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# MOLECULAR CHARACTERISATION AND BIOMASS AND METABOLITE PRODUCTION OF *LACTOBACILLUS REUTERI* LPB P01-001: A POTENTIAL PROBIOTIC

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## ABSTRACT

*Lactobacillus reuteri* LPB P01-001 was isolated from the gastrointestinal tract of wild swine and was characterised by biochemical testing and sequencing of gene 16S rRNA. A simple and low-cost culture medium based on cane sugar (2.5% p/v) and yeast extract (1% p/v) was used in the production of this probiotic. The fermentative conditions were a) pH control at 6.5 and b) no pH control; both were set at 37°C in a 12 L slightly stirred tank bioreactor. Fermentation parameters such as the specific growth rate, productivity and yield of biomass, lactic and acetic acid levels were determined. *L. reuteri* LPB P01-001 behaves as an aciduric bacteria because it grows better in a low pH medium without pH control. However, the lactic acid production yield was practically half (9.22 $g.L^{-1}$) of that obtained under a constant pH of 6.5, which reached 30.5 $g.L^{-1}$ after 28 hours of fermentation. The acetic acid production was also higher under pH-controlled fermentation, reaching 10.09 $g.L^{-1}$ after 28 hours of fermentation. These parameters may raise the interest of those committed to the efficient production of a probiotic agent for swine.

**Key words:** Probiotic, *Lactobacillus reuteri*, Molecular characterisation, Fermentation parameters, Lactic and acetic acids.

## INTRODUCTION

*Lactobacillus reuteri* is an obligatorily heterofermentative lactic acid bacteria, a microaerophilic, and is a common inhabitant of the gastrointestinal tract of humans (28, 36) and animals such as pigs (12, 24, 33), turkeys, chickens, and monkeys (24). *L. reuteri* also belongs to the predominant microflora of fermented cereal products and meat (14, 15, 25).

Some species of *L. reuteri* produce the enzyme invertase, which is used in converting sugar from sucrose (17, 26). In addition, *L. reuteri* also produces a large amount of glucan and fructan exopolysaccharides, which are considered prebiotics (22). These prebiotics have been investigated with regards to antitumour activity (52), immunomodulation (55), and cholesterol reduction (50). In recent years, there has been considerable interest in the use of probiotic microorganisms and organic acids as alternatives to antibiotics in feeds to reduce antibiotic residues in the carcass, among other excellent benefits such as diarrhea control and immunostimulation (2, 37, 42, 48, 54, 61). The antimicrobial effect exerted by

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*Lactobacillus* arises from its production of many compounds, mainly organic acids, peroxide hydrogen, bacteriocins and reuterin (1, 16, 45, 47, 58, 49).

*Lactobacilli* strains require a nutritional complex frequently found in media containing fermentable carbohydrates, amino acids, vitamins, nucleic acids precursors, and minerals to produce significant biomass (8, 11, 25), which results from a series of highly coordinated enzyme-catalysed events. Lactic acid bacteria are generally cultivated on MRS medium (11).

One of the most important parameters for cultivating acid lactic bacteria is the pH of the fermentation environment (7, 56). *Lactobacilli* are microorganisms with an optimal pH around 5.5 – 6.2, and their growth generally occurs at pH 5.0 or lower; their growth rate is often reduced at a neutral pH or in media that are initially alkaline (25). The limitations of growth and acid production by the end-product are well known. Kashket (27) reported growth inhibition in *Lactobacillus* by cytoplasm acidification via the produced acid. Additionally, the energy gained by lactate production is no longer available for cell growth, but it is used to some extent for the maintenance of pH homeostasis.

Some of the important aspects for industrial production of probiotics are related to the microorganism itself, the cost of nutrient substrates, and the processes used in their production and recovery are a few important aspects of *Lactobacilli* in the industrial production of probiotics. In addition, parameters for scale optimisation and amplification are necessary. According to Schmidell (56), different phases of the process must be evaluated, such as the kinetics of growth, and primary or secondary metabolite production, as well as separation, recovery and formulation of the products. The fermentative process requires monitoring parameters of the culture system as a function of the fermentation time (23).

In a spontaneous fermentation process, lactic acid bacteria evolve in non-pH-controlled conditions, but for other applications (e.g., lactic acid or biomass production), it may be necessary to achieve pH control (20). The optimal pH for the growth of various strains of lactic bacteria has been previously determined as has the correlations between pH and lactic acid concentration (18, 38).

*L. reuteri* LPB P01-001 has been isolated from the gastrointestinal tract of wild swine and fulfils some important requirements for use as a probiotic: it is non-pathogenic, non-toxigenic, bile-resistant, and tolerant to gastric acidity, and it produces antimicrobial compounds with the ability to reduce pathogens, normal inhabitants of the gut, that are host-specific. Furthermore, technological aspects include the ability of *L. reuteri* LPB 01-001 to withstand lyophilisation, freeze-drying processes and the final formulation of the product (43, 44). However, more criteria presented by FAO/WHO (13) must be determined, which include the following: antioxidant activity, ability to modulate the immune response and adherence in intestinal tissue.

Thus, the present work aimed to develop a low-cost culture medium and to determine convenient growth conditions for the potential probiotic *L. reuteri* LPB P01-001 strain, to perform its molecular identification, and to evaluate fermentation parameters such as biomass yield and production of lactic and acetic acids, which are also considered to be inhibitory substances for pathogens.

## MATERIALS AND METHODS

### Isolation and biochemical characterisation of *L. reuteri* LPB P01-001

*Lactobacillus* strains were obtained from the gastrointestinal tract of swine by plating on MRS agar (Merck) with 5% bile (40), and the colonies were submitted to Gram staining and catalase testing. Their biochemical phenotypic properties were studied by means of sugar fermentation and other biochemical reactions using the API 50 CH gallery system (Biomerieux) for *Lactobacilli* identification (5, 35). The strain was characterised as an acid and bacteriocin producer

(43, 44), according to growth inhibition tests.

**Antimicrobial activity**

To study its antimicrobial activity, the isolated *L. reuteri* LPB P01-001 was grown in MRS broth (Merck) and centrifuged. The supernatant, after being sterilised by filtration, was tested against two pathogenic strains: *Staphylococcus aureus* coagulase positive (ATCC 14458) obtained from CEPPA, Brazil, and a swine haemolytic *Escherichia coli* LME21, from Enrietti's Lab, Brazil. These indicator strains were grown in Tryptone Soy Broth at 37ºC for 24 h and were used for testing the supernatant in Muller Hinton (Difco) broth and on agar plates.

Fermented MRS broth was centrifuged to remove bacterial cells (6000 rpm for 30 min), and the resulting supernatant was concentrated to 10% of the original volume under vacuum at 45ºC. The pH of the material was adjusted to 5.5, and the sample was filtered through a sterile 0.22 µm membrane (44).

**Inhibition of indicator pathogenic strains in liquid media**

Sterile supernatant was added in the same amount to double-concentration Mueller-Hinton broth and then inoculated with the above-mentioned testing strains (OD 0.040). The absorbance at 620 nm was periodically recorded to monitor the growth inhibition effect (44).

**Molecular identification of the *L. reuteri* LPB P01-001 strain**

Confirmatory molecular tests were carried out according to the following protocol: total genomic DNA was isolated following Young and Blakesley's method (63), and the oligonucleotide primers p27f (31) and p1401r (21) were used for PCR. The reaction was carried out in a BioRad (Thermal Cycler) with 50 µL of DNA (50-100 ng), 0.2 mmol $L^{-1}$ dNTP mixture, 1.5 mmol $L^{-1}$ $MgCl_2$, 0.4 µmol $L^{-1}$ of each primer and 2 U of Taq DNA polymerase (Invitrogen). Amplification was conducted using an initial denaturation step at 95ºC for 2 min, followed by 30 cycles of 1 min at 94ºC, 1 min at 55ºC, and 3 min at 72ºC, and a final step of 5 min at 72ºC.

Fragments of amplified 16S rRNA were purified using a column (GFX PCR DNA and Gel Band Purification Kit, Amersham Biosciences), and the DNA was eluted in 30 µL of sterile ultrapure water. The samples were submitted to a sequencing process in a mega BACE 1000 apparatus (Amersham Biosciences). The primers used were p10f, p1100r (30), p765f (60), and p782r (9).

Partial sequences of 16S rRNA were compared with the 16S rRNA of related microorganisms included in the RDP (Ribosomal Database Project, Wisconsin, USA; 10; 34) and Genbank (41). Matrices of evolution distances were calculated according to Kimura's model (29), and a phylogenetic tree was constructed by following the Neighbor-Joining's method (53) using the RDP software.

**Inoculum preparation and bioreactor fermentation**

The preparation of the inoculum began with its reactivation from MRS agar. The strain was transferred to MRS broth and kept at 37°C for 48 hours. After its reactivation, the inoculum was transferred at a proportion of 10% (v/v) in relation to the total volume of the culture medium. After preliminary studies in flasks (44), the composition of the chosen E3 medium was 1% p/v yeast extract and 2.5% v/v total cane sugar, due to its low cost as compared to commercial MRS medium (unpublished data). The dry weight of biomass obtained using the E3 medium was similar to the biomass achieved in commercial MRS medium, approximately 1.3 $g.L^{-1}$, but E3 medium is 33 times cheaper than commercial medium MRS. Bench fermentation experiments were conducted under the same temperature and inoculation conditions; the fermentation period was 28 hours.

Fermentation experiments were carried out in a 12 L stirred tank bioreactor (New Brunswik) under the following conditions: a) one at a constant pH of 6.5 and another without pH control, starting at pH 4.65 in the first case; to maintain a

constant pH, 3N sodium hydroxide was added automatically; b) slight agitation at 80 rpm was used in both cases, to prevent cells from settling at the bottom of the bioreactor and to incorporate the fewest number of air bubbles, considering the microaerophilic requirements of the strain; c) the temperature was kept at 37°C. The experiments were done in triplicates and statistically analysed.

**Evaluation of kinetic parameters**

Fermented samples were collected to determine the following parameters: pH, sugar consumption, biomass, and organic acid levels.

**Determination of reducing sugar**

The reducing sugar levels in the fermented samples were determined by following Somogyi-Nelson's method (39, 57), which is based on the colourimetric reaction of sugars with a cupro-alkaline reactive, which – in presence of molybdic arsenic – forms a blue-coloured compound whose maximal absorbance occurs at 535 nm. For the standard curve, a glucose solution containing 100 µg of sugar per mL of solution was used. The non-reducing sugar levels (sucrose) in the fermented samples were determined after sample hydrolysis (4) and were then determined again as described above.

**Biomass determination**

The biomass concentration in fermented samples was determined gravimetrically after filtration through a 0.22 µm pore PVDF membrane and a drying step at 80°C for 24 hours until a constant weight was reached (40).

**Organic acid identification and quantification**

Lactic and acetic acid levels were determined using HPLC chromatography with an HPX87H column, operated under the following conditions: column temperature: 60ºC, mobile-eluent phase: $H_2SO_4$, mobile phase concentration: 5 mM, outflow of the mobile phase: 0.6 $mL.min^{-1}$, pump pressure: 48 $kg/cm^2$, volume sample: 50 µL, dilution: 1:5, time retention of standards: glucose: 9.38 min, lactic acid: 12.74 min, and acetic acid: 14.92 min.

**Analyses of biomass and metabolite production during the fermentative process**

The specific growth rate µ ($h^{-1}$) was determined using the angular coefficient of the best correlation of the exponential phase of biomass growth, where the neperian logarithms of the biomass (X) concentration (LnX) ($g.L^{-1}$) versus fermentation time (h) were plotted (3).

The yield of biomass in relation to the consumed substrate ($Y_{X/S}$) – a substrate conversion factor – can be obtained from Equation 1:

$$\mathbf{Y_{X/S}\ (g.g^{-1}) = (X_f\text{-}X_i)/(S_i\text{-}S_f)}$$

where: $X_f$ = final biomass concentration; $X_i$ = initial biomass concentration; $S_i$ = initial sugar concentration; $S_f$ = final sugar concentration.

The productivity of biomass ($P_X$) is defined by Equation 2:

$$\mathbf{P_X\ (g.L^{-1}.h^{-1}) = \mu.X_f}$$

where: µ = specific growth rate; $X_f$ = final biomass concentration.

The yield of metabolic products ($Y_{P/S}$) was calculated using Hiss' formula (23), expressed by Equation 3:

$$\mathbf{Y_{P/S}\ (g.g^{-1}) = (P_f\text{-}P_i)/(S_i\text{-}S_f)}$$

where: $P_f$ = final product (lactic acid + acetic acid concentrations); $P_i$ = initial product (lactic acid + acetic acid concentrations); $S_i$ = initial sugar concentration; $S_f$ = final sugar concentration.

## RESULTS AND DISCUSSION

**Isolation and biochemical characterisation of *L. reuteri* LPB P01-001**

The *L. reuteri* LPB P01-001 strain has probiotic potential due to its bile resistance (5% bile) and phenol resistance (0.4%), isolated from healthy swine gut, and such features

are important characteristics for surviving in the intestine (unpublished data). Tolerance to bile and phenol (phenols can be formed in the gut by bacteria that have deaminated aromatic amino acids from the diet or can be produced by endogenous proteins) is important for improved survival rates, but not necessarily for multiplication in the intestine (62).

This strain has a bacillary morphology and reacts positively to Gram staining. After biochemical characterisation, the strain was presented as catalase (-); thus, it belongs to the *Lactobacillus* genus, which does not produce catalase to decompose hydrogen peroxide (25). The strain is a lactic acid bacteria because it is a lactic acid producer, as detected by HPLC. It is heterofermentative due to its production of products other than lactic acid, including acetic acid and ethanol from glucose (25), substances with possible antimicrobial activity. *L. reuteri* LPB P01-001 produces $CO_2$ from hexose and presents better growth at 37-45°C with slight growth at 15°C. The results of the API 50 CH gallery system (Biomerieux) are not conclusive regarding identification of the *Lactobacillus fermentum* species, due to limitations of the biochemical method (5). According to Bergey's Manual (25), the *Lactobacillus fermentum* species cannot be distinguished from the *Lactobacillus reuteri* species by means of simple physiological tests. Other parameters may distinguish the species, such as: % mol guanine and cytosine, diamino acid levels of peptidoglycan or the electrophoretic mobility of lactic acid dehydrogenase.

**Molecular characterisation**

The isolated strain was molecularly identified, and the sequence was analysed using the BLAST routine of the Genbank and the Sequence Match of the RDP. The partial sequence of 16S rRNA from LPB P01-001 and the phylogenetic tree are presented in Figures 1 and 2. The partial sequence of 16S rRNA from sample LPB P01-001 is 98% similar to sequences of 16S rRNA from the *Lactobacillus reuteri* lineages available in the databases. Additionally, it presented similar but lower percentages (i.e., 96 to 97%) when compared to other *Lactobacillus* species, such as *Lactobacillus vaginalis* (ATCC49540$^T$), *Lactobacillus pontis* (LTH 2587$^T$), *Lactobacillus panis* (DSM 6035$^T$) and *Lactobacillus antri* (DSM 16041$^T$). The phylogenetic analysis confirmed a closer phylogenetic proximity of the LPB-P01-001 sample with the *Lactobacillus reuteri* species. *Lactobacillus reuteri* LPB P01-001 has been deposited at the Bioprocess Engineering and Biotechnology Department.

CACCAGTGAGTGGCGGACGGGTGAGTAACACGTAGGTAACCTGCCCCGGAGCGGGGGATACATTTGGAAACAGAA
ATACCGCATAACAACAAAAGCCACATGGCTTTTGTTTTGAAAGATGGTTCGGCTATCACTCTGGGATGGACCTGCGG
TGCATTAGCTAGTTGGTAAGGTAACGGCTTCCAGGCGATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACAAT
GGAACTGAGACACGGTCCATACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGCAAGCCTGATGG
AGCAACACCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTGGAGAAGAACGTGCGTGAGAGTAAC
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CAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTGCTTAGGTCTGATGTGAAAGCCTTCGGCTTA
ACCGAAGAAGTGCATCGGAAACCGGGCGACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGTGGAAT
GCGTAGATATATGGAAGAACACCAGTGGCGAAGCGGCTGTCTGGTCTGCAACTGACGCTGAGGCTCGAAAGCATGG
GTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTATACGATGAGTGCTAGGTGTTGGAGGTTTCCGCCCTTC
AGTGCCGGAGCTTACGCATTAAGCACTCCGCCTGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGG
TGCCCGCACAAGCGGTGGAGCATGTGTGTTTAATTCGACAGCTACGCGAAGAACCTTACCAGGTCTTGACATCTTGC
GCTAACCTTAGAGATAAGGCGTTCCCTTCGGGGACGCACTGACAGGTGGTGCATGGTCGTCGTCAGCTCGTGTCGTG
AGATGTTGGGTTAAGTCCCGCAACGAGCG

**Figure 1.** Partial sequence of 16S rRNA of sample LPB P01-001

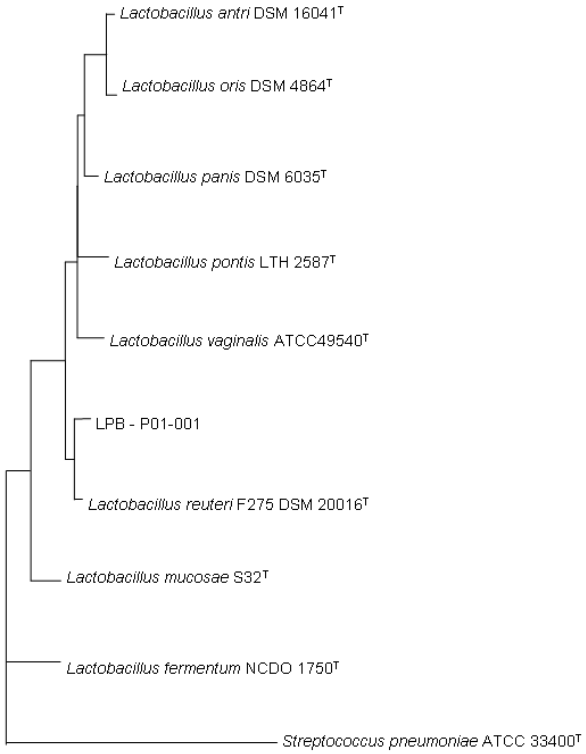


**Figure 2.** Phylogenetic tree showing the phylogenetic relations between sample LPB P01-001 and the lineage of related microorganisms based on 16S rRNA sequences

**Kinetic parameters of *L. reuteri* LPB P01-001**

The growth kinetic parameters of *L. reuteri* LPB P01-001 in 12 L bench fermentation experiments with E3 medium under controlled and uncontrolled pH were determined for up to 12 hours at the end of the exponential growth phase, as can be seen in Figures 3A and B. The biomass increased after 2.8 h in both conditions, and the sugars were rapidly and almost completely consumed during the fermentation time with pH control (Figure 3A). The total sugar concentration decreased within 28 hours of fermentation, varying from 23.03 $g.L^{-1}$ to 0 $g.L^{-1}$. The initial biomass concentration was 0.19 $g.L^{-1}$, and 1.52 $g.L^{-1}$ of biomass was achieved after 28 hours of fermentation under pH control (Figure 3A), while 14.76 $g.L^{-1}$ was obtained without pH-controlled fermentation after 26 hours (Figure 3B).

*L. reuteri* LPB P01-001 showed the usual growth behaviour, with duplication times from 2.8 – 4.0 h, depending on the initial pH and the control conditions, indicating the adequacy of the simple and low-cost E3 medium. In Figure 3B, the sugar consumption is shown to decrease slowly in the fermentation experiment without pH control when compared to fermentation with pH control.

Figures 3A and 3B show the biomass production, sugar consumption, and pH variation during *L. reuteri* LPB P01-001 fermentation. In the experiment represented in Figure 3A, the pH was kept constant at 6.5 during the fermentation time. It was found that the sugars were readily consumed during 28 hours of fermentation, while in the uncontrolled pH experiment (Figure 3B), the pH changed from 4.65 to 3.9 after 8 h. Although the sugar was not completely consumed, similar biomass contents were obtained in the experiments with and without pH control (Table 1 and 2). The kinetic parameters for biomass and lactic and acetic acid production are shown in Tables 1 and 2.

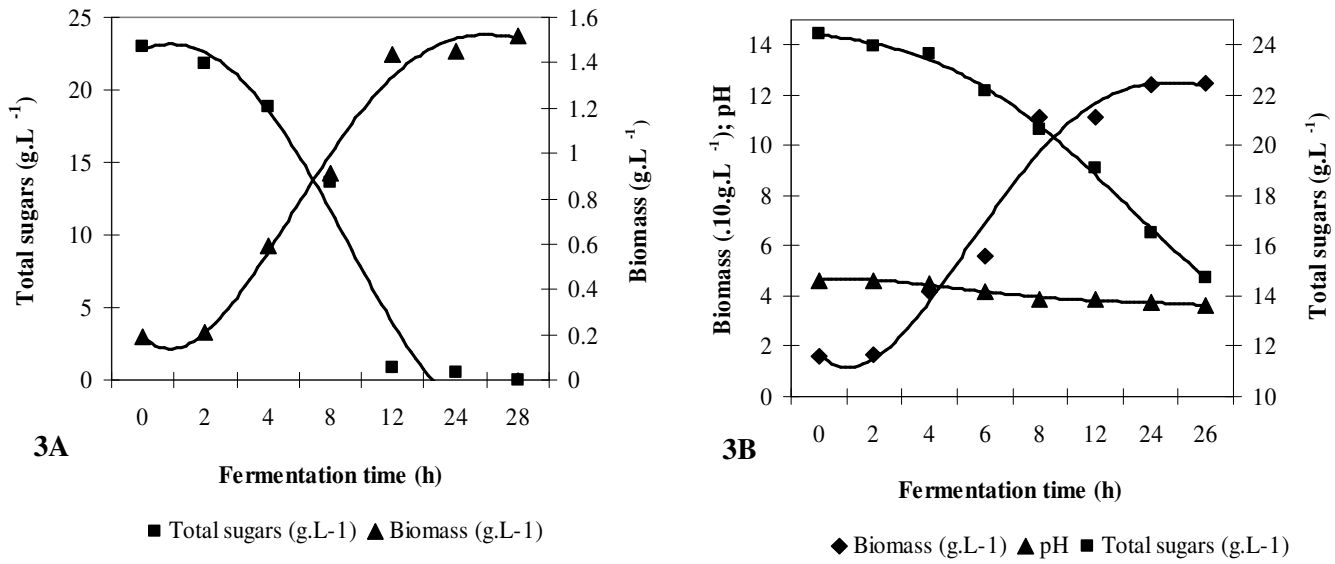


**Figure 3.** A) Profile of sugar consumption ($g.L^{-1}$) and biomass growth ($g.L^{-1}$) of *L. reuteri* LPB-P01-001 with pH control; B) Profile of pH, sugar consumption ($g.L^{-1}$), and biomass growth of *L. reuteri* LPB P01-001 without pH control

**Table 1.** Analysed and calculated parameters for *L. reuteri* LPB-P01-001 under pH-controlled fermentation (total sugar, biomass, lactic acid, acetic acid, yields and productivities)

| Fermentation Time | Total sugars ($g.L^{-1}$) | Biomass produced ($g.L^{-1}$) | Lactic acid produced ($g.L^{-1}$) | Acetic acid produced ($g.L^{-1}$) | Biomass productivity $P_X$ ($g.L^{-1}.h^{-1}$) | Biomass yield $Y_{X/S}$ ($g.g^{-1}$) | Lactic acid yield $Y_{P/S}$ ($g.g^{-1}$) | Acetic acid yield $Y_{P/S}$ ($g.g^{-1}$) | Lactic acid productivity $P_{ac}$ ($g.L^{-1}.h^{-1}$) | Acetic acid productivity $P_{aa}$ ($g.L^{-1}.h^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 23.03 | 0.19 | 9.516 | 2.29 | - | - | - | - | - | - |
| 4 | 18.86 | 0.592 | 11.344 | 4.49 | 0.100 | 0.096 | 0.44 | 0.53 | 0.457 | 0.55 |
| 8 | 13.57 | 0.91 | 16.431 | 5.21 | 0.039 | 0.076 | 0.73 | 0.31 | 0.86 | 0.365 |
| 12 | 0.81 | 1.44 | 16.530 | 6.04 | 0.104 | 0.056 | 0.31 | 0.17 | 0.58 | 0.31 |
| 28 | 0 | 1.52 | 30.5 | 10.09 | 0.048 | 0.057 | 0.91 | 0.34 | 0.75 | 0.28 |

**Table 2.** Parameters for *L. reuteri* LPB-P01-001 fermentation without pH control (total sugar, biomass, lactic acid, acetic acid, yields and productivities)

| Fermentation Time | Total sugars ($g.L^{-1}$) | Biomass produced ($g.L^{-1}$) | Lactic acid produced ($g.L^{-1}$) | Acetic acid produced ($g.L^{-1}$) | Biomass productivity $P_X$ ($g.L^{-1}.h^{-1}$) | Biomass yield $Y_{X/S}$ ($g.g^{-1}$) | Lactic acid yield $Y_{P/S}$ ($g.g^{-1}$) | Acetic acid yield $Y_{P/S}$ ($g.g^{-1}$) | Lactic acid productivity $P_{ac}$ ($g.L^{-1}.h^{-1}$) | Acetic acid productivity $P_{aa}$ ($g.L^{-1}.h^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 24.43 | 0.16 | 7.98 | 3.74 | - | - | - | - | - | - |
| 4 | 23.62 | 0.415 | 8.63 | 3.80 | 0.063 | 0.31 | 0.80 | 0.07 | 0.16 | 0.015 |
| 8 | 20.65 | 1.115 | 9.17 | 3.99 | 0.12 | 0.25 | 0.31 | 0.066 | 0.15 | 0.03 |
| 12 | 19.08 | 1.12 | 9.22 | 4.13 | 0.08 | 0.18 | 0.23 | 0.07 | 0.10 | 0.03 |

The specific growth rate μ ($h^{-1}$) was determined using the angular coefficient of the best correlation of the neperian logarithm of biomass (X) concentration (LnX) ($g.L^{-1}$) versus fermentation time (h) plot. The biomass and acid metabolite data obtained from bioreactor were statistically treated, and the corresponding kinetic parameters were determined, as can be seen in Tables 1 and 2. The specific growth rate (μ) for pH-controlled fermentation was 0.177 $h^{-1}$, while for the non-pH controlled fermentation was 0.302 $h^{-1}$. The production of lactic acid and acetic acid was higher in the pH control experiment, possibly due to the higher sugar consumption in this case (Figures 4A and B).

The kinetics of organic acid production under pH control and uncontrolled fermentation are presented in Figures 4A and B.

To obtain more accurate parameter values, some data were evaluated using an application of the general substrate consumption balance equation (23) shown in Equation 4:

$$\Delta S/\Delta t = (1/Y_{x/s})\ \Delta X/\Delta t + mX \qquad (4)$$

The maintenance coefficient for pH-controlled fermentation (pH=6.5) was 0.50 $g.L^{-1}.h^{-1}$, while in uncontrolled fermentation, it was 0.13 $g.L^{-1}.h^{-1}$. The maintenance coefficient value obtained for *L. reuteri* LPB P01-001 in E3 medium was higher than that reported for *Lactobacillus rhamnosus* (6, 51). When the general substrate consumption balance is used, the resulting parameters are more accurate (23).

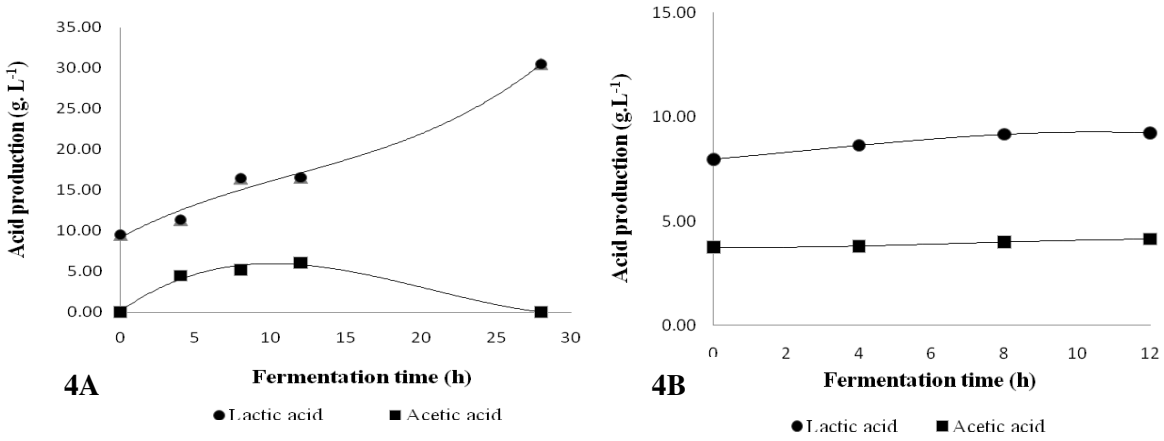


**Figure 4.** A) Profile of lactic and acetic acid production by *L. reuteri* LPB P01-001 fermentation with pH control; B) Profile of lactic and acetic acid production by *L. reuteri* LPB P01-001 fermentation without pH control.

When extracellular metabolites are synthesised – as in this work – the parameter is of interest (46). From Equation 4, it can be deduced that:

$$\Delta S = 1/Y_{x/s}\ \Delta X + mX\ \Delta X \qquad (5)$$

Considering Equation 5 and the values calculated, it was estimated that, for the pH-controlled fermentation, 59% of the energy was used for biomass synthesis and 41% was used for self-maintenance, including the synthesis of metabolic acids. Under an uncontrolled pH, the values were 70% and 30%, respectively.

Although the maintenance coefficient represents the production of metabolites and the energy required for their

production, the value does not specifically express the type or kinetics of the metabolic acids produced. Therefore, we determined the kinetics of lactic and acetic acid production under different pH conditions, as shown in Tables 1 and 2. It has previously been reported that glucose consumption is lower at pH values lower than 5.5 and that a longer time is required to attain the maximum lactic acid concentration (59). A similar trend may occur for these fermentation conditions with cane sugar in E3 medium, since the growth at a constant pH of 6.5 produced about twice as much organic acid as that obtained at an uncontrolled pH (equivalent to 4.65) (Tables 1 and 2).

The results for *L. reuteri* LPB P01-001 show that, at an uncontrolled pH, a reduction in the rate of sugar consumption occurred (Figure 3B), and a poor production of lactic and acetic acids with reference to those obtained at a constant pH of 6.5 was observed, confirming other reported data (59).

Narayanan *et al.* (38), Giraud *et al.* (18), and Girauld *et al.* (19) reported a higher yield in lactic acid in relation to the yield in biomass for *Lactobacillus* during culture at pH 5-6. These results are in accordance with the yield observed for *L. reuteri* LPB P01-001 (Table 3).

**Table 3.** Biomass and lactic acid yields ($Y_{X/S}$ and $Y_{P/S}$) found in the literature for *Lactobacillus* strains

| Strain | Culture conditions | $Y_{X/S}$ ($g.g^{-1}$) | $Y_{P/S}$ ($g.g^{-1}$) | Reference |
|---|---|---|---|---|
| *L. rhamnosus* | pH 6.2, 40ºC, glucose-yeast extract | 0.1 | 0.88 | 35 |
| *L. reuteri* LPB P01-001 | pH 6.5, 37ºC, 1% yeast extract; 2.5% sugar cane | 0.056 | 0.31 | 41 |
| *L. plantarum* | pH 6.0, 30ºC, MRS liquid medium | 0.22 | 0.75 | 17 |
| *L. plantarum* | pH 5.0, 55ºC, MRS | 0.19 | 0.81 | 16 |

Considering the results presented in Figures 4A and B, it is clear that the lactic and acetic acids present a zeroth-order kinetic pattern:

$$dA / dt = k$$

where: A: concentration ($g.L^{-1}$); t: time (h); k: kinetic constant ($g.L^{-1}.h^{-1}$).

For the pH-controlled fermentation, the aciduric *L. reuteri* LPB P01-001 strain synthesised a higher quantity of lactic and acetic acids, corresponding to 20.98 $g.L^{-1}$ and 7.8 $g.L^{-1}$ after 28 hours of fermentation. These results are quite different from those obtained under an uncontrolled pH, in which case, the values for lactic and acetic acids were only 1.25 $g.L^{-1}$ and 0.39 $g.L^{-1}$, much lower than those obtained at a constant pH of 6.5. At a controlled pH, the lactic acid production was 16.78 times higher than under uncontrolled pH fermentation. The results are similar to those for acetic acid production, where – at a controlled pH – the production was 20 times higher than the acetic acid production during uncontrolled pH fermentation. Coincidentally, lactic acid production was predominant over that of acetic acid, a typical characteristic of heterofermentative lactic acid bacteria.

*L. reuteri* LPB P01-001 isolated from pigs can produce substances that produce inhibition of the frequent pathogens *S. aureus* (ATCC 14458) and haemolytic *E. coli*. The growth inhibition % for *Escherichia coli* haemolytic swine and *Staphylococcus aureus* determined using the supernatant of MRS broth fermentation for *Lactobacillus reuteri* LPB P01-001, in Mueller-Hinton medium adjusted to different pH values, was 96.7% at pH 5.5, the inhibition was 28.9% at pH 6.5 and 22.5% inhibition was observed at pH 7.0 for *Escherichia coli* haemolytic swine; and values of 96.1% at pH 5.5, 21.9% at pH 6.5, and 15.9% at pH 7.0 were found for *Staphylococcus aureus* (Table 4). A freeze-dried product containing viable lactic acid bacteria LPB P01-001 reuterin

producer (unpublished data) and metabolic organic acids may be useful for swine nutrition and disease protection (42, 58, 61). At present, these types of agents are increasingly used to replace antibiotics as growth promoters in animals. The kinetics of *L. reuteri* LPB P01-001 fermentation with 1% yeast extract and 2.5% total cane sugar in a 12 L bioreactor at 37ºC were determined. Under controlled and uncontrolled pH, the biomass and lactic and acetic acid production were evaluated. Fermentation parameters such as the specific growth rate μ ($h^{-1}$), productivity $P_x$ ($g.L^{-1}.h^{-1}$), yield of biomass $Y_{x/s}$ ($g.g^{-1}$) and metabolic organic acid yields $Y_{P/S}$ ($g.g^{-1}$) in lactic and acetic acid are shown below. Similar results regarding a pH effect on *Lactobacillus* microbial growth were reported by LeBlanc *et al.* (32), who indicated that the bacterial growth of *Lactobacillus* was not noticeably affected by the pH of the fermentation medium.

**Table 4.** Inhibition (%) of indicator pathogenic strains in liquid media

| pH | *Escherichia coli* LME21 | *Staphylococcus aureus* ATCC14458 |
|---|---|---|
| 5.5 | 96.7 | 96.1 |
| 6.5 | 28.9 | 21.9 |
| 7.0 | 22.5 | 15.9 |

In fermentation carried out under a controlled pH of 6.5, greater organic acid production was obtained, although the biomass growth was similar in both experiments (Tables 1 and 2).

The pH effect on the total sugar consumption at uncontrolled pH is very strong and accounts for the low final yield of the lactic and acetic acids (19); this effect is displayed in Table 2.

The corresponding values for biomass yield ($Y_{x/s}$) calculated after 12 h are 0.056 (pH-controlled fermentation) and 0.18 $g.g^{-1}$ (uncontrolled fermentation), with the greater value obtained for the case in which the initial pH was 4.65 and the fermentation pH was not controlled. Growth rates are often reduced under neutral pH conditions (25), and this effect was observed when *L. reuteri* LPB P01-001 was grown at pH 6.5.

## CONCLUSION

These results demonstrate that *L. reuteri* LPB-P01-001 is an interesting aciduric strain because it grows better under acidic conditions. The biomass yield was higher in the culture medium without pH control. However, the yield of lactic and acetic acids was higher in the fermentation experiments conducted at a constant pH of 6.5. Culture conditions with a constant pH of 6.5 are better for metabolic acid production, but when maximal biomass productivity is the goal, a lower pH should be used. Further studies may determine the optimal balance of these two parameters for use in industrial production of this potentially probiotic strain. Although the biomass production presented lower values, the biomass production cost is lower using sugar cane as compared to that of commercial media such as MRS synthetic medium; thus, sugar cane is a low-cost substrate alternative for *L. reuteri* probiotic production.

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