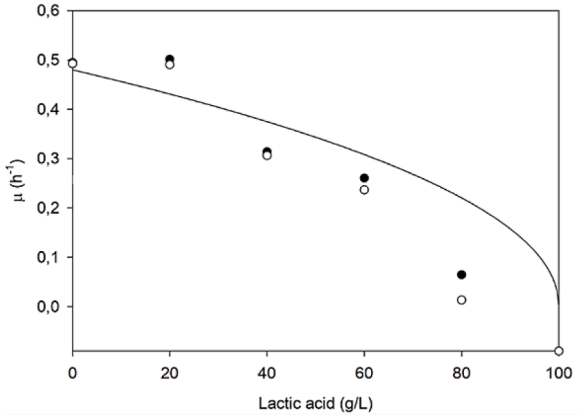

Fig. 2. Product inhibition. Evaluation of lactic acid inhibition on the growth of *L. fermentum* in bottle experiments with an initial glucose concentration of 30 g/L, pH 6.5 and temperature of 37 °C. The experiment was run in duplicate.

Initial batch processes with glucose at different concentrations (10, 30 and 50 g/L) were tested and summarized in table 3. During the first 8 h growth rate, glucose consumption and lactic acid production were evaluated. Batch processes with 30 g/L of initial glucose demonstrated the highest CFU/mL of about $1 \times 10^9 \pm 0.14$ after 8 h of growth. This value did not change after 16 h, however it decreased to 7.4×10^9 after 24 h of growth (supplementary Fig 1).

Table 3 summarises bioreactor experiments in batch with pulse and batch mode. The highest concentration of viable cells was obtained during batch experiments with an initial concentration of glucose equal to 30 g/L. An $Y_{x/s}$ of about 0.17 g/g was obtained in the batch experiment with a starting concentration of about 10 g/L of glucose and it gradually decreased with 30 and 50 g/L of glucose initially in the medium, and when about 50±5 g/L of glucose were provided by adding a concentrated pulse after the first 8 h of growth (batch with pulse, Table 3). On the other hand, production of LA and ethanol proportionally increased with higher glucose concentrations in the medium, as indicated by higher $Y_{LA/x}$ in the different experiments.

Batch processes with 30 g/L of glucose and an almost doubled amount of nitrogen sources (20 g/L yeast extract and 15 g/L soy peptone) led to a final concentration of viable cells of about 1.52±0.26 $\times 10^9$ and an $Y_{x/s}$ of about 0.13±0.02 g/g (, OD_{max} 9.8 ± 1.0; LA 18.0 ± 2.0 g/L; Et 5.6 ± 1.5 g/L).

The batch process on medium 2 containing 30 g/L of glucose was then run on a Biostat D100 Sartorius bioreactor with 60 L of working volume, according to the scale-up strategy described in the materials and methods section. As shown in Table 3 all data were in line with those obtained on the 2 L scale. A slightly lower viability was obtained on the 150 L scale at the end of the fermentation (16 h), however, the value was within the variability interval observed on the 3 L scale.

The EPSs produced by *L. fermentum* and secreted in the broth were quantified at the end of all fermentation processes and results are reported in table 3. Higher concentrations of EPSs were obtained in batch processes with 50 g/L of glucose, whereas no polysaccharide could be detected when only 10 g/L of carbon source were present the medium.

Table 3
Fermentation experiments performed on Biostat CT plus (3 L) and Biostat 100 (150 L) bioreactors.

Process	OD max (600 nm)	Viability (CFU/mL)	Dry weight (g/L)	LA (g/L)	Et (g/L)	$Y_{x/s}$ (g/g)	$Y_{LA/x}$ (g/g)	$Y_{Et/x}$ (g/g)	EPS (glucose equivalents) (mg/mL)
Batch									
10 g/L	5.1 ± 0.4	$3.6 \pm 0.6 \times 10^8$	1.7 ± 0.1	6.5 ± 0.7	3.0 ± 0.6	0.17±0.04	3.8 ± 0.1	1.7 ± 0.2	n.d
30 g/L	6.3 ± 0.3	$1.0 \pm 0.1 \times 10^9$	2.4 ± 0.3	19.5 ± 1.3	6.7 ± 1.4	0.09±0.01	8.3 ± 1.3	2.8 ± 0.7	0.450±0.040
50 g/L	7.7 ± 1.5	$6.9 \pm 1.7 \times 10^8$	2.7 ± 0.5	26.8 ± 1.5	9.1 ± 1.1	0.07 ±0.001	9.8 ± 1.1	3.4 ± 0.2	0.630±0.006
Batch_P	7.3 ± 0.2	$6.1 \pm 0.9 \times 10^8$	2.7 ± 0.3	34.5 ± 0.7	9.6 ± 0.8	0.05 ±0.006	12.8 ± 1.7	3.6 ± 0.7	0.400±0.004
Scale up									
Batch 150L	6.8	8.0×10^8	2.6	21.4	8.2	0.10	8.2	3.1	0.47

EPS, exopolysaccharides; Batch_P, batch with pulse; LA, lactic acid; Et, ethanol.

3.3. Concentration and spray drying of probiotic biomass

Eleven L of broth were microfiltered and diafiltered with PBS in about 2 h. The initial flux was 40 LHM and it decreased by about 4-fold at the end of the process due to cake formation. Overall, the microfiltration and diafiltration steps yielded a 6.5-fold concentrated bacterial retentate, containing about 30 g of dry biomass. After addition of trehalose and sucrose the concentrated biomass was spray dried resulting in a survival of about 60% after drying. The recovered biomass showed a viability of $5.7 \pm 1.2 \times 10^{10}$ CFU/g of powder.

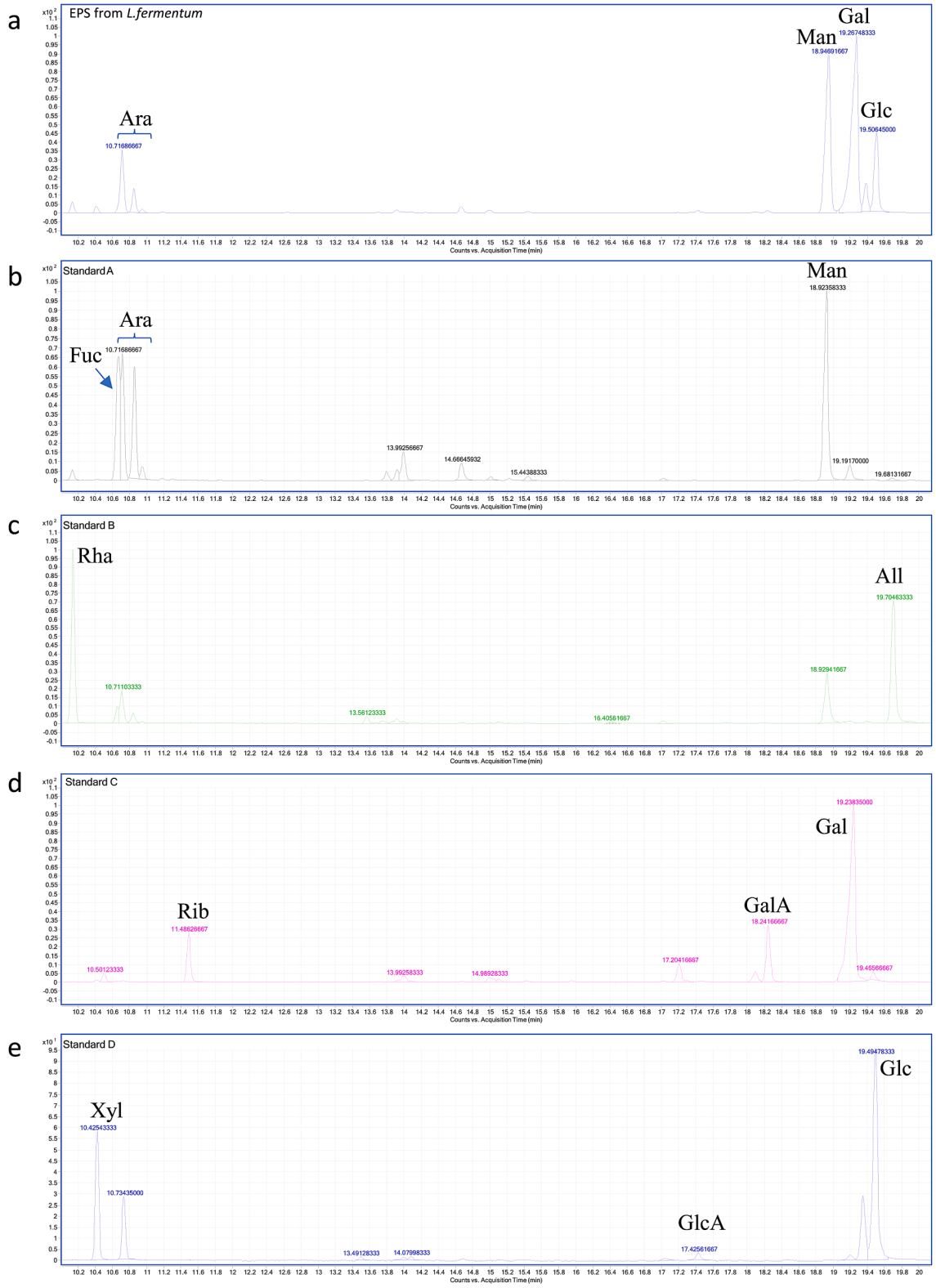

Fig. 3. GC–MS chromatograms of acetylated methyl glycosides of EPS from *L. fermentum* (a), and standard sugars (b-e). Ara, arabinose; Man, mannose; Gal, galactose; Glc, glucose; Fuc, fucose; Rha, rhamnose; All, allose; Rib, ribose; GalA, galacturonic acid; Xyl, xylose; GlcA, glucuronic acid.

3.4. Chemical characterization and NMR study of EPSs

The glycosyl analysis of the recovered and partially purified EPSs indicated that the polysaccharides contained, as main constituents, mannose, galactose, glucose, and arabinose, as revealed by the comparison between the GC–MS chromatogram of standards with that of the isolated polymer (Fig. 3).

In addition, ^{1}H and 2D ^{1}H, ^{13}C DEPT-HSQC NMR experiments were obtained (Fig. 4).

In the anomeric region of both spectra, many signals appeared, suggesting a mixture of exopolysaccharides. However, a more accurate analysis of the DEPT-HSQC experiment revealed some signals suggesting a mixture of at least two polysaccharides, one of which is a mannan whereas the other could be identified as a heteropolysaccharide (HePS). The structure of the last could be very similar to the neutral polysaccharide isolated from *Lactobacillus delbrueckii* ssp. *bulgaricus* LBB.B26 [50]. In fact, the carbon signal at δ 110 ppm correlating in the DEPT-HSQC spectrum with the anomeric proton at δ 5.18 ppm, together with signals of carbon nuclei in the range of δ 80–85 ppm could be assigned to galactofuranose units, due to the typical downfield shift of these signals [8]. A methylation analysis on the EPSs mixture, indicated terminal glucose, terminal galactose, terminal mannose, terminal arabinose, 4-substituted glucose, 6-substituted mannose, and 3, 6-substituted galactose units. Some of these units could belong to the HePS, thus confirming the previous hypothesis. Finally, albeit the hexose units are frequent in lactobacilli EPS [12], the presence of arabinose has been detected and associated to bacterial stress resistance [44].

3.5. Effect of L. fermentum on H. pylori

The data obtained (Fig. 5a, b and c) show that *L. fermentum* can almost completely inhibit the inflammatory state strongly induced by *H. pylori* in the displacement assay. In fact, the genes encoding proinflammatory cytokines IL-1α, IL-6 and IL-8 are strongly downregulated in the presence of *L. fermentum* compared to infection with *H. pylori* alone. IL-1β, TGF-β and TNF-α were unmodulated. Therefore, the probiotic, which by itself can reduce the basal expression of proinflammatory molecules and increase antimicrobial defenses by inducing the expression of HBD-2, may improve the conditions of the gastric mucosa when damaged by *H. pylori*.

3.6. Effects of H. pylori and L. fermentum on the gastric epithelium morphology

Scanning electron microscope observation at high magnification (10–13 KX) revealed the typical rod-like shape of *L. fermentum* (Fig. 6 LB panel), while *H. pylori* shows the characteristic gull-wing shape (Fig. 6 HP panel). Low magnification images (1500X) demonstrate different cell morphologies: *L. fermentum* doesn't alter the flattened physiological aspect of the cell monolayer, while the presence of the pathogen causes cell surface ruffles and loss of flattened shape.

4. Discussion

L. fermentum is a widely studied probiotic, however, distinct strains often differ in terms of physiology and biological properties [6, 11, 35–37, 42, 48, 54, 57, 60]. The aim of this study was to develop an improved fermentation process and demonstrate its suitability for scale-up and subsequent concentration, including the drying process, for the potential industrial production of viable biomass by using a *L. fermentum* strain, that was newly isolated from buffalo milk, as test example. The work focused on the identification of an alternative to the commonly used MRS medium, that could support growth up to the pilot scale, despite the absence of complex animal derived constituents. In fact, regulatory (safety), ethical and religious issues increasingly limit the use of ingredients of animal origin and steer product manufacturing towards the search for safer solutions.

A very critical point regarding the potential commercial use of probiotics is the delivery of an adequate number of viable cells in the target area. This is affected by the survival rate during the manufacturing

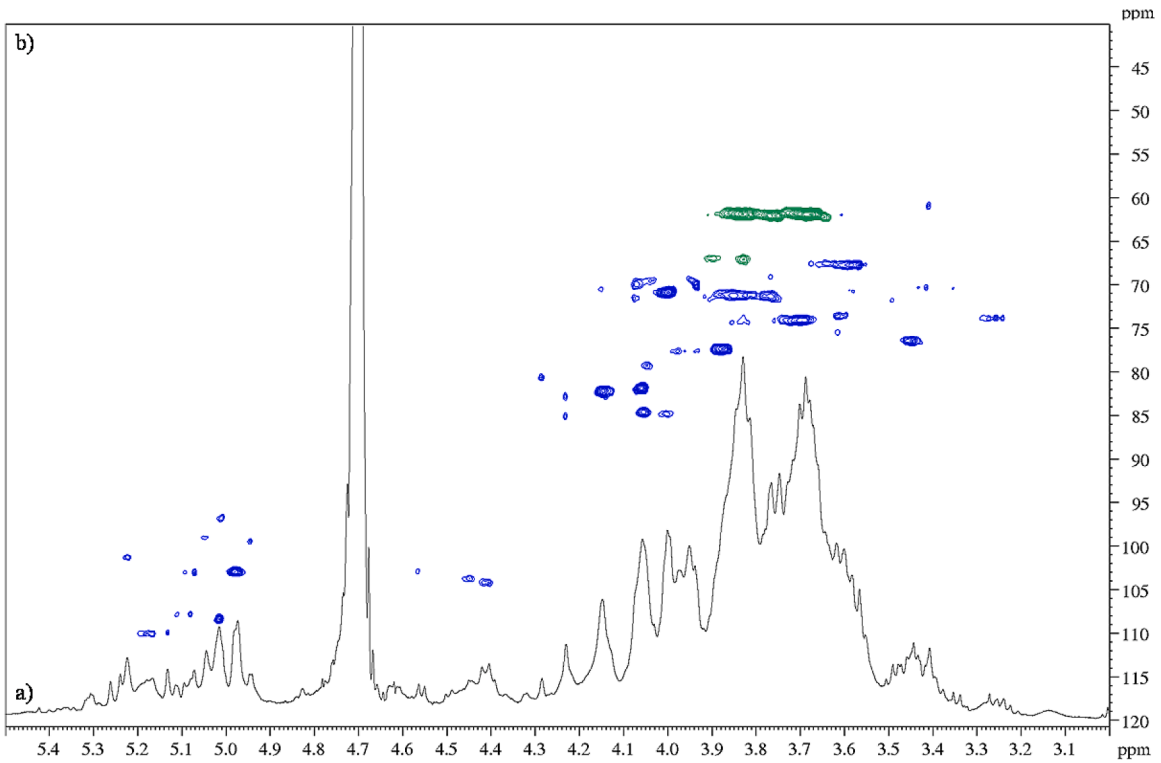

Fig. 4. a) ^{1}H NMR spectrum of *L. fermentum* EPS. b) DEPT-HSQC NMR experiment of *L. fermentum* EPS. The spectra were recorded in D_2O at 298 K, at 600 MHz.

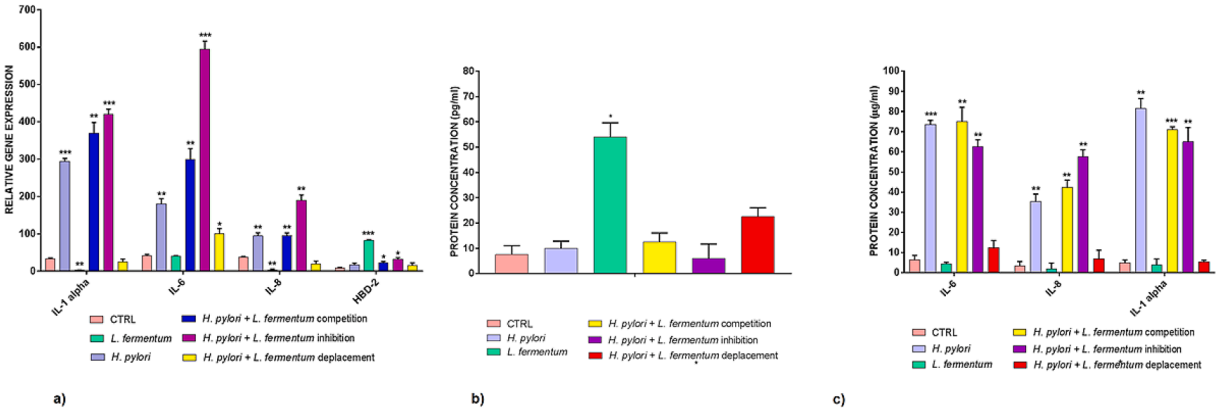

Fig. 5. Real-Time PCR (a) and ELISA (b and c) show the expression levels of proinflammatory cytokines and HBD-2 in AGS cells infected with *H. pylori* and/or *L. fermentum*. Data are expressed as relative mRNAs expression (A) and protein concentration (C) in each group and are representative of three different experiments ± SD. Significant differences are indicated by *$p<0.05$, **$p<0.01$, ***$p<0.001$.

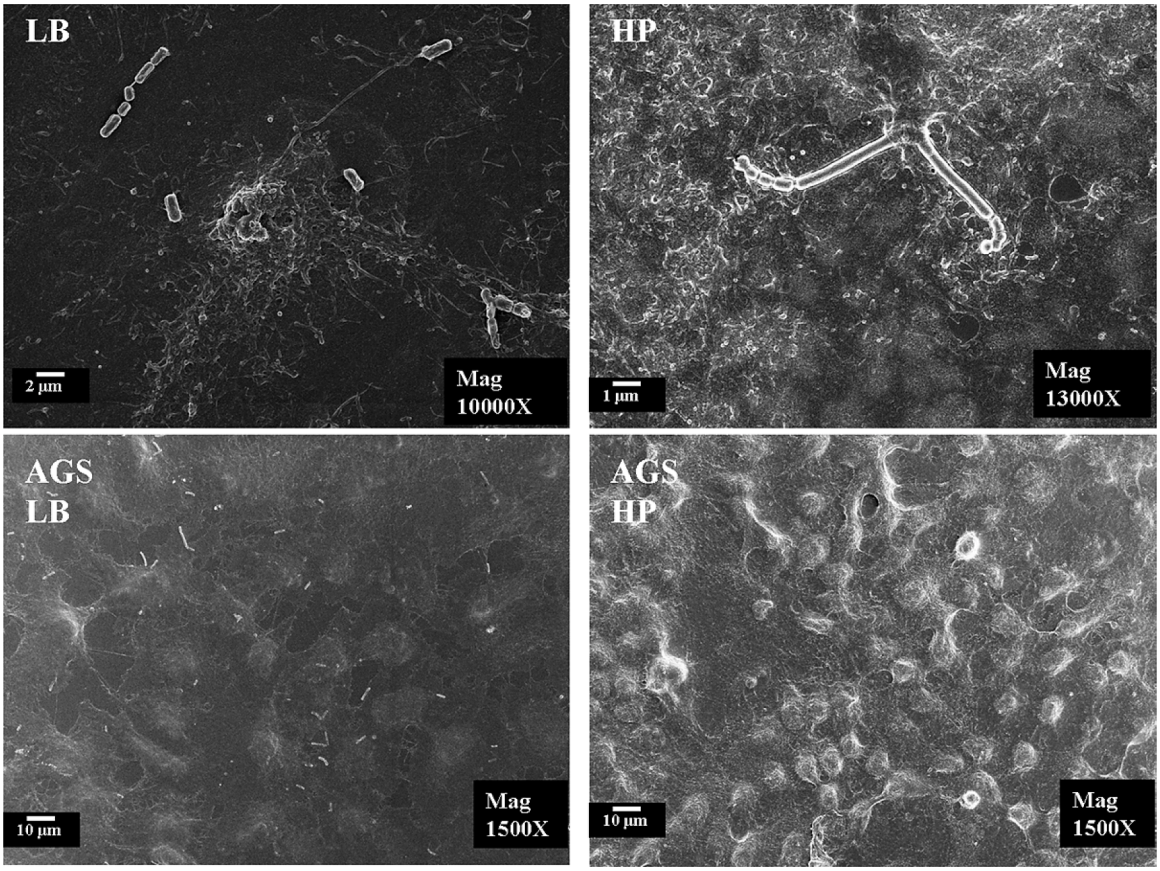

Fig. 6. Microorganism and co-culture micrograph obtained by scanning electron microscopy. LB, *L. fermentum*; HP, *H. pylori*; AGS and LB or HP indicates co-culture. Different magnifications highlight bacteria or cells morphology.

process, and during transit in the gastrointestinal tract. Therefore, it is quite important to obtain high densities of viable cells during fermentation processes and further processing.

Media with different composition were tested in small scale bottle experiments. The elimination of beef extract and the replacement of bactocasitone or casein, which are typical but animal derived complex nitrogen sources, with soy peptone in medium 2 were demonstrated to be superior with respect to achieved biomass concentration as well as viability. Higher concentration of YE, as well as lower C:N ratios, and the presence of additional elements for growth (eg. $MgSO_4$, NaCl, ascorbic acid, sodium citrate) (Wayah and Philip. 2018) in this medium compared to medium 1, 4 and 5 greatly improved fermentation performance. This not only shows that it is possible to eliminate and/or replace animal derived media components, but that these traditionally used media are clearly not optimal for commercial manufacturing processes of *L. fermentum* and most likely also of other LAB. Among all carbon sources tested glucose and maltose mostly improved cell density, however the latter yielded a significantly lower concentration of viable cells (two tailed non homoscedastic, t-student $p<0.00001$, Anova $p<0.05$). In a different study [10, 57] *L. fermentum* Ogi E1 also showed similar cell densities on these two substrates, whereas compared to the results presented in this study *L. fermentum* GA715 showed lower

biomass concentrations. In general, both strains showed a different ranking for the same carbon sources, which together with our results indicate a high diversity on the efficiency for using different carbon sources within the same species.

Fermentation process duration is a key aspect of industrial applications. Preliminary 24 h bottle and bioreactor experiments demonstrated that the maximum concentration of viable cells was obtained after 8 h of growth (it remained constant until 16 h, whereas it greatly decreased after 24 h of growth). These data indicate the possibility to shorten the process, thereby greatly improving the overall productivity.

Therefore, different initial concentrations of glucose (10, 30, 50 g/L) were next evaluated in 3 L bioreactors, in batch mode, with 8 h processes. With up to 30 g/L of glucose in the medium viability increased, whereas no further improvement was observed with higher substrate concentrations. Interestingly, a decreasing $Y_{x/s}$ trend and an increase of $Y_{LA/x}$ and $Y_{Et/x}$, were observed with higher initial glucose concentrations (10, 30, 50 g/L). Even splitting substrate feeding (25 g/L initially in the medium and 25 g/L at the end of the exponential phase) did not improve cell viability, suggesting a major carbon flux towards LA and ethanol instead of biomass. Considering an average bacterial biomass elemental composition equal to $CH_{1.66}N_{0.20}O_{0.27}$—$CH_2N_{0.24}$ $O_{0.33}$ [7], and nitrogen content in medium 2, the moles of N needed to support the production of biomass in the presence of 30 and 50 g/L of glucose might have been insufficient, generating a nitrogen limitation condition. For this reason, batch processes with 30 g/L of glucose and an 80% higher total nitrogen concentration were performed. Results showed an improvement of the number of viable cells of about 50% (two tailed non homoscedastic t-student, $p<0.005$) and a 39% improvement of the $Y_{x/s}$, probably indicating the need of additional N source to further support biomass production and slightly decrease lactic acid secretion. These data are also in accordance with results obtained with *L. fermentum* Ogi E1 on starch and yeast extract, since the authors found that increasing the concentration of YE in the medium above 20 g/L (C_g:N_g equal to 1:1) did not improve biomass production [11]. Since, as indicated for the first time in this study, *L. fermentum* tolerates high LA concentrations (Pm=100 g/L) as other LAB, strategies that allow administration of higher amounts of carbon source should be investigated to maximize biomass production.

One of the necessary technological characteristics of probiotics is their "ability to be produced at large scale" [23]. Therefore, the development of biotechnological processes up to the pilot scale and the use of a suitable drying process are important steps to industrial application. Considering the lack of literature on this aspect, the batch process was scaled to the 150 L reactor. Considering the different reactor geometry of the 3 and 150 L fermenter, a constant power input and tip speed were maintained in the two reactor set-ups. This approach allowed to obtain very similar results to that observed on lab scale experiments. Further downstream processing was performed to simulate a complete manufacturing cycle. Biomass concentration on hollow fibers was the most critical point of the downstream process [4]. Clogging of the membrane, either due to production of EPS, or to the lumen of the hollow fibers, allowed processing of about 11 L of broth. The low volume caused a partial loss of dried sample in the spray-drier chamber thereby reducing the final yield of recovered powder. However, this problem can be avoided on industrial scale by increasing the surface area of filtration modules and the volume of spray dried sample. Among the available drying techniques, spray-drying is one of the most predominant in the dairy industry since it guarantees lower energy costs and higher sustainability [30]. However, due to the high inlet and outlet temperatures, and considering the sensitivity of probiotics, variable tolerance among different strains and operating conditions is observed [30]. Notwithstanding the stressful microfiltration treatment, the *L. fermentum* strain isolated in this work showed 60% survival after spray-drying, demonstrating a good resistance to harsh conditions that prevail during the process.

The partially purified EPSs isolated from the supernatant of *L. fermentum* cultures were subjected to a partial characterization by GC–MS and NMR spectroscopy. Results indicated that the EPS fractions contained a mixture of a homopolysaccharide, such as a mannan, and a heteropolysaccharide. The last resulted to be very similar to that already characterised from another strain of *Lactobacillus* [50], due to the distinguishable signals in the NMR experiments of the galactofuranose units and to the identification of the same attachment points reported for the neutral polysaccharide from *L. delbrueckii* in the methylation analysis. The detection of mannan could be due to the presence of residues of medium components (yeast extract) as often previously found [9, 24], whereas the arabinose content is quite rare in lactobacilli [44].

In healthy individuals, the gastric epithelium, owing to the shape and polarization of its cells and to cell-cell and cell-matrix adhesions, represents the first barrier of defense against pathogens. *H. pylori*, colonizing the gastric mucus, manages to disassemble this epithelial barrier and induce an inflammatory state that can sometimes lead to the onset of neoplastic changes [58]. Clinical treatment consists in the standard triple therapy (lansoprazole, clarithromycin, and metronidazole), which may cause serious side effects and increased antibiotic resistance [1, 56]. The use of *Lactobacillus* spp. instead of antibiotics could avoid this drawback [22] and *L. fermentum* is interesting in this respect. In fact, recent studies demonstrated the ability of *L. fermentum* UCO-979C to improve immune response and resistance against infections caused by *Helicobacter pylori* [26]. The *L. fermentum* strain newly isolated in this work was evaluated during *H. pylori* infection to demonstrate whether it could improve the inflammatory state of the intestinal epithelium.

For this purpose, competition, inhibition, and displacement assays in which AGS cells were infected with *L. fermentum* or *H. pylori* alone, or co-infected at different times, were conducted. The expression of proinflammatory cytokines, soluble mediators of natural immunity and the immune response [41], and of Human β-defensin-2 (HBD-2), inducible antimicrobial peptide active against Gram-positive and Gram-negative bacteria, fungi, and the envelope of some viruses, and involved in the innate immune response [20], was evaluated.

Results showed that *L. fermentum* by itself can increase the production of HBD-2 by gastric epithelial cells, which is of great importance as it induces the enhancement of antimicrobial defenses and mechanisms of innate immunity; it was also shown to have a strong anti-inflammatory effect by downregulating the expression of proinflammatory cytokines in the displacement assay, therefore following infection with *H. pylori*.

The neoplastic changes induced by *H. pylori* are caused mainly by a rearrangement of the actin filaments of the cytoskeleton, following the formation of protrusions and massive stress fibers in gastric epithelial cell cultures that destroy cell-cell junctions, altering cell morphology [59].

In a second set of experiments, it was verified that *L. fermentum*, unlike *H. pylori*, does not induce any morphological changes in the structure of the gastric epithelium as shown by SEM analysis.

Differently from *H. pylori, L. fermentum* manages to interact with the gastric epithelial cells, even at the level of the cell-cell junctions, preserving their structure.

5. Conclusions

This work presents a comprehensive approach towards the development of a potential probiotic production process demonstrating the suitability of the newly isolated *L. fermentum* strain to upstream and downstream process development. Exopolysaccharides have been partially purified showing peculiar structural features as found by NMR analyses.

Moreover, the biological activity of the newly isolated *L. fermentum* strain was evaluated in a gastric epithelial cell model demonstrating defensin upregulation and *H. pylori* inhibition, also modulating inflammatory cytokines.

Author contributions

DC, SD and AF drafted the manuscript; SD, MV and DC conducted fermentation experiments; SD conducted downstream processing; AD, SD and MV performed cell viability counts; AF conducted biological assays; AC and MMC characterized the EPSs and drafted the related manuscript sections; MC performed SEM experiments; DC, CS and GD conceived the study.

Supplementary files

Supplementary figure 1- Viability monitoring during growth in bottle and batch experiments at different time points.

Declaration of Competing Interest

The authors declare non conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2022.e00732.

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