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Production of butyric acid by different strains of *Lactobacillus plantarum* (*Lactiplantibacillus plantarum*)

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ABSTRACT

The butyric acid content produced by three *Lactobacillus plantarum* (*Lactiplantibacillus plantarum*) strains (FP37, FP38 and FP48) deposited as probiotic strains was determined. The experimentation was carried out to evaluate whether the production of butyric acid was due to lipolytic activity for the presence of specific lipases or was related to the fermentation of saccharides. Therefore, the three strains were inoculated in different substrates with and without the addition of fat (tributyrin). The free butyric acid contents were determined by gas chromatography after distilling each culture medium. The results showed that butyric acid production occurred mainly in the substrates supplemented with fat and therefore it was linked to the activity of specific *L. plantarum* lipase. The L. *plantarum* FP48 strain produced the highest amount of butyric acid in fat substrates and could be a useful strain in the development of dairy products with functional properties.
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1. Introduction

Butyric acid is a saturated and short-chain fatty acid composed of four carbon atoms that causes the characteristic smell of rancid butter. It is naturally produced by anaerobic bacteria with applications in the food and pharmaceutical industries. In its ester form is used as a food flavour additive to increase fruit-like fragrance and its various derivatives are used as ingredients in drugs to treat several diseases (Jiang et al., 2018).

Different effects of butyric acid on health have been demonstrated. In the gut, butyrate produced by intestinal microflora can play a regulatory role in transepithelial fluid transport, improves the possible inflammatory state of the mucosa, and modulates visceral sensitivity and intestinal motility (Xiao et al., 2021). It has a trophic, beneficial and protective role in the colonic mucosa, and it is a protective agent against inflammatory bowel diseases (Tralongo et al., 2014).

These anti-inflammatory properties seem to be present not only when butyrate is produced by the gastrointestinal microbiota, but also when butyrate is taken up orally (Kratz, Baars, & Guyenet, 2013; Manrique Vergara & González Sánchez, 2017). In in vitro models, anticarcinogenic effects of butyrate are observed: the addition of butyrate leads to inhibition of proliferation, induction of apoptosis, or differentiation of tumour cells (Raman, Ambalam, & Doble, 2016). It was also found that butyric acid can reduce blood cholesterol levels in broiler chickens (Mansoub, 2011). Moreover, recent opinions suggest that microbial short chain fatty acids and butyrate could be a supportive treatment for patients with Coronavirus SARS-CoV-2 infection (Archer & Kramer, 2020; Patil, Bhandary, Haridas, Sarathkumar, & Shetty, 2021; Sarkar, Borah, & Sharma, 2020).

Coppola, Avagliano, Calignano, and Berni Canani (2021) found that butyrate could prevent obesity and related metabolic diseases both by endogenous production and by exogenous intake through butyrate supplements. A randomised clinical trial suggested that oral butyrate supplementation may be effective in the treatment of paediatric obesity (Coppola et al., 2022). Due to these characteristics, enhancing the intake of butyric acid in diet is an interesting approach to prevent several diseases. Butyrate dietary intake could be increased by the consumption of dairy products where butyrate is present due to microbial anaerobic fermentation of fibre, including cellulose, in the ruminant gut.

Butyric acid is present in milk fat in quantity ranging between 3 and 4.6% (Vaseji, Mojgani, Amirinia, & Iranmanesh, 2012). It was also found in different types of cheese such as Grana Padano and

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Parmigiano Reggiano cheese. In this latter the content of butyric acid was found at values ranging from 3.4 to 144 mg 100 g^{-1} fat in function of the stage of ripening (Summer et al., 2017). It occurs in butter at approximately 3% and in goats' milk cheese at approximately 1–1.8% (USDA, 2006). At the typical fat content (4%) of dairy products, the butyric acid can have relevant clinical effects on body mass and metabolic health (Kratz et al., 2013).

In nature the majority of butyric acid is generated in the colon of mammals by bacterial fermentation of dietary fibre, undigested starch, and proteins (Guilloteau et al., 2010; Wang et al., 2019; Warman, Jia, & Kato, 2022), but it can also be produced by host lipases from the triglyceride tributyrin, used as a prodrug to deliver biologically active butyrate (Miyoshi et al., 2011; Stilling et al., 2016). The butyrate-producing bacteria are anaerobic microorganisms and belonging to the genera *Clostridium, Butyrvibrio, Butyribacterium, Eubacterium, Fusobacterium, Megasphera,* and *Sarcina* (Fu, Liu, Zhu, Mou, & Kong, 2019). These microorganisms, via Embden–Meyerhof–Parnas (EMP) pathway, can oxidise sugar, and occasionally amylose and pectin, to pyruvate. Pyruvate is then oxidised to acetyl-CoA with the production of CO_2 and H_2. Part of the acetyl-CoA is converted into acetic acid and the other part generates acetoacetyl-CoA, which is reduced to butyryl-CoA, the precursor of butyrate (Ciani, Comitini, & Mannazzu, 2013). For these reasons, in recent years several studies have been conducted on the possibility of use of these microorganisms, especially Clostridia, in bioreactors to produce butyric acid from fibre-rich by-products (Akhtar et al., 2018; Fu, Yang, Wang, Wang, & Tang, 2017; Oh et al., 2019). However, Clostridia are pathogenic (Zaragoza, Orellana, Moonen, Moutafis, & Marcellin, 2019), and this limits their use in the food industry.

Lactic acid bacteria (LAB) are, instead, commonly used in the food industry because of their acceptance as "generally recognised as safe" status for human consumption and they too are able to produce butyric acid (Özcelik, Kuley, & Özogul, 2016). In particular *Lactobacillus plantarum* (*L. plantarum*), recently reclassified as *Lactiplantibacillus plantarum* (Zheng et al., 2020), is a major widespread species among LAB due to its ecological niches, existing in dairy products and fermented foods. Many strains of *L. plantarum* have studied for their probiotic effects and for this reason they were used for the development of therapeutic and functional foods (Seddik et al., 2017).

Probiotics are live microorganisms, which when administered in adequate amounts, could exert a health benefit on the host. The beneficial effects are often strain specific. Cebeci and Gürakan (2003) characterised 13 strains of *L. plantarum* for their probiotic properties, in particular they studied tolerance to acid and bile salts, ability to ferment fructooligosaccharides, β-galactosidase activity and susceptibility to antibiotics. *L. plantarum* strains were found in natural whey starter cultures used for the manufacture of mozzarella cheese (De Angelis et al., 2008) and were isolated also from different types of cheese (Fernandes et al., 2017; Ngamsomchat et al., 2022; Zago et al., 2011).

Previous studies reported that *L. plantarum* was capable of producing butyric acid (Pessione et al. 2015; Özcelik et al., 2016) and FP37, FP38 and FP48 strains were included as probiotic in public repositories also for their ability to produce butyric acid during fermentation. As for all LAB species, the butyrogenic capability of *L. plantarum* could be related to the fermentation of saccharides via EMP pathway, but it is known that *L. plantarum* is also a source of strain-specific intracellular esterases and lipases (Esteban-Torres, Mancheño, de las Rivas, & Muñoz, 2015; Lopes et al., 2002), able to hydrolyse esters containing short-chain fatty acids such as those occurring in milk fat. So far the butyrogenic capability of *L. plantarum* is not assigned to any specific metabolic pathway and is highly dependent on the substrate type (Botta et al., 2017). When L. *plantarum* was used as a probiotic in a co-culture with other LAB strains to inoculate milk for cheese-making, it was expected that short-chain fatty acids would form by the bacterial fermentation of lactose, that is to be included amongst the non-digestible carbohydrates with respect to probiotics (Kuczyńska, Wasilewska, Biczysko, Banasiewicz, & Drews, 2011). Therefore, in cheese, there are two independent ways that would cause butyric acid formation, one by the action of lipolytic enzymes and the other by fermentation from carbohydrates.

This work is a preliminary study on the ability of *L. plantarum*, present in different types of dairy products, to produce butyric acid by evaluating whether the production is due to lipolytic activity for the presence of specific lipases or is related to the fermentation of saccharides. To this aim the FP37, FP38 and FP48 strains, classified as probiotic also for their ability to produce butyric acid, were inoculated in different substrates with and without the addition of tributyrin, the fat used to test the lipase activity, and the butyric acid content was determined.

2. Materials and methods

2.1. Fermentation test

The following *Lactobacillus plantarum* (*Lactiplantibacillus plantarum*) strains from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH collection were used for the experiments with all types of substrates: *L. plantarum* FP37 (DSM 22098), *L. plantarum* FP38 (DSM 22099), *L. plantarum* FP48 (DSM 22100).

The lyophilised microbial strains were revitalised by individual inoculation in 10 mL of de Man, Rogosa and Sharpe broth (MRS) and incubated at 30 °C for 16 h. The purity of the microbial strains was verified by inoculating 10 μL of each strain into MRS agar plate by the spread/streak technique and then incubating at 30 °C for 72 h. Portions of the microbial strains were stored in MRS broth cultures with 20% glycerol at −25 °C, while other portions (working cultures) were prepared in 30 mL of MRS broth, incubated for 24 h at 30 °C and used for inoculation in the fermentation tests.

The working cultures from the FP37, FP38 and FP48 microbial strains were inoculated at 3×10^6 cfu mL^{-1} in the substrates skimmed milk (SM), MRS broth, tryptone soya broth (TSB) and synthetic fat-free medium (SFF) for 24 h at 30 °C.

SM is a skimmed ultra high temperature sterilised milk purchased from the local market and has the following nutritional composition: 4.9% carbohydrates, 3.3% protein, 0.5% fat and 0.1% salt. MRS (Oxoid, Basingstoke, UK) is composed of peptone, beef extract, yeast extract, glucose, sorbitan monooleate, dipotassium hydrogen phosphate, sodium acetate $3H_2O$, triammonium citrate, magnesium sulphate $7H_2O$, and manganese sulphate $4H_2O$. TSB (Oxoid) is composed of pancreatic digest of casein, enzymatic digest of soya bean, sodium chloride, dipotassium hydrogen phosphate, and glucose. SFF was created in our laboratory by mixing dextrose, peptone, and yeast nitrogen base (patent pending).

To evaluate the butyrogenic capacity of the microbial strains as a function of the fat media, the culture media, MRS, TSB and SFF were added to 30 g L^{-1} tributyrin (Sigma–Aldrich, Milano, Italy) using a T-25 Ultra Turrax Ika-Werke (Staufen, Germany), and 3×10^6 cfu mL^{-1} working cultures were inoculated into them. The uninoculated substrates were incubated under the same conditions and used as controls.

The tests were carried out in triplicate in sterile glass bottles. The aliquots taken under sterile conditions from the test bottles at

0, 6 and 24 h of fermentation were collected and submitted to chemical analysis.

2.2. Fat extraction from substrate

All solvents and reagents used in the determinations were supplied by Sigma–Aldrich (Milano, Italy).

2.2.1. Fat extraction from SM

The milk fat content of SM was determined after extraction with ethyl ether from an acid solution, which was followed by evaporation and weighing of the residue, as reported by IDF (1986) with some modifications. A 160 mL homogeneous sample aliquot was subjected to extraction in the following manner: it was centrifuged at 7000×*g* for 10 min to favour cream surfacing. The surfaced cream was recovered, and 1 mL of hydrochloric acid, 10 mL of ethanol and 20 mL of a mixture of ethyl ether:heptane at a ratio of 1:1 (v/v) were added; the whole mixture was shaken for a few minutes to homogenise and then centrifuged at 2500×*g* for 10 min. The separated supernatant containing the lipid fraction was filtered into a flask using a filter-coated funnel in which 2 g of anhydrous sodium sulphate was placed. The extractions of the remaining samples were repeated, another 20 mL of the ether-heptane mixture was added, and these results were then centrifuged and filtered as described above. The extracted fat was dried using a removing solvent with a Rotavapor Labourota 4000-efficient (Heidolph Instrument, Germany) at 50 °C. The amounts of extracted fat were expressed as percentages of weight by volume.

2.2.2. Fat extraction from MRS, TSB and SFF

The fat contents of MRS, TSB and SFF were determined according to the method of Folch, Lees, and Stanley (1957), with some modifications. Approximately 7 g of sample was weighed in a test tube, and 10 mL of deionised water was added. After shaking, 10 mL of a chloroform-methanol 2:1 (v/v) mixture was added and centrifuged at 8000×*g* for 10 min. The supernatant was transferred to a 100-mL flask. The extraction steps were repeated three times. The extract was dried with a Rotavapor Labourota 4000-efficient (Heidolph Instrument, Germany) at 40 °C and recovered with hexane. Then, 3 mL of saturated NaCl solution was added to the test tube. This was centrifuged at 8000×*g* for 10 min. An additional 3 mL of hexane was added two times. The supernatant was taken and filtered on filter paper with anhydrous sodium sulphate. The solvent was removed, and the quantity of extracted fat was weighed. The quantity of extracted fat was expressed as a percentage (w/w).

2.3. pH and optical density

The pH and optical density measurements were conducted with a Crison pH metre (Basic 20 Instruments, Barcelona, Spain) and a UV–Visible scanning spectrophotometer (Shimadzu, Milan, Italy) at 600 nm, respectively, using 10-mL aliquots that were obtained under sterile conditions from the test bottles at 0, 6 and 24 h of fermentation. Optical density measurements of the OD_{600} values were used to determine the microbial growth levels in the solutions (cfu mL^{-1}).

2.4. Determination of free butyric acid

The free butyric acid contents in the substrates were determined at the end of the fermentation test by gas chromatography coupled with mass spectrometry (GC/MS) after steam distillation with a Cazenave distiller. A 20-mL volume of substrate, 200 mg of $CaCl_2$ and 0.5 mL of antifoam were added to the distiller tube. The distillate (40 mL) was recovered in 1 mL of 1 N NaOH solution. At the end of distillation, 1 mL of 1 N HCl was added. Then, 2 mL of diethyl ether containing 2-methyl-3-heptanone (10 mg kg^{-1}) as an internal standard was added to 10 mL of distillate. After centrifugation at 8000×*g* for 5 min, 1 μL of the ether solution was injected into the GC–MS. The same procedure was performed on standard aqueous solutions of butyric acid that were prepared at different concentrations to create a calibration curve. For the analysis, a Hewlett-Packed 6890 N gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with an HP-5MS (J&W Scientific, Folsom, CA, USA) capillary Column 30 m × 0.25 mm i. d. × 0.25 μm film thickness was used as described by Manzo et al. (2019). The chromatographic conditions were modified as follows: helium as the transport gas with an initial oven temperature of 45 °C for 2 min, which was followed by an increase of 6 °C min^{-1} up to 80 °C, then an increase of 20 °C min^{-1} up to 210 °C. The injector was set to splitless mode and maintained at a temperature of 250 °C. Butyric acid was identified by comparison with the mass spectrum provided by the NIST database and with the RI of the authentic compound. The method detection limit (LOD) was 0.25 ppm, and the minimum quantifiable concentration (LOQ) was 0.50 ppm of butyric acid.

2.5. Statistical analysis

All determinations and experiments were performed in triplicate, and the results are presented as the average values (± standard deviations) of three replicates. One-way analysis of variance (ANOVA) and Tukey's multiple-range tests ($p \leq 0.05$) were carried out on the data using XLSTAT software (Addinsoft, New York, NY, USA).

3. Results and discussion

3.1. Butyric acid production in sterilised skimmed milk

The butyric acid quantities released in the sterilised milk samples that were fermented for 24 h with the three lactic acid bacteria strains were determined; these samples exhibited differences depending on the culture strain used (Table 1). In this substrate the fat content was found to be 0.47% and the butyric acid content varied from 1.7 mg kg^{-1} (FP48) to 10.5 mg kg^{-1} (FP37), which implied a strain-dependent relationship.

In addition to butyric acid (C4), the volatile water-soluble fatty acids, caproic (C6), caprylic (C8) and capric (C10), were extracted by steam distillation of fermented milk that was inoculated with the lactobacilli strains. The detection of long- and medium- and short-chain fatty acids in the fermented milk suggests that lipoprotein lipases might be responsible for hydrolyses of fatty acids from milk triacylglycerols. In fact, C10 and C12 fatty acids, which are known to be located on milk triglycerides at the sn-2 position, and C4–C8 represent the main components of volatile fatty acids that are likely produced by cell-associated esterase activity, which is a similar result to that already reported in previous research (Corsetti & Gobbetti, 2002). Data obtained from this study also confirm the previous findings by Esteban-Torres et al. (2015), which demonstrated that *L. plantarum* had a halotolerant lipase that is located extracellularly or associated with whole cells, which is especially attractive in food fermentations. We also found 0.5 mg kg^{-1} butyric acid in the uninoculated control sample, which was incubated under the same conditions, and was probably released by the lipolytic activity of bacterial lipase that may survive in sterilised milk (Martins, Ahmad, Silva, & Andrade, 2015).

Moreover the acidifying capacities of the three bacterial strains that were tested in sterilised skimmed milk varied (Fig. 1). The

Table 1
Fat and free butyric acid content in different substrates inoculated with *L. plantarum* strains (FP37, FP38 and FP48) and uninoculated (control) for 24 h.[a]

Substrate	Fat (%)	Butyric acid (mg kg^{-1})			
		Control	FP37	FP38	FP48
SM	0.47 ± 0.04^{b}	0.51 ± 0.08	10.56 ± 0.09^{d}	6.11 ± 0.21^{cd}	1.74 ± 0.16^{d}
MRS	0.03 ± 0.01^{c}	n.d.	12.91 ± 0.21^{d}	10.50 ± 0.11^{c}	16.75 ± 0.31^{c}
MRS + tributyrin	3.03 ± 0.02^{a}	n.d.	46.72 ± 0.32^{a}	26.90 ± 0.34^{a}	113.90 ± 0.84^{a}
TSB	0.02 ± 0.01^{c}	n.d.	7.73 ± 0.10^{d}	6.10 ± 0.19^{cd}	6.70 ± 0.22^{cd}
TSB + tributyrin	3.02 ± 0.02^{a}	n.d.	33.23 ± 0.17^{b}	19.80 ± 0.23^{b}	97.50 ± 0.51^{b}
SFF	n.d.	n.d.	n.d.	n.d.	n.d.
SFF + tributyrin	3.00 ± 0.02^{a}	n.d.	21.73 ± 0.19^{c}	9.45 ± 0.18^{c}	48.87 ± 0.61^{c}

[a] Abbreviations are: SM, skimmed milk; MRS, De Man Rogosa and Sharpe broth; TSB, tryptone soya broth; SFF, synthetic fat-free medium; n. d., not detected. Different superscript letters in the same column indicate statistically significant differences ($P < 0.05$).

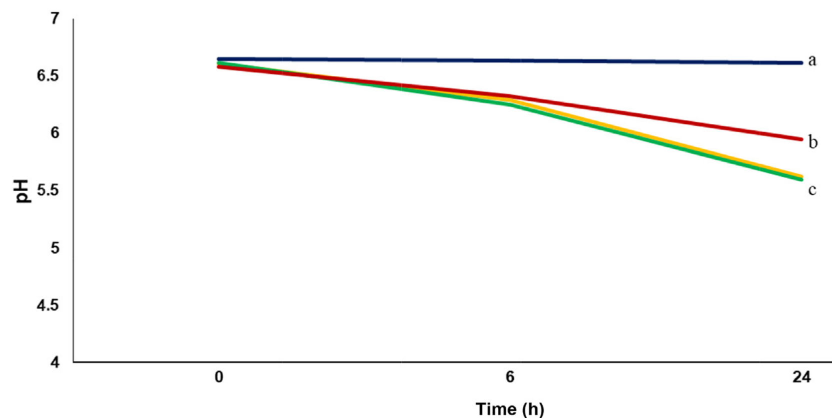

Fig. 1. pH value during the fermentation process of skimmed milk inoculated with *L. plantarum* strains (——, FP37; ——FP38; ——; FP48) and uninoculated (control, ——). Different letters indicate statistically significant differences between the strains ($P < 0.05$).

initial pH value of 6.60 decreased to 5.90 for the FP48 strain and to 5.50 for the FP37 and FP38 strains in parallel with the formation of butyric acid for all bacterial strains. In fact, the FP48 strain, which showed a lower acidifying capacity, produced the lowest amount of butyric acid (1.7 mg kg^{-1}), and the results showed opposite patterns for the FP37 and FP38 strains. Monitoring the initial pH was important because it could affect the production of butyric acid, as reported by Calero, Lagoa-Costa, Fernandez-Feal, Kennes, and Veiga (2018) who found an increase in butyric acid by decreasing the pH from 6 to 5 of cheese whey used in a sequencing batch reactor.

Although the common metabolic pathways for lactic acid production have been identified (Zhang & Vadlani, 2015), it is clear that these strains have different metabolic pathways for butyrate production, and this may influence a cheese manufacturer's choice of the strain depending on the cheese to be produced. For example strains that maximise the production of butyric acid should be avoided in Mozzarella cheese, while in other types of cheese, specially ripened cheese, could be employed. In any case, the butyric acid concentrations should be below the threshold (46 mg kg $^{-1}$) that is associated a rancid taste in dairy products, as reported by Scanlan, Sather, and Day (1965).

3.2. Butyric acid production in synthetic substrates

To discriminate between fat lipolysis or saccharide fermentation by *L. plantarum* strains, fermentation tests using the FP37, FP38 and FP48 strains were performed using the synthetic substrates, MRS, TSB and SFF, that contained <0.03% fat (Table 1). Subsequently, the fermentation tests were performed with the same substrates after supplementation with the same quantity of tributyrin, which is a synthetic triglyceride that does not exist in milk fat and is routinely used to test the ability of microbial strains to synthesise lipases (de Los Angeles Camacho-Ruiz, Mateos-Díaz, Carrière, & Rodriguez, 2015).

After evaluating the pH changes of lactobacilli grown in MRS broth after 24 h, the FP48 strain showed a pH decrease from approximately 6 to 4.6, while the FP37 and FP38 strains showed more pronounced decreases, which reached the lowest pH values (3.80) with the same kinetics (Fig. 2). For the tributyrin-enriched culture medium, the pH changes for the three *lactobacilli* strains after 24 h were essentially the same, with FP48 being the worst acidifying strain.

In contrast, the pH levels decreased more markedly in the TBS medium inoculated with the FP48 strain (pH change from 6.85 to 5.65 after 24 h) (Fig. 3). This could be explained by the fact that pH decreases in media are a means to evaluate the adaptation of a strain to the environment. This adaptation is a complex phenomenon that depends on many variables, such as the strain used and the ingredients in the medium, as reported by Bevilacqua, Corbo, Mastromatteo, and Sinigaglia, 2008. Compared with FP48, the other two strains of *lactobacilli*, FP37 and FP38, showed more restrained acidification trends as the final pH at 24 h stopped at 6.75 regardless of the presence of tributyrin (Fig. 3). When fermenting the studied strains in parallel using the same substrates (TSB and MRS) supplemented with tributyrin, the presented acidification kinetics were comparable with those exhibited without the addition of tributyrin with similar final pH values (Figs. 2 and 3). In general, compared with the MRS medium, the strains showed a lower ability to acidify the TSB medium. This could be linked to the presence of MRS and, thus, to the absence of yeast extract in the TSB medium, which positively influences the production of biomass and therefore the growth of microbial strains (Bevilacqua et al., 2008). All strains exhibited good and similar performances in the MRS and TBS substrates and exhibited counts higher than 7 log cfu

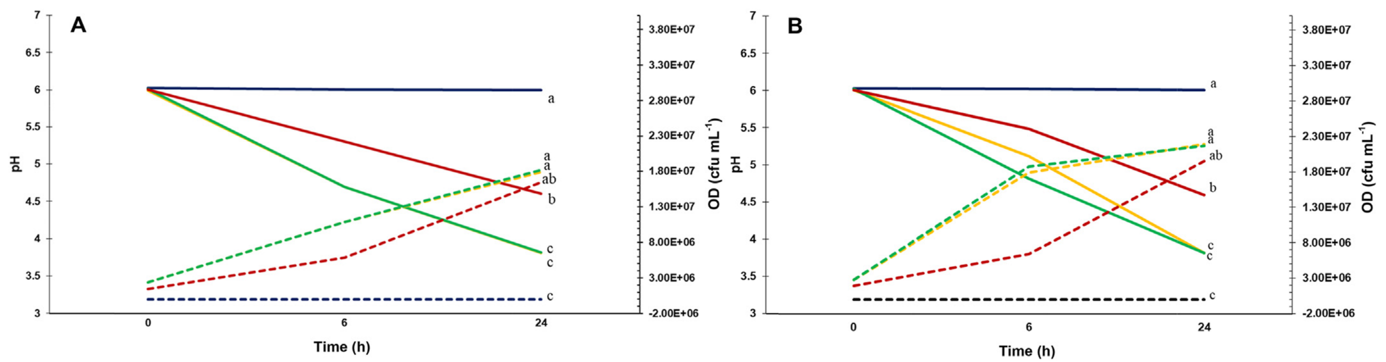

Fig. 2. pH values (solid lines) and microbial growth (dashed lines; cfu mL^{-1}) during fermentation for 24 h in (A) MRS and (B) MRS with tributyrin inoculated with *L. plantarum* strains (—, - -, FP37; —, - -, FP38; —, - -, FP48) and uninoculated (control, —, - -). Different letters indicate statistically significant differences between the strains for each parameter ($P < 0.05$).

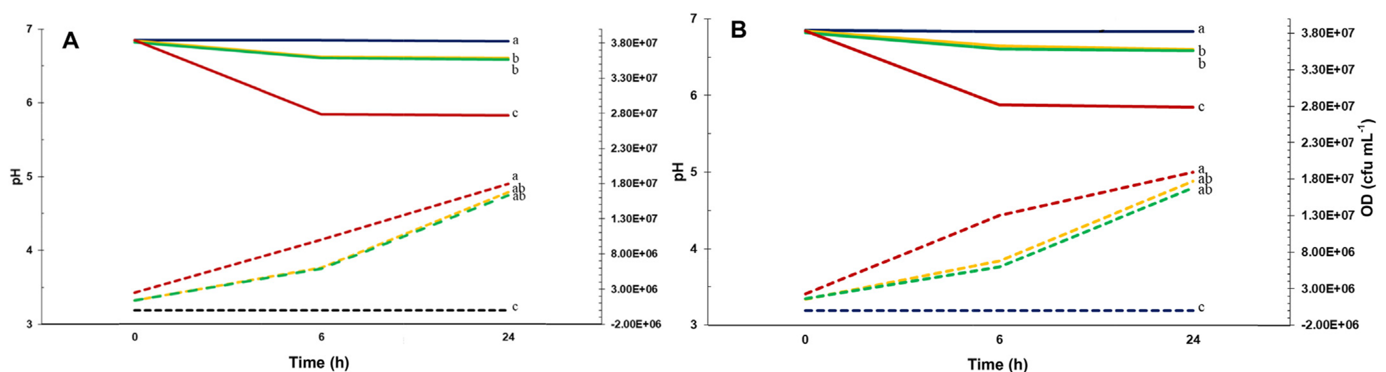

Fig. 3. pH values (solid lines) and microbial growth (dashed lines; cfu mL^{-1}) during fermentation for 24 h in (A) TSB and (B) TSB with tributyrin inoculated with *L. plantarum* strains (—, - -, FP37;—, - -, FP38; —, - -, FP48) and uninoculated (control, —, - -). Different letters indicate statistically significant differences between the strains for each parameter ($P < 0.05$).

g^{-1} for up to 24 h of fermentation (Figs. 2 and 3). In the MRS substrate, the strains with the highest growth were FP37 and FP38. In TSB broth, the trend was different, and the FP48 strain showed the highest growth. This further confirms the hypothesis, which is advanced on the basis of the pH values, that the FP37 and FP38 strains adapt and therefore grow better in the MRS substrate, while FP48 grows better in the TSB substrate. Additions of tributyrin did not significantly affect either the microbial growth or trends in pH values in either the MRS or TBS medium.

However, significant changes in butyric acid production were observed when the strains were grown in MRS broth, as shown in Table 1. Compared with the results obtained with sterilised milk, the strains grown in tributyrin-enriched MRS broth released higher amounts of butyric acid, which were in the 26.9–113.9 mg kg^{-1} range. Furthermore, the behaviour of the strains was reversed because *L. plantarum* FP48, which was the worst producer of butyric acid in milk, became the best producer in the MRS culture medium and also for the FP38 strain, which changed from best and became the worst. The control sample contained trace amounts of butyric acid (0.5 mg kg^{-1}).

In TSB broth supplemented with tributyrin, the levels of butyric acid produced varied in the range from 19.8 to 97.5 mg kg^{-1} depending to the strains grown. *L. plantarum* FP48 and FP38 were confirmed as the best and worst butyric acid producers, respectively. The elevated non-esterified fatty acid concentrations can be explained by the presence of lipases demonstrating strong fatty acid specificity towards butyric acid occupying the sn-1 and sn-3 positions of tributyrin. *L. plantarum*-associated lipases have been reported to exhibit increased specificity from short-to medium-chain fatty acids (Gobbetti, Fox, Smacchi, Stepaniak, & Damiani, 1996). The presence of 6.1–7.7 mg kg^{-1} butyric acid in TSB broth and 10.5–16.7 mg kg $^{-1}$ in MRS without tributyrin supplementation was attributed to the presence of 0.03–0.02% fat in the meat and yeast extract, which were both ingredients of the culture media that underwent lipolysis.

Since the synthetic media MRS and TSB used as culture broths for the growth curve experiments showed trace concentrations of triglycerides (0.03–0.02% fat), to verify whether the butyric acid production by the LAB strains was due to lipolysis or polysaccharide fermentation, we used SFF medium prepared with fat-free ingredients for bacterial growth as previously described. Using fully defatted culture media, the acidification and growth kinetics similar to those of MRS medium were obtained (Fig. 4), which confirmed the positive influence of yeast extract on microbial proliferation, especially of the FP37 and FP38 strains.

The results (Table 1) indicate that all strains were not butyric acid producers when a fat source was missing in the culture medium. Conversely the three LAB strains released butyric acid in substrates containing synthetic tributyrin, which confirmed that the butyric acid production in milk is derived exclusively from the lipases associated with the bacterial strains.

Fermentations in defatted synthetic media supplemented with tributyrin produced butyric acid levels via the triglyceride hydrolysis in the range of 1.7–113 mg kg $^{-1}$. Specifically, the FP48 strain was the most productive strain (113 mg kg^{-1}) in MRS supplemented with tributyrin, while the butyric acid yields from fermentation by the FP37 and FP38 strains in the same medium were considerably lower, at 46 and 26 mg butyric acid kg $^{-1}$, respectively.

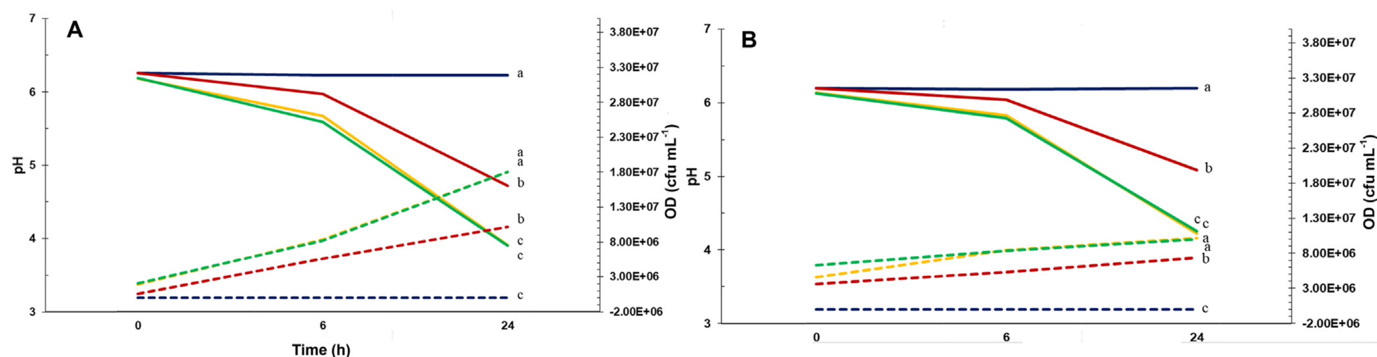

Fig. 4. pH values (solid lines) and microbial growth (dashed lines; cfu mL^{-1}) during fermentation for 24 h in (A) fat-free medium and (B) fat-free medium with tributyrin inoculated with *L. plantarum* strains (—, – –, FP37; —, – –, FP38; —, – –, FP48) and uninoculated (control, —, – –). Different letters indicate statistically significant differences between the strains for each parameter ($P < 0.05$).

4. Conclusions

The probiotic FP37, FP38 and FP48 strains of *Lactobacillus plantarum* (*Lactiplantibacillus plantarum*) were able to grow in culture media and produce butyric acid in a strain-dependent metabolic manner. The butyrogenic capability of the three strains tested was related to lipase-mediated triglyceride hydrolysis and not to saccharide fermentation. This was demonstrated by the use of a synthetic fat-free medium, where saccharide was present but butyric acid was not produced at all. Accordingly, in the media supplemented with tributyrin, the butyric acid releases were significantly higher and were clearly attributed to lipase-mediated triglyceride hydrolysis associated with *L. plantarum* strains. The low butyric acid contents in the MRS and TSB substrates could be due to trace amounts of fat. This result substantiates previous findings in the literature indicating that *L. plantarum* is a good source of lipases and esterases since lipolytic and esterase activities share a common catalytic mechanism.

Ultimately, among the three strains of *L. plantarum* deposited as probiotics, FP48 was the strongest butyric acid-producing strain in fat substrates. Due to the beneficial properties on human health of butyric acid reported in literature, FP48 could be a useful strain in the development of dairy products with functional properties.

Author contributions

Alessandra Aiello: formal analysis, writing—original draft preparation, conceptualization. **Fabiana Pizzolongo:** conceptualization, methodology, investigation, writing—review and editing, supervision. **Lucia De Luca**: data curation, visualization. **Giuseppe Blaiotta**: formal analysis. **Maria Aponte**: supervision. **Francesco Addeo**: conceptualization, writing—original draft preparation. **Raffaele Romano**: conceptualization, resource, project administration, funding acquisition. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

None.

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