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Relationships between the use of Embden Meyerhof pathway (EMP) or Phosphoketolase pathway (PKP) and lactate production capabilities of diverse *Lactobacillus reuteri* strains[§]

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The aims of this study is to compare the growth and glucose metabolism of three *Lactobacillus reuteri* strains (i.e. DSM 20016, DSM 17938, and ATCC 53608) which are lactic acid bacteria of interest used for diverse applications such as probiotics implying the production of biomass, or for the production of valuable chemicals (3-hydroxypropionaldehyde, 3-hydroxypropionic acid, 1,3-propanediol). However, the physiological diversity inside the species, even for basic metabolisms, like its capacity of acidification or glucose metabolism, has not been studied yet. In the present work, the growth and metabolism of three strains representative of the species diversity have been studied in batch mode. The strains were compared through characterization of growth kinetics and evaluation of acidification kinetics, substrate consumption and product formation. The results showed significant differences between the three strains which may be explained, at least in part, by variations in the distribution of carbon source between two glycolytic pathways during the bacterial growth: the phosphoketolase or heterolactic pathway (PKP) and the Embden-Meyerhof pathway (EMP). It was also shown that, in the context of obtaining a large amount of biomass, DSM 20016 and DSM 17938 strains were the most effective in terms of growth kinetics. The DSM 17938 strain, which shows the more significant metabolic shift from EMP to PKP when the pH decreases, is more effective for lactate production.

Keywords: *Lactobacillus reuteri*, microbial growth, acidification kinetics, glucose metabolism, Embden-Meyerhof pathway, Phosphoketolase pathway, lactate production

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Introduction

Lactic acid bacteria (LAB) constitute a heterogeneous group of Gram-positive bacteria that share the common trait of rapidly converting carbohydrates into lactate, leading to an acidification of their environment. LAB used as transformation agents for milk and other food products have several technological roles (Rodriguez *et al*., 2012). For instance, they participate in the formation of flavors and rheological qualities of cheeses and fermented milks (Hugenholtz and Smid, 2002; Stanton *et al*., 2005). They also extend the shelf-life of foods, lowering the pH and producing antimicrobial compounds (Gilliland, 1990; Itoh, 1992) such as lactate, bacteriocins (Hansen, 2002), reutericyclin, N-acylated tetramic acid (Cotter *et al*., 2005). This variety of synthesized inhibitory compounds also prevents development of undesirable or pathogenic bacteria (Casas and Dobrogosz, 2000), and is at the origin of efficient protective roles of LAB in many fermented foods. These bacteria are widely distributed in natural habitats, ranging from many fermented foods to intestinal tracts of humans and mammals. Most of the LAB thus contribute to the natural population of the human gastrointestinal tract, some of them provoking a beneficial effect on the host, called the probiotic effect (Gilliland, 1990). Among these LAB, *Lactobacillus reuteri* resides in the gastrointestinal tract of humans and animals such as poultry, swine and other mammals (Casas and Dobrogosz, 2000; Savino *et al*., 2007) and is considered as a probiotic species (Ahrné *et al*., 1998). This bacterium is of particular interest due to its antimicrobial behavior and, which is linked to the production of a substance called reuterin (Talarico *et al*., 1988; Morita *et al*., 2008) explaining for a great part its probiotic mode of action. In addition, *L. reuteri* is relevant for the biotechnological synthesis of valuable chemical products and building blocks from glycerol such as 1,3-propanediol (1,3-PDO) (Talarico *et al*., 1990; Drozdzynska *et al*., 2011), 3-hydroxypropionaldehyde (3-HPA) (Lüthi-Peng *et al*., 2002a, 2002b; Vollenweider *et al*., 2003; Doleyres *et al*., 2005; Rütti *et al*., 2011) or 3-hydroxypropionic acid (3-HP) (Jiang *et al*., 2009; Luo *et al*., 2011).

Various species of LAB employ different glycolytic pathways to metabolize carbohydrates (glucose, fructose, sucrose…) into the common, three-carbon intermediate stage of glycolysis (dihydroxyacetone phosphate, glyceraldehyde 3-phosphate, pyruvate…). Homofermentative LAB convert carbohydrates into lactate as an end-product using the Embden-Meyerhof pathway (EMP), whereas heterofermentative LAB produce lactate, acetate, ethanol and carbon dioxide using the phosphoketolase pathway (PKP). When fructose was pre-

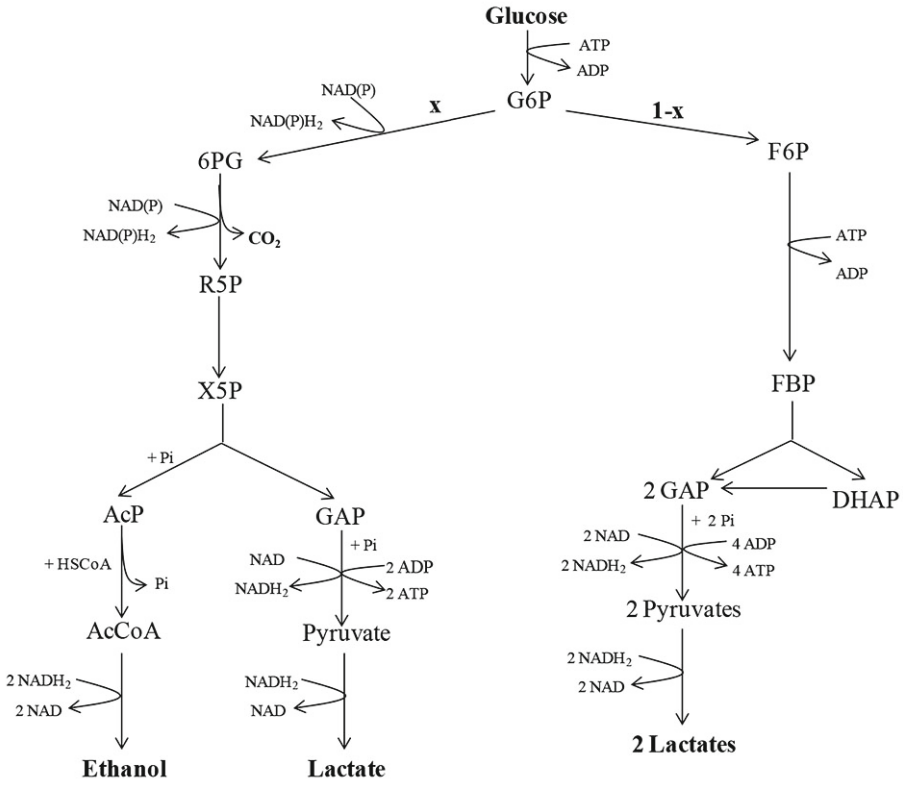

Fig. 1. Pathways of glucose metabolism in *L. reuteri*. The phosphoketolase pathway (PKP) is displayed on the left while the Embden-Meyerhof pathway (EMP) is on the right (G6P, glucose-6-phosphate; 6PG, 6-phosphogluconate; R5P, ribulose-5-phosphate; X5P, xylulose-5-phosphate; AcP, acetyl-phosphate; AcCoA, acetyl-coenzyme A; F6P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde-3-phosphate). Glucose consumption is equal to x through the PKP and to 1-x through the EMP.

sent, the redox balance could be maintained by the production of mannitol, which enabled the formation of acetate instead of ethanol (Årskold *et al.*, 2008). The PKP has a lower energy yield in the form of ATP compared to that of the EMP (2 ATP for the EMP vs. only 1 for the PKP) but it is used by LAB to ferment pentoses (Kandler, 1983). As demonstrated by Årskold *et al.* (2008), *L. reuteri*, which displays a heterofermentative metabolism, simultaneously exploits these two operating glycolytic pathways starting from glucose (Fig. 1). These findings have been recently corroborated by van Niel *et al.* (2012) who expressed the relative importance of each pathway in glucose metabolism during the exponential phase of growth (87.8% for the PKP flux and 12.2% for the EMP flux in the case of the ATCC 55730 strain). The EMP is therefore used as a shunt to provide energy.

To the best of our knowledge, the combined approach between the characterization of bacterial growth and the study of glucose metabolism has never been performed on various strains of *L. reuteri* to evaluate potential intra-species diversity. In this framework, the present study focuses on comparing three strains of *L. reuteri,* namely DSM 20016, DSM 17938 and ATCC 53608 under batch growth conditions, mainly in terms of growth and metabolism. To this aim, complementary approaches were carried out to compare these three strains at the genomic and the metabolic levels. Growth kinetics and bacterial cultivability were thus characterized while acidification kinetics was studied. In parallel, the growth medium composition was assessed by high performance liquid chromatography (HPLC) and the evolution in the use of each pathway during bacterial growth was discussed.

Materials and Methods

Lactobacillus reuteri strains

The DSM 20016 strain (Deutsche Sammlung Von Microorganismen, GmbH), also classified as ATCC 53609, is the type strain of *L. reuteri* and was purchased from the Pasteur Institute Collection. The ATCC 53608 strain was ordered from the American Type Culture Collection (ATCC). The SD 2112 strain, also called ATCC 55730, had been cured of two independent plasmids carrying unwanted antibiotic resistance traits resulting in the daughter strain DSM 17938 (Rosander *et al.*, 2008). This strain was obtained from BioGaia AB.

Chemicals

Unless otherwise specified, chemicals were purchased from Sigma-Aldrich. All solutions and media in contact with *L. reuteri* were autoclaved at 110°C for 20 min.

Bioinformatics analyses

Three different *L. reuteri* strains (DSM 20016, ATCC 53608 and SD 2112) were chosen and bioinformatic tools were used (i.e. Kegg pathways, Artemis, Clustal W) to compare them at a genomic level based on the sequencing and annotation data (Rosander *et al.*, 2008; Frese *et al.*, 2011). The aim was to compare the selected strains from the genomic and phenotypic points of view in order to try to explain the differences in metabolic behavior, particularly glucose metabolism through EMP and PKP.

Inoculum preparation

Stock cultures of *L. reuteri* strains were kept at -80°C in a solution containing 25% (w/v) glycerol. For inoculum preparation, 500 µl of frozen *L. reuteri* stock was placed in 10 ml of de Man, Rogosa, and Sharpe medium (MRS: polypeptone 10.0 g/L, meat extract 10.0 g/L, yeast extract 5.0 g/L, glucose 20.0 g/L, Tween 80 1.08 g/L, dipotassium phosphate 2.0 g/L, sodium acetate 5.0 g/L, ammonium citrate 2.0 g/L, magnesium sulfate 0.2 g/L, manganese sulfate 0.05 g/L, Biokar Diagnostics) in a sealed tube. Then, 1 ml of this tube was transferred into a sealed tube containing 9 ml broth, and incubated anaerobically at 37°C for 16 h (20 h for the ATCC 53608 strain). This suspension was used as pre-culture. This operation was repeated three times to obtain three independent pre-cultures. After 16 h (20 h for the ATCC 53608 strain), the cell concentrations of the pre-cultures were checked by measuring the optical density at 600 nm (OD_{600nm}) with a spectrophotometer (Beckman, DU 640 B).

Initiation of growth and growth monitoring

In order to avoid glucose limitation (Supplementary data Fig. S1), subsequent cultures were performed in MRS broth with the previous composition, but supplemented with glucose (20 g/L). A part of the total glucose disappeared during the sterilization process, probably due to Maillard reactions. In fact, the measured glucose concentration after sterilization was 30 g/L. Three Schott bottles containing 250 ml of supplemented MRS broth were inoculated with pre-cultures to an initial OD_{600nm} of 0.1 (corresponding to a cell concentration of around 10^8 CFU/ml). Cultures were then incubated in batch mode at 37°C under anaerobic conditions created during the culture. The bacterial growth kinetics was followed by sampling 1 ml of culture and measuring the OD_{600nm}, enabling determination of maximal specific growth rate μ_{max} (in h^{-1}) and generation time t_g (in h). The maximal specific growth rate was determined using the semi-logarithmic plot of OD_{600nm} versus time. The stationary phase was considered as reached when the OD_{600nm} did not significantly vary between two consecutive measurements. Reported data correspond to the mean values (± standard deviations) of dual measurements from three independent batch cultures.

Determination of cell cultivability

The concentration of cultivable cells (expressed in CFU/ml) was evaluated by plating serially diluted samples in physiological water (NaCl 150 mM, GPR Rectapur) onto MRS agar medium (MRS with 15 g/L agar; Biokar Diagnostics). Plates were incubated under anaerobic conditions (GENbox anaer, bioMérieux) at 37°C for 48 h. Reported results are expressed as the means ± standard deviations of duplicates from three independent experiments.

Acidification measurements

The acidification activity of bacteria during their growth at 37°C was followed in triplicate using the CinAc® device (BioVal, Spinnler and Corrieu, 1989). Cultures were carried out in 150 mL flasks of MRS broth supplemented with glucose, seeded at OD_{600nm} = 0.1. The time necessary to reach different pH values ($t_{pH5.5}$, $t_{pH5.2}$, $t_{pH5.0}$, $t_{pH4.5}$, $t_{pH4.0}$, in h) as well as the acidification kinetics (pH as a function of time) and the acidification rate (dpH/dt as a function of time or pH) were chosen to characterize the acidification activity of bacterial suspensions during microbial growth. Results were reported as the means ± standard deviations of three acidification kinetics for each strain.

Analysis of substrates and metabolites

Each sample (900 µl collected each hour during growth monitoring) was mixed (50% v/v) with trichloroacetic acid 21% (v/v), in order to precipitate proteins and therefore to stop fermentation reactions (Prolabo) and then centrifuged at 13,000 × *g* for 2 min at 4°C before filtration through a 0.22 µm pore-size filter (Millipore). The supernatant was stored at 4°C. Propionic acid 1% (w/w) was used as an internal standard and added at 50% (v/v) to an equal volume of the sample, before HPLC analysis. Glucose, lactate, acetate and ethanol quantifications were performed at least in replicate and expressed as the means ± standard deviations of duplicates from three independent experiments. Separation was performed on a Biorad Aminex HPX-87H column (300 mm × 7.8 mm; Bio-Rad) equipped with a cation H^+ Micro-Guard column (30 mm × 4.6 mm; Bio-Rad) at a H_2SO_4 (0.005 M) flow rate of 0.6 ml/min (600 pump; Waters Associates, Millipore) and a temperature of 50°C. Quantification was performed by a Waters ultra-violet (UV) detector set at 210 nm and a Waters 2414 refractive index (RI) detector put in series, with external standards of known amounts of commercially pure substances prepared fresh in filtered, deionized water. Results were analyzed by Millennium software (Waters Associates).

Growth and metabolism data analysis

The fitting of the bacterial growth curves allowed obtaining growth descriptors in order to compare the three strains. This fitting was performed using the modified Gompertz equation (1) (Zwietering *et al.*, 1990), with X = biomass concentration expressed in OD_{600nm}, X_0 = initial biomass concentration (OD_{600nm} at t = 0 h), A = asymptotic value of ln (X/X_0), λ = lag phase duration (h), μ_{max} = maximal specific growth rate (h^{-1}). For the fitting of the glucose consumption kinetics and those of the lactate and ethanol production kinetics, the modified Gompertz equation was applied to ($S_0 - S(t)$) and ($P(t) - P_0$), respectively (expressed in g/L; equations 2 and 3). In these equations, μ_{max} was replaced by r_{Smax} or r_{Pmax}, the maximal rates of consumption and production (in g/L/h), respectively. The A parameter represents the asymptotic value ($S_0 - S_{final}$) or ($P_{final} - P_0$) (in g/L), respectively. For each strain, the product yields ($Y_{lactate/glucose}$, $Y_{ethanol/glucose}$, $Y_{CO2/glucose}$, expressed in mol/mol) were calculated using the formula (4) and (5) and the fitted concentrations of glucose, ethanol and lactate determined as described above.

The percentage of use of each glycolytic pathway (PKM and EMP) was calculated using equations (6) and (7). The percentage of use of the PKP was calculated from the ratio of ethanol concentration produced through this pathway to the sum of both product concentrations divided by 2 because one mole of glucose produces 2 moles of products

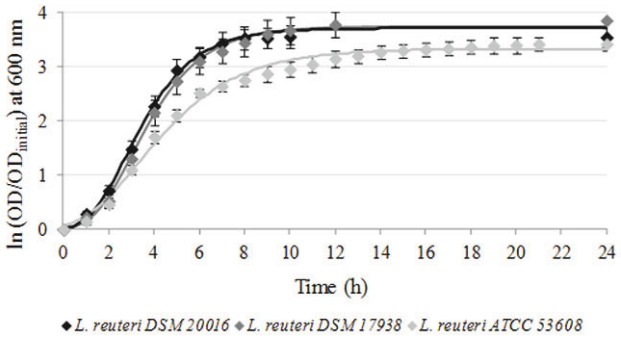

Fig. 2. Growth curves of the three *L. reuteri* strains cultivated in MRS medium supplemented with 20 g/L of glucose. Solid lines correspond to a data fitting performed by the modified Gompertz method. Reported data correspond to the mean values (± standard deviations) of dual measurements from three independent batch cultures. Maximal specific growth rates were equal to 0.82, 0.80, and 0.50 h^{-1}, respectively for the DSM 20016, DSM 17938 and ATCC 53608 strains.

(lactate or ethanol).

$$\ln\left(\frac{X}{X_0}\right) = A \cdot \exp\left(-\exp\left[\frac{\mu_{max}.\exp(1)}{A}(\lambda - t)+1\right]\right) \quad (1)$$

$$S_0 - S(t) = A \cdot \exp\left(-\exp\left[\frac{rs_{Smax}.\exp(1)}{A}(\lambda - t)+1\right]\right) \quad (2)$$

$$P(t) - P_0 = A \cdot \exp\left(-\exp\left[\frac{r_{Pmax}.\exp(1)}{A}(\lambda - t)+1\right]\right) \quad (3)$$

$$Y_{lactate/glucose} = \left(\frac{[lactate]_{final} - [lactate]_{initial}}{[glucose]_{initial} - [glucose]_{final}}\right) \quad (4)$$

$$Y_{ethanol/glucose} = \left(\frac{[ethanol]_{final} - [ethanol]_{initial}}{[glucose]_{initial} - [glucose]_{final}}\right) \quad (5)$$

$$\text{Use of PKP (\%)} = \left(\frac{2 \cdot [ethanol]_{produced}}{[ethanol]_{produced} + [lactate]_{produced}}\right) \text{x } 100 \quad (6)$$

$$\text{Use of EMP (\%)} = 100\,(\%) - \text{use of PKP (\%)} \quad (7)$$

Results

Study of growth kinetics under batch conditions on glucose

As displayed in Fig. 2, for the DSM 20016 strain, the exponential phase started after 2 h and lasted 2.5 h. The high seeding rate (OD_{600nm} = 0.1, i.e. 10^8 CFU/ml) may explain the brevity of the latency and the exponential phases. In these culture conditions, the maximal specific growth rate of the DSM 20016 strain was equal to 0.82 h^{-1} (Table 1), corresponding to a generation time of 0.85 h. The stationary phase was reached after 10 h. The growth profile of the DSM 17938 strain was comparable to the DSM 20016 in terms of beginning and duration of the exponential phase, specific growth rate of 0.80 h^{-1} and generation time of 0.87 h (Table 1). The stationary phase was reached after 12 h. After 24 h, the maximal cell concentration of the DSM 17938 culture was similar to that of the DSM 20016 strain. In contrast, the ATCC 53608 strain showed significant differences in terms of growth kinetics. The exponential phase started after 2 h but lasted 3.5 h (Fig. 2), with a specific growth rate of 0.50 h^{-1} corresponding to a generation time of 1.39 h (Table 1). The stationary phase was reached after 21 h. For the ATCC 53608 strain, a slight difference between the fitting and the experimental data was observed between 8 and 12 h (Fig. 2), which may be due to the flocculation of bacterial cells observed for this strain during growth.

The ATCC 53608 strain displayed clearly the slowest growth performance under the performed conditions, while the two other strains grew faster. Furthermore, the maximal biomass concentrations were similar for the DSM 20016 and the DSM 17938 strains, while inferior for the ATCC 53608 strain. All these results indicated that *L. reuteri* DSM 20016 and DSM 17938 were very close in terms of high growth performance on glucose.

Table 1. Growth kinetics characteristics of the three *L. reuteri* strains

	DSM 20016	DSM 17938	ATCC 53608
Maximal OD_{600nm}	4.42 ± 0.09	4.31 ± 0.11	3.04 ± 0.06
latency phase (h)	1.12 ± 0.13	1.31 ± 0.15	0.87 ± 0.11
μ_{max} (h^{-1})	0.82 ± 0.04	0.80 ± 0.05	0.50 ± 0.01
t_g (h)	0.85 ± 0.04	0.87 ± 0.05	1.39 ± 0.02

Study of acidification kinetics during growth on glucose

With the aim to better understand the differences in growth kinetics between the three *L. reuteri* strains, the acidification kinetics were further assessed using the CinAc® device. This system aims at monitoring in real time the pH evolution of several simultaneous cultures of acidifying microorganisms. During the experiment, various kinetic parameters are automatically calculated. The obtained values characterize the strains used and their conditions of implementation. The following specific parameters were evaluated: V_{max} (i.e. the maximal acidification rate), t_{Vmax} (i.e. the time to reach V_{max}), pH_{Vmax} (i.e. the pH corresponding to V_{max}) and t_{pH} (time to reach a given pH). According to Picque *et al.* (1992), these values are relatively constant for a given LAB strain, at least in a given range of biomass concentrations, and allow characterizing and differentiating the acidification behavior of studied strains.

For the three *L. reuteri* strains, the parameters characterizing the acidification kinetics and the times necessary to reach different pH values are summarized in Supplementary data Table S1. The time required to reach a pH of 5.5 from an initial pH of 6.2 was comparable for all three strains ($t_{pH5.5}$ comprised between 2.25 h and 2.53 h). From pH 5.5 and more clearly from pH 5.2, the acidification kinetics significantly varied depending on the strain (Fig. 3A). At pH 4.5, the deviation between strains increased as $t_{pH4.5}$ varied from 5.30 h for the DSM 20016 strain to 13.37 h for the ATCC 53608 strain. A minimum pH of about 4.0 was reached for the three strains, but the time to reach it was dependent on the strain (15.27 h for DSM 20016, 17.67 h for DSM 17938 and 35.22 h for ATCC 53608; Fig. 3A). A ranking based on the acidification performance of the three studied strains un-

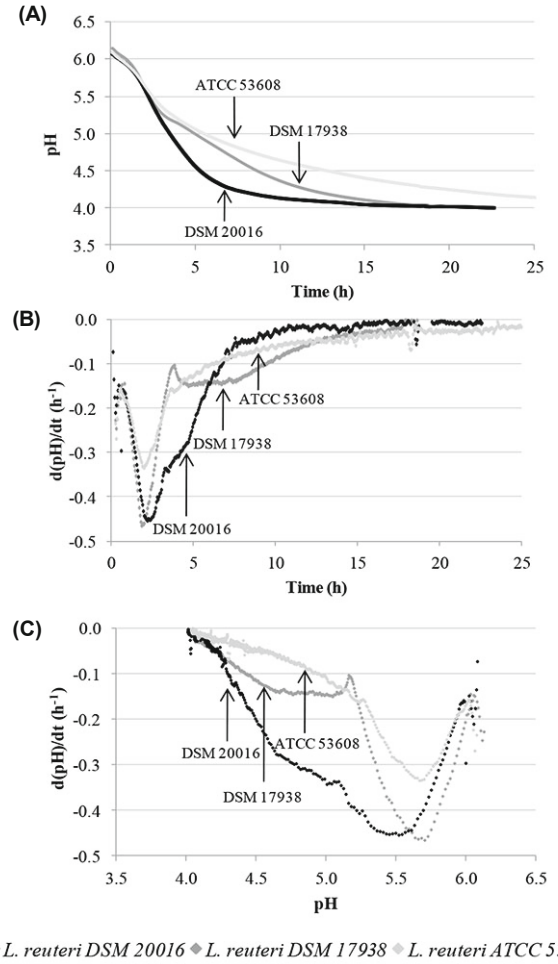

Fig. 3. Acidification kinetics of the three *L. reuteri* strains during their growth in MRS medium supplemented with 20 g/L of glucose: (A) pH profile as a function of growth time; (B) acidification rate profile as a function of growth time; (C) acidification rate profile as a function of pH. Measures were stopped when minimum pH (here pH = 4.0) was reached, that is an acidification rate equal to zero.

der the performed growth conditions can thus be established: the DSM 20016 strain was the quickest, followed by the DSM 17938 strain while the ATCC 53608 strain was significantly slower.

As observed on Fig. 3C, the pH_{Vmax} values were similar for the three strains, being equal to 5.68 for the DSM 17938 and ATCC 53608 strains and to a slightly lower value of 5.46 for the DSM 20016 strain. On the contrary, significant differences were observed between the strains in the case of the V_{max} absolute values (0.46 h^{-1} and 0.47 h^{-1} for the DSM 20016 and the DSM 17938 strains respectively, vs. 0.34 h^{-1} for the ATCC 53608 strain, Fig. 3B). The acid stress resistance of the DSM 20016 strain to low pH is also shown by the range in pH where the acidification rate remains over 50% of V_{max} (1.35 pH units), though for the ATCC 53608 this value was only of 0.71 pH units and 0.69 pH units for the DSM 17938.

Besides, the time at which this maximal acidification rate was reached (i.e. t_{Vmax}) differed for the three bacterial strains (Fig. 3B), as it was lower for the DSM 17938 and ATCC 53608 strains (1.87 h and 1.92 h, respectively) than for the DSM 20016 strain (2.30 h). However, the study of the duration where the speed of acidification stays over $V_{max}/2$ clearly shows that the high acidification rate was maintained longer for the DSM 20016 strain (237 min) than for the ATCC 53608 (168 min) and DSM 17938 (114 min).

Glucose consumption and products formation during batch growth

As shown in Fig. 4, for the three *L. reuteri* strains, the glucose consumption was related to the production of lactate and ethanol. Moreover, it is noteworthy that there was no production of acetate (results not shown) during glucose fermentation in the three cases. The production of CO_2 has been observed but not measured. In comparing Figs. 2 and 4, lactate and ethanol production was observed throughout bacterial growth and further. Indeed, these concentrations increased until 22 h of culture for the DSM 20016 strain, while production continued at least until 24 h of culture

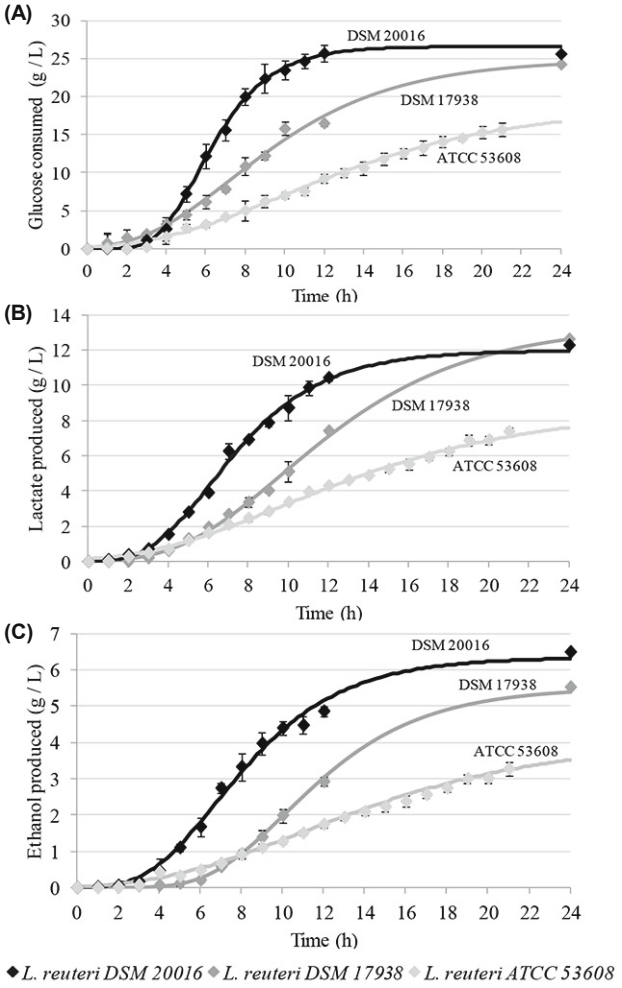

Fig. 4. Evolution of (A) glucose concentration, (B) lactate, and (C) ethanol concentrations (determined by HPLC) as a function of growth time for the three *L. reuteri* strains. At the beginning of the growth, [glucose] = 30 g/L for each strain. Solid lines correspond to a data fitting by the modified Gompertz method.

Table 2. Characterization of glucose consumption and metabolites production for the three studied *L. reuteri* strains

		DSM 20016	DSM 17938	ATCC 53608
Glucose	r_{Smax} (g/L/h)	4.84 ± 0.10	2.02 ± 0.10	1.07 ± 0.08
	t r_{Smax} (h)	5.6	7.4	10.0
Lactate	r_{Pmax} (g/L/h)	1.44 ± 0.06	0.96 ± 0.03	0.46 ± 0.01
	t r_{Pmax} (h)	6.2	9.8	10.0
	$Y_{lactate/glucose}$ (mol/mol)	0.90	1.08	0.91
Ethanol	r_{Pmax} (g/L/h)	0.70 ± 0.04	0.55 ± 0.04	0.22 ± 0.02
	t r_{Pmax} (h)	7.0	10.2	10.0
	$Y_{ethanol/glucose}$ (mol/mol)	0.93	0.87	0.82

for the DSM 17938 and the ATCC 53608 strains. These results corroborate the acidification profile (Fig. 3A). As initial glucose concentration in the medium was equal to 30 g/L, this substrate was not totally consumed by any of the three strains (Fig. 4A). The end of the bacterial growth was thus not caused by a lack of glucose but probably by an inhibition due to the low pH conditions and/or the presence of lactate and ethanol.

Lactate and ethanol production yields are shown on Table 2. In the case of lactate, the DSM 20016 and ATCC 53608 strains exhibited the lowest yields of conversion (0.90 and 0.91 mol/mol, respectively), while the DSM 17938 strain displayed the highest (1.08 mol/mol). Due to the stoichiometry of the metabolic pathways, these yields are equal to those of ATP produced. Moreover, the yield of ethanol production is different from one strain to another. The ATCC 53608 strain had the lowest value (0.82 mol/mol), whereas the DSM 17938 and the DSM 20016 strains showed higher values (0.87 and 0.93 mol/mol, respectively). Significant differences were also observed in the case of maximal rates of substrate consumption and metabolite production (Table 2). The rates r_{Smax} and r_{Pmax} were the lowest in the case of the ATCC 53608 strain and the highest for the DSM 20016 strain while the DSM 17938 strain displayed intermediate values. In the case of the DSM 20016 and DSM 17938 strains, the time needed to reach these maximal rates (t r_{Smax} and t r_{Pmax}) varied (Table 2). On the contrary, the time to reach r_{Smax} and r_{Pmax} was the same (10.0 h) for the ATCC 53608 strain.

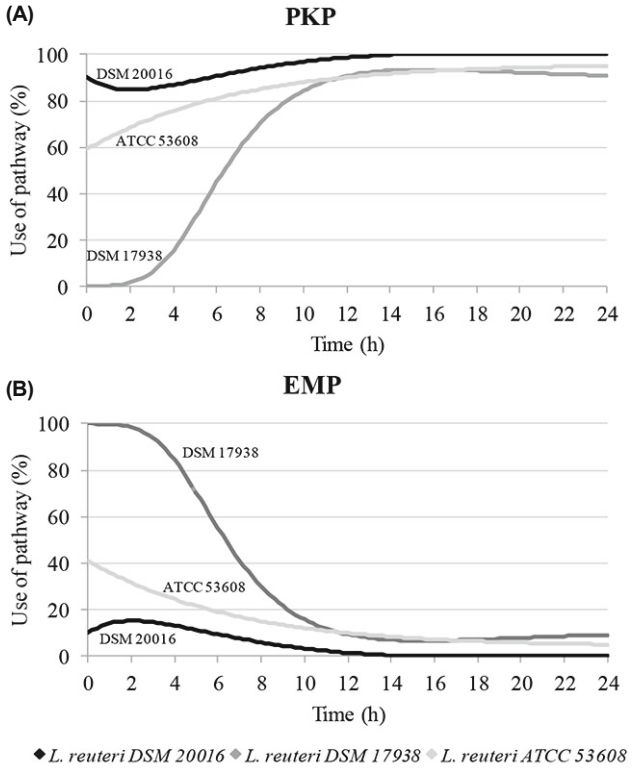

Fig. 5. Evolution of the glucose consumption between the PKP and the EMP as a function of bacterial growth time.

Balance in the glycolytic pathways

The heterofermentative *L. reuteri* simultaneously exploits the PKP and the EMP starting from glucose as initial substrate. By calculating the ratio of ethanol concentration produced to the sum of products divided by 2, the relative percentages of glucose consumed in each pathway can be determined for each strain (Fig. 5). It is notable that, for each strain, the relative importance of these two pathways varied all along the growth. Furthermore, the profile of the orientation through each pathway significantly varied when comparing the three strains (Fig. 5).

In the case of the DSM 17938 strain, the proportion of PKP use moved from 0 to 93% during bacterial growth. The increasing use of this pathway started after 2 h, which corresponds to the beginning of the exponential phase of growth (Figs. 2 and 6). After 14 h, a slight slowdown in the use of this pathway was observed (Fig. 5) together with a slowdown in the kinetic of acidification (Fig. 3A) and therefore in the production of lactate (Fig. 4B). For the ATCC 53608 strain, the use of PKP increased moving from 59 to 95% between 0 and 12 h (Fig. 5) but no correlation with the growth curve was observed. For both strains, there was therefore an increase in the use of this pathway over time. The profile of the DSM 20016 strain appeared to be different as two phases can be distinguished: in the first, the use of the PKP slightly decreased at the expense of the EMP (between 0 and 3 h, Fig. 6), and in the second (between 3 and 24 h) the percentage of glucose metabolized through the PKP increased again. Between 0 and 3 h, the decrease in pH was important (from 6.2 to 5.2, Fig. 3A) and the acidification rate was maximal ($dpH/dt = -0.46\ h^{-1}$, Fig. 3B), which is consistent with the fact that the EMP is maximally used at this time of growth. All the three strains predominantly used the PKP at the end of bacterial growth.

Genomic comparison of the two glycolytic pathways for the three *L. reuteri* strains studied

Given the differences in the use of the two glycolytic pathways between the three strains, differences in the genes involved in these pathways could be expected. Nevertheless, no significant differences appeared after the comparison of the genes involved in the EMP and PKP. Although all of these genes are common to the three strains studied, the genomic organization was not the same and there also may be differences in the level of expression. Transcriptomic and proteomic analyses could help to better understand these various metabolic behaviors.

Discussion

This work was focused on characterizing three strains of *L. reuteri* (DSM 20016, DSM 17938, and ATCC 53608) in terms of growth and metabolism. These three strains are located on different branches of the phylogenetic dendrogram and are therefore representative of the intra-species variability. The comparison was performed based on complementary methodological approaches carried out at different levels (genomic, growth kinetics, acidification performance and metabolic behavior). This was done in order to get insights that would help in strain selection, bearing in mind the diversity of application of the species studied in this work. Growth and acidification kinetics showed significant differences between the three strains. Moreover, it was shown that the predominance use between PKP and EMP differed between *L. reuteri* strains. It is noteworthy to observe that the orientation of glucose consumed through each pathway depends not only on the fermentation time in batch mode but also, and even though it concerns the energy production, on the strain within the species. To the best of our knowledge, such a comparative study of growth and metabolism between *L. reuteri* strains has not been published yet. Indeed, only few studies concerning these characteristics of *L. reuteri* appear in the literature (Årskold *et al.*, 2008; van Niel *et al.*, 2012), characterizing only one strain (i.e. ATCC 55730) and little is known in the field to allow comparison between different *L. reuteri* strains.

The end-products of a fermentation performed by heterolactic LAB depend upon the starting substrate, as hexoses lead to lactate and ethanol, whereas pentoses lead to acetate and lactate (Årskold *et al.*, 2008). For instance, starting from glucose, all redox equivalents could only be directed to lactate and ethanol production to balance the pool and, consequently, no acetate could be formed (Årskold *et al.*, 2008). *L. reuteri* growth on glucose is therefore limited by a redox imbalance, which results in ethanol formation rather than the energetically more favorable acetate formation (Stevens *et al.*, 2011).

Although few results are available in the literature concerning glucose fermentation by *L. reuteri* (Årskold *et al.*, 2008; van Niel *et al.*, 2012), acidification kinetics presented here generated interesting information. As *L. reuteri* is a strictly heterofermentative LAB and produces lactate, CO_2 and ethanol from glucose, the decrease in pH was attributed to the production of lactate. The comparison of the acidification profiles obtained for the three strains was consistent with bacterial growth performance. The observed differences in acidification and growth kinetics may be linked to various lactate resistance behaviors between strains. As in the other well characterized LAB, the lactate production significantly slows down after the bacterial growth (Figs. 2 and 4A), indicating that lactate is a primary metabolite provoking growth inhibition. Similar results were highlighted by van Niel *et al.* (2012) in the case of the ATCC 55730 strain. It has been evidenced here that, among the three studied strains, the DSM 20016 and the ATCC 53608 ones are the two most different strains in terms of lactic fermentation, though the DSM 17938 shows an intermediate behavior under the performed experimental conditions.

Concerning the use of each glycolytic pathway, since the work of Årskold *et al.* (2008), it has been commonly assumed that the heterofermentative *L. reuteri* ATCC 55730 strain uses both the PKP (hetero-fermentation) and the EMP (homofermentation) to metabolize glucose into lactate, ethanol and CO_2. This work demonstrated that the main flux is through the PKP, while the EMP is used as a shunt (representing about 27% of the total flux starting from 50 g/L of glucose during the exponential phase). In addition, van Niel *et al.* (2012) recently expressed the relative percentages of glucose consumption through each pathway for the ATCC 55730 strain (PKP flux: 87.8% ± 7.4%, EMP flux: 12.2% ± 7.4%). These values were estimated for the period of maximum growth rate according to the metabolic flux analysis described by Årskold *et al.* (2008). It seemed interesting to check the distribution of the carbon flux through other strains when the fermentation proceeds. Even if some features (i.e. increase in the PKP and decrease in the EMP after the peak of lactate production as shown by comparing the HPLC and the acidification profiles) were similar for the three *L. reuteri* strains, the profile of the orientation through each pathway during glucose consumption significantly varied between them (Fig. 5). Whatever the strain, the flux through the EMP was more important during the acceleration phase (mainly in the case of the DSM 17938 strain), in the course of which, significant amounts of glucose remained in the medium, whereas the "heterolactic" behavior through the PKP was more observed when high levels of lactate were present in the culture medium. It is nevertheless worth to note that some of the results found at the beginning of bacterial growth (i.e. before 2 h) are probably excessive. The estimation of the percentages of use of the PKP and EMP were calculated from the ethanol and lactate concentrations quantified in the growth medium. However, at the start of the growth phase, the low concentrations measured can lead to errors in this estimation. Moreover, as MRS is a complex medium, a part of the ethanol produced could come from substrates other than glucose (citrate and threonine from peptones, meat extract and yeast extract). The estimated quantity produced from these sources is low compared to the final concentrations of ethanol produced (between 3.5 and 6.5 g/L, depending on the strain). Therefore, the observed differences between the metabolic behaviors of the various strains cannot be explained by this estimated part. Although the DSM 17938 and DSM 20016 strains had a similar behavior in terms of growth kinetics (Fig. 2 and Table 1), the DSM 20016 and ATCC 53608 strains were the closest in terms of use of the glycolytic pathways (Fig. 5). Conversely, the DSM 17938 strain shows an almost complete shift from EMP to PKP when pH is going down and surprisingly does not show any alteration in the lactate production observed and reached the best yield of lactate production among the three strains. This strain seems to adopt a particularly adaptable metabolism in a changing environment.

From a stoichiometric point of view, during growth of LAB, the EMP yields 2 moles of lactate and 2 moles of ATP per mole of glucose consumed (glucose + 2 ADP → 2 lactates + 2 ATP). For the PKP, the overall stoichiometric reaction is: glucose + 1 ADP → 1 lactate + 1 ethanol + 1 CO_2 + 1 ATP. Thus, the stoichiometry of the metabolic pathways shows that the molarity of ATP produced is equal to that of lactate

produced. Therefore, the PKP efficiency in terms of ATP synthesis is about half that of the EMP. Nevertheless, the advantage of the PKP is that it generates $NADPH_2$, an essential co-factor for reduction reactions used in biosynthesis (in particular in the synthesis of fatty acids and steroids), as well as ribulose-5-phosphate, which is the precursor for the synthesis of nucleotides and nucleic acids. In addition, in producing less organic acids, the PKP limits the acid inhibition of bacteria by their own metabolism and the growth ends up exhausting glucose while permitting the production of energy, even though it gives a lower ATP yield than EMP. Regarding the thermodynamics, the free-energy of the EMP is $\Delta_{EMP} = -205.2$ kJ/mol and the theoretical possible ATP yield based on the energy balance of the reaction is therefore $205.2 / (2 \times 51.8) = 1.98$ (51.8 kJ/mol being the free-energy necessary for ATP synthesis from ADP; Werner *et al*., 2010). In a similar way, the free-energy of the PKP is $\Delta_{PKP} = -262.14$ kJ/mol and the theoretical possible ATP yield is thus of 2.53 (Werner *et al*., 2010). It is therefore more interesting from a thermodynamic point of view to use the PKP during bacterial growth rather than the EMP. Nevertheless, the calculated values of the thermodynamic ATP yield (i.e. 1.98 for the EMP and 2.53 for the PKP) do not fit with the stoichiometric values (i.e. 2 for the EMP and 1 for the PKP) in the case of the PKP. The observed differences may be explained by the ecological niche of LAB. Indeed, during evolution, some pathways such as the PKP may not have reached their optimal ATP net production because energy yield is not their only and main function (Werner *et al*., 2010) and PKP was however selected because of the environmental constraints (e.g. pentose availability, requirement of $NADPH_2$ production or precursor synthesis, exhaustion of the sugars in the medium, limitation of acid production).

In summary, the results of our work suggest that the orientation of the intracellular energy substrates depends on each *L. reuteri* strain. The phenotypic variability is important between these strains and significant differences were observed even for strains of the same *Lactobacillus* species. Homofermentative LAB ferment hexoses to lactate by the EMP. At slow growth and low glycolytic flux rates the homofermentative bacteria shift to mixed acid fermentation with formate, acetate, ethanol and lactate as the products. The change is caused by regulation of the lactate dehydrogenase and pyruvate formate lyase activities, which are subject to control by the catabolic and anabolic flux rates and changes in the $NADH/NAD^+$ ratios (Garrigues *et al*., 1997, 2001). For example, *Lactococcus lactis* normally employs homolactic fermentation. However, if glucose is limited, a shift towards a form of heterolactic fermentation is observed, in which 1 mole of glucose is metabolized into 1 mole of acetate, 2 moles of formate and 1 mole of ethanol. The shift between EMP and PKP could also be caused at least in part by regulation of the lactate dehydrogenase (leading to the synthesis of NAD^+) which is subjected to control by the catabolic and anabolic flux rates. Moreover, in the presence of lactic acid at low pH (pH 4.8) and low growth rate, it has been shown in *Lactobacillus plantarum* a significant up-regulation of genes involved in the PKP (e.g. transketolase, transaldolase and alcohol dehydrogenase) (Pieterse *et al*., 2005). A transcriptomic approach would allow confirming the origin of the observed differences in the metabolic shift between *L. reuteri* strains.

Conclusion

The present study contributes to a better understanding of a poorly characterized LAB - *L. reuteri*, which has many application interests. Taken together, the results emphasize the intra-species variability of this species. The EMP pathway producing more lactate, the shift to the PKP pathway is clearly a way to reduce acid production and probably extends the possibility of bacterial survival in acidic environment. The DSM 17938 strain, showing the highest initial EMP level and then the highest PKP level was also the most efficient for lactate production (concentration and yield from a glucose containing medium, quick acidification kinetics). The regulation process at the origin of the shift observed in *L. reuteri*, helping its adaptation to acidic environments, warrants a further in-depth study.

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