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# Diversity of *Lactobacillus reuteri* Strains in Converting Glycerol into 3-Hydroxypropionic Acid

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**Abstract** The present study aims at comparing the performances of three *Lactobacillus reuteri* strains (DSM 20016, DSM 17938, and ATCC 53608) in producing 3-hydroxypropionic acid (3-HP) from glycerol and at exploring inhibition phenomena during this bioconversion. Differences were highlighted between the three strains in terms of 3-HP production yield, kinetics of substrate consumption, and metabolite production. With a maximal productivity in non-optimal conditions (free pH) around 2 $g.L^{-1}.h^{-1}$ of 3-HP and 4 $g.L^{-1}.h^{-1}$ of 3-hydroxypropionaldehyde (3-HPA) depending on the strain, this study confirmed the potential of *L. reuteri* for the biotechnological production of 3-HP. Moreover, the molar ratios of 3-HP to 1,3-propanediol (1,3-PDO) obtained for the three strains (comprised between 1.25 and 1.65) showed systematically a higher 3-HP production. From these results, the DSM 17938 strain appeared to be the most promising strain. The impact of glycerol bioconversion on the bacteria's physiological state (a decrease of around 40 % in DSM 17938 cells showing an enzymatic activity after 3 h) and survival (total loss of cultivability after 2 or 3 h depending on the strains) was revealed and discussed. The effect of each metabolite on

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*L. reuteri* DSM 17938 was further investigated, displaying a drastic inhibition caused by 3-HPA, while 3-HP induced lower impact and only at acidic pH.

**Keywords** 3-Hydroxypropionic acid · *Lactobacillus reuteri* · Glycerol bioconversion · Inhibitory activity

## Introduction

In recent years, the tremendous growth of biodiesel manufacturing industries resulted in a large production of available glycerol as by-product. Therefore, development of processes of glycerol bioconversion into high added-value products has become economically viable [1, 2]. One such chemical, namely 3-hydroxypropionic acid (3-HP), holds an important place in the current list of the top 10 platform chemicals among renewable biomass products [3]. 3-HP is a three-carbon non-chiral organic molecule, which is the isomer of lactic acid (2-HP). It contains two functional groups (carboxyl and β-hydroxyl) making it highly reactive and suitable for production of polymer materials through chemical synthesis [4] or bioproduction (e.g., poly(3-hydroxypropionate) or poly(3-hydroxybutyrate-co-3-hydroxypropionate) [5–7]). 3-HP can also be used as a crosslinking agent for polymer coatings, metal lubricants, and antistatic agents for textiles [8]. Moreover, it is a precursor for several key chemical compounds, such as 1,3-propanediol ($C_3H_8O_2$), acrylic acid ($C_3H_4O_2$), methyl acrylate ($C_4H_6O_2$), acrylamide ($C_3H_5NO$), ethyl 3-HP ($C_5H_{10}O_3$), malonic acid ($C_3H_4O_4$), propiolactone ($C_3H_4O_2$), and acrylonitrile ($C_3H_3N$) [8–10]. This platform molecule or its derivatives can therefore be widely employed for the industrial production of many commercially valuable chemical compounds such as resins, adhesives, plastics, and latex. Currently, the high price and the potential toxicity of 3-HP synthesis [11] explain its low volume of production [12] which limits its use for mass markets. Despite the economic importance of 3-HP for its various applications, studies focusing on obtaining this building block by microbiological pathways are recent. Scientific studies demonstrated that few microorganisms, among which the Gram-positive probiotic *Lactobacillus reuteri*, are able to naturally produce 3-HP by bioconversion of glycerol [13–15]. With the aim of increasing the 3-HP concentrations, several studies lean on metabolic engineering of bacterial hosts such as *Klebsiella pneumoniae* (16.0 $g.L^{-1}$ [16], 22.7 $g.L^{-1}$ [17]) and *Escherichia coli* (14.3 $g.L^{-1}$ [18], 57.3 $g.L^{-1}$ [19], 42.1 $g.L^{-1}$ [20]). Nevertheless, although unmodified and without achieving these levels of production, *L. reuteri* is a very interesting candidate among the 3-HP-producing bacteria (GRAS status, ability to synthesize vitamin B12, glycerol not used as carbon source for growth but only as electron acceptor, avoiding the presence of undesirable by-products in the bioconversion medium). However, only the work of Dishisha et al. [15] focused on *L. reuteri* as 3-HP producer using the DSM 20016 strain. Moreover, bioconversion performances and mass balance analyses of various *L. reuteri* strains have never been studied.

The metabolic pathway of 3-HP biosynthesis from glycerol by *L. reuteri* comprises two steps (Fig. 1): a first enzymatic reaction in which glycerol is converted into 3-hydroxypropionaldehyde (3-HPA) by a coenzyme B12-dependent glycerol dehydratase (GDH, [21–24]). 3-HPA is subsequently oxidized in 3-HP through a three-step reaction. First a CoA-dependent propionaldehyde dehydrogenase (PduP) requires $NAD^+$ and leads to 3-HP-CoA and NADH, $H^+$. Then a phosphotransacylase (PduL) converts 3-HP-CoA into 3-HP-phosphate and simultaneously releases the Coenzyme A. Actually, a propionate kinase (PduW)

leads to 3-HP and ATP [13, 15, 25]. 3-HPA can also be reduced in 1,3-propanediol (1,3-PDO) by a 1,3-PDO oxidoreductase (PduQ) requiring NADH, $H^+$ [26] (Fig. 1), the ratio between 3-HP and 1,3-PDO depending in part on the NADH, $H^+$ availability. The whole enzymes involved in this glycerol metabolic pathway are encoded in the propanediol utilization (pdu) operon [25, 27]. Among the products of this metabolic pathway, only the toxicity of 3-HPA towards the producing strains has been studied in details [28–31]. Concerning 3-HP, one recent study [32] displayed that this organic acid produced by an endophytic marine fungi exerted an antibacterial activity against *Staphylococcus aureus* and *Salmonella typhi*, which was attributed to its free carboxylic acid group. In another work [33], minimum inhibitory concentration (MIC) for 3-HP on *E. coli* was estimated to be 15 $g.L^{-1}$. However, to the best of our knowledge, there are no studies focusing on the evaluation of the toxic effects of 3-HP on *L. reuteri*. At last, the 1,3-PDO was demonstrated to have no toxic effect when added in a wide concentration range from the beginning of glycerol fermentation by *Clostridium butyricum* [34].

In this framework, our study aims at comparing three *L. reuteri* strains (DSM 20016, ATCC 53608 and DSM 17938) in terms of glycerol bioconversion performances and mass balance analyses including the use of $^{13}C$ NMR. In addition, the physiological state of producing

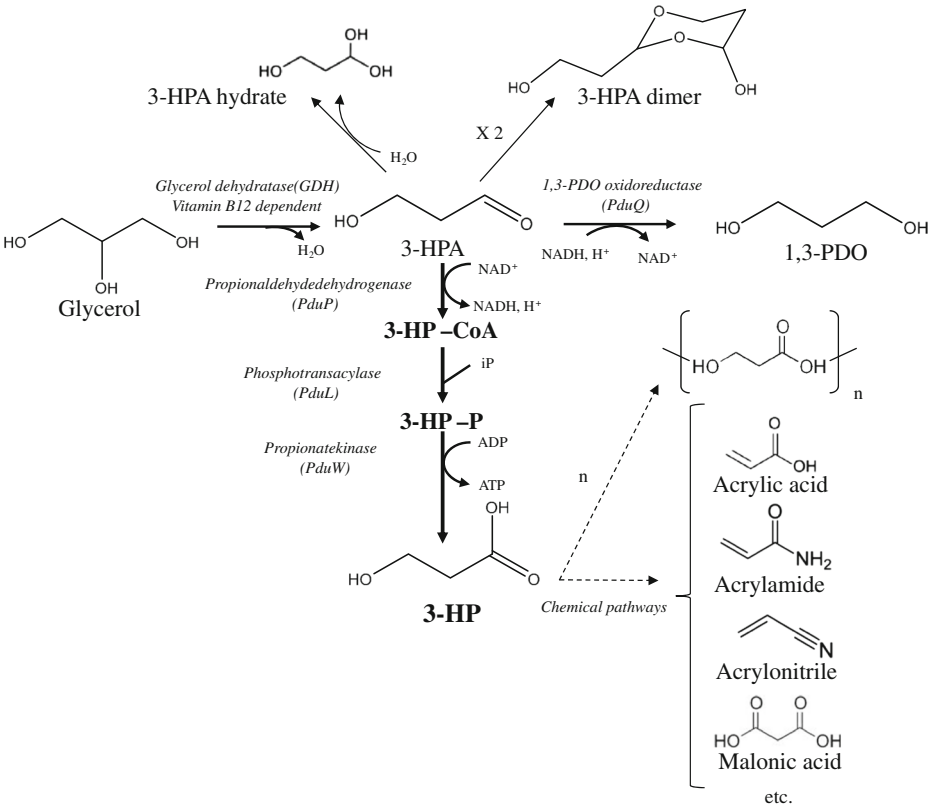


**Fig. 1** Metabolic pathway of 3-HP biosynthesis from glycerol in *L. reuteri* and some potential applications of this platform chemical. *Bold arrows* correspond to metabolic pathway, *simple arrows* to chemical equilibrium of 3-HPA in the system, and *dashed arrows* to chemical pathways leading to potential 3-HP derivatives

bacteria (i.e., cell viability using flow cytometry and cultivability by plate counting) was characterized during bioconversion. Moreover, the potential toxic effect of each metabolite of the glycerol bioconversion pathway (i.e., 3-HP, 3-HPA, and 1,3-PDO) on the strain DSM 17938 was investigated. For the first time, the level of performance of the three studied strains was discussed considering the differences observed in the glycerol metabolism and in the cell resistance to the main resulting products.

## Materials and Methods

### Bacterial Strains and Growth Conditions in Schott Bottles

The DSM 20016 strain (Deutsche Sammlung Von Microorganismen, GmbH, Braunschweig, Germany), the type strain of *L. reuteri*, was purchased from the Pasteur Institute Collection (Paris, France). The ATCC 53608 strain was ordered at the American Type Culture Collection (ATCC, Rockville, Maryland, USA). The ATCC 55730 strain (also called SD 2112) was cured from two plasmids, resulting in the daughter strain DSM 17938 [35], which was obtained from BioGaia AB (Stockholm, Sweden). All solutions and media in contact with *L. reuteri* were autoclaved at 110 °C for 20 min.

Bacterial growth was performed anaerobically, at 37 °C, in Schott bottles filled up with 500 mL of de Man, Rogosa, and Sharpe (MRS) medium (Biokar diagnostics, Beauvais, France) supplemented with 20 $g.L^{-1}$ of glucose (initial pH = 6.2). The bottles were inoculated at an initial optical density at 600 nm ($OD_{600\ nm}$) of 0.1 (corresponding to a cell concentration of $10^8$ $CFU.mL^{-1}$) with a preculture performed in MRS for 8 h. These cultures were conducted until the beginning of the stationary phase of growth. Cells were harvested by centrifugation (5000×*g* for 10 min at 4 °C) and washed twice with 0.1 M potassium phosphate buffer (pH 6.5) to ensure no carry-over of growth metabolites.

### 3-HP Production from Glycerol in Schott Bottles

Cells were re-suspended in Schott bottles (250 mL) containing 250 mL of 200 mM glycerol solution (18.2 $g.L^{-1}$ in distilled water, initial pH = 6.5) to an $OD_{600\ nm}$ of 20 (corresponding about $2.0.10^{10}$ $CFU.mL^{-1}$). In parallel, negative controls were performed by resuspending bacterial cells in distilled water without glycerol. The whole of the cell suspensions were incubated for 4 h at 37 °C. Samples were harvested each hour and immediately treated for the analyses described below. Results were reported as the means ± standard deviations of three experiments for each strain.

### Acidification Kinetics

The 3-HP production during glycerol bioconversion was followed in line using the CinAc device, which continuously measured the pH values of microbial suspensions (BioVal-Process, Grignon, France; [36, 37]). Bioconversions were carried out in 150-mL flasks of glycerol (200 mM in distilled water), seeded with harvested cells at $OD_{600\ nm}$ = 20 as described above. Results were reported as the means ± standard deviations of three acidification kinetics for each strain.

## Glycerol Bioconversion in Bioreactor

Growth cultures performed in Schott bottles as described above were used to inoculate a 7-L bioreactor (Setric Génie Industriel, Toulouse, France) containing 5 L of supplemented MRS at an initial $OD_{600\ nm}$ of $10^{-5}$ (corresponding to a cell concentration of $10^4$ CFU.mL$^{-1}$). Stirrer speed was maintained at 100 rpm, temperature at 37 °C, and pH at 6 by addition of 10 N KOH. Growth was conducted for 16 h until the beginning of the stationary phase of growth and cells were harvested as described above and re-suspended in 500 mL of distilled water. Biotransformation of glycerol was subsequently done in a 5-L Biostat®-A bioreactor (Sartorius France, Dourdan, France) with a 2.5-L working volume. The process was started by resuspending in 2.0 L solution containing 45.5 g of glycerol, the 500 mL of harvested *L. reuteri* cells, to reach an $OD_{600\ nm}$ of 13 (around $1.3.10^{10}$ CFU.mL$^{-1}$) and a final glycerol concentration of 18.2 g.L$^{-1}$ (200 mM). Glycerol biotransformation was performed at 37 °C, 100 rpm, and at pH 6 regulated with 10 N KOH. Samples were harvested each 30 min and immediately treated for measuring the metabolite concentrations. The experiment was conducted for 3 h and stopped after the end of the KOH addition and therefore of the 3-HP production. These experiments were duplicated.

## Cell Cultivability

The concentration of viable and cultivable cells (i.e., cultivability, expressed in CFU.mL$^{-1}$) was monitored during glycerol bioconversion by plating samples serially diluted in physiological water (NaCl 150 mM) onto MRS agar medium (MRS supplemented with 15 g.L$^{-1}$ agar; Biokar Diagnostics, Beauvais, France). Plates were incubated under anaerobic conditions (GENbox anaer, BioMérieux, Marcy l'Etoile, France) at 37 °C for 48 h. Reported results were expressed as the means ± standard deviations of two samples from three independent experiments.

## Evaluation of the Cell Physiological State by Flow Cytometry

Carboxyfluorescein diacetate (cFDA) contained in Chemchrom V8 (Biomérieux, Marcy l'Etoile, France) was used to assess *L. reuteri* viability (i.e., esterase activity assimilated to enzymatic activity) whereas the nucleic acid dye, propidium iodide (PI), made it possible to quantify dead cells (i.e., loss of membrane integrity), according to the method of Rault et al. [38]. Harvested cell suspensions of the DSM 17938 strain were diluted in Mac Ilvaine buffer pH 7.3 (0.1 M citric acid (Fisher Chemical, Elancourt, France); 0.2 M disodium dihydrogenophosphate (J. T. Baker, Deventer, The Netherlands)) to reach approximately $10^6$ cells.mL$^{-1}$. One milliliter of each diluted suspension was supplemented with 10 μL of PI (1.496 mM in distilled water, Sigma-Aldrich, Buchs, Switzerland) and 10 μL of Chemchrom V8 (formerly diluted at 10 % *v*/*v* in DMSO) and incubated for 10 min at 40 °C.

Flow cytometry analyses were performed with a BactiFlow flow cytometer (Partec, Sainte-Geneviève-des-Bois, France) equipped with a specific volumetric counting system allowing the expression of the cell concentration in number of cells per milliliter, an air-cooled argon ion laser emitting at 488 nm, and four filters: a forward-angle light scatter (FSC), a side-angle light scatter (SSC), both combined with a diode collector, a 530-nm band-pass filter (515 to 545 nm) to collect the green fluorescence of carboxyfluorescein (FL1 channel), and a 630-nm long-pass filter to collect the red fluorescence of propidium iodide (FL2 channel) with photomultiplier tubes. Milli-Q quality water was used as sheath fluid. Data were collected and analyzed with

the Flowmax software (Partec, Sainte-Geneviève-des-Bois, France). Analyses were performed by using logarithmic gains, specific detector settings, and compensation settings adjusted on samples with unstained *L. reuteri* cells in order to eliminate cellular autofluorescence. A combination of FSC and SSC was used to discriminate the bacteria from the background. The statistical tables given by the Flowmax software indicated the concentrations (in cells.mL$^{-1}$) and percentages of total and stained cells detected by FSC, SSC, FL1, and FL2 channels. Each result was the average ± standard errors of two measurements on each sample from at least two independent experiments.

## Data Mining and Sequence Analysis

The genome sequences of the *L. reuteri* strains DSM 20016 (accession no. CP000705.1), ATCC 53608 (accession no. GCA_000236455.1), and ATCC 55730 (i.e., the mother strain of the DSM 17938; accession no. CP002844.1) were obtained from GenBank. All predicted proteins or enzymes were searched against a non-redundant protein database (NCBI) using BLASTP. The ATCC 55730 strain was always chosen as a reference for nucleotide or amino acid sequence comparison, and the two other strains were compared to this reference strain.

## 3-HPA Synthesis and Quantitative Analysis of Substrates and Metabolites

Unless otherwise specified, chemicals were purchased from Sigma-Aldrich (Buchs, Switzerland). 3-HP was purchased from TCI-Europe (Zwyndrecht, Belgium). 3-HPA was chemically synthesized in our laboratory. 1,2,4-Butanetriol (9.4 mmol) was dissolved in dioxane (0.05 M). Water (4.7 mL) and sodium periodate (37.6 mmol) were then added, and the mixture was magnetically stirred at 1,100 rpm at room temperature. Reaction was followed by thin-layer chromatography (TLC) (9/1 ethyl acetate/methanol) until completion. The crude mixture was then filtered on Celite® and the latter washed with dioxane. The solvent was removed under reduced pressure, and the crude oil was purified by flash chromatography (100 % ethyl acetate) to provide 3-HPA as a colorless viscous oil.

Glycerol, 3-HP, 3-HPA, and 1,3-PDO concentrations were determined by high-performance liquid chromatography (HPLC). Separation was performed on a Biorad Aminex HPX-87H column (300 mm × 7.8 mm; Biorad, Richmond, USA) equipped with a cation $H^+$ Micro-Guard column (30 mm × 4.6 mm; Biorad) at a controlled temperature of 50 °C. Each sample (900 μL) was mixed (50 % *v*/*v*) with trichloroacetic acid 24 % (*v*/*v*) (Prolabo, Paris, France) then centrifuged at 13,000×*g* for 2 min at 4 °C and filtered through a 0.22-μm pore-sized filter (Millipore, Guyancourt, France). Citric acid (10 g.L$^{-1}$ in deionized water) was used as internal standard and added at 50 % (*v*/*v*) to the sample just before the analysis. A twenty-microliter volume of each sample was injected in a 5 mM $H_2SO_4$ mobile phase flowing at 0.6 mL.min$^{-1}$ (600 pump; Waters Associates, Millipore, Molsheim, France). Quantification was performed by a Waters ultraviolet (UV) detector set at 210 nm for 3-HP (retention time = 13.1 min) and a Waters 2414 refractive index (RI) detector put in series for glycerol, 3-HPA, and 1,3-PDO (retention time = 13.4, 15.0, and 17.7 min, respectively; Online Resource 1a and b). Injection of external standards of known amounts of fresh pure substances in filtered and deionized water permitted to establish calibration curves. Because of co-elution of glycerol and 3-HP between 13 and 14 min, the 3-HP concentration was estimated through the UV detector. The RI area corresponding to this 3-HP concentration was then calculated thanks to the corresponding RI calibration curve. The quantification of the glycerol was determined by difference between the total area of the RI peak

and the calculated contribution of 3-HP in this peak surface. Results were analyzed by the Empower software (Waters Associates). All quantifications were expressed as the means ± standard deviations of three independent experiments.

### Identification of the Products of the Glycerol Bioconversion by $^{13}C$ NMR Analysis

The glycerol bioconversion was followed in situ by nuclear magnetic resonance (NMR) to identify precisely the metabolites produced and especially the molecules of the 3-HPA system. The $^{13}C$ spectra were acquired on a 75-MHz Bruker spectrometer (Fourier 300, Bruker Biospin, Wissembourg, France) with a spectral width of 25,000 Hz and a relaxation delay of 2 s. Fields were stabilized by locking on $D_2O$ (Euriso-Top, Saint-Aubin, France). $^{13}C$ glycerol was also purchased from Euriso-Top. After the end of bacterial growth, cells were washed as described above and re-suspended in 50 mL of $^{13}C$ glycerol (200 mM in distilled water) at an $OD_{600\ nm}$ of 20. 0.9 mL of bioconversion medium and 0.1 mL of $D_2O$ were introduced in NMR tubes, and glycerol bioconversion was followed in situ at 37 °C during 3 h with one spectrum acquisition every 30 min.

## Results

### Monitoring of the Acidification Kinetics During Glycerol Bioconversion

To characterize and compare the 3-HP production over time between the three *L. reuteri* strains, and since 3-HP is the only organic acid produced, the kinetics of acidification during glycerol bioconversion was assessed using the CinAc system. This device aims at monitoring in real time and automatically the pH evolution of several simultaneous growth or bioconversion media of acidifying microorganisms. Figure 2 displays the pH evolution during glycerol bioconversion performed by the three studied strains. For each strain, one "control" flask (cells suspended in distilled water without glycerol) was included in the experimental design. In the case of this negative control, the slight decrease in pH observed at the beginning was due to the

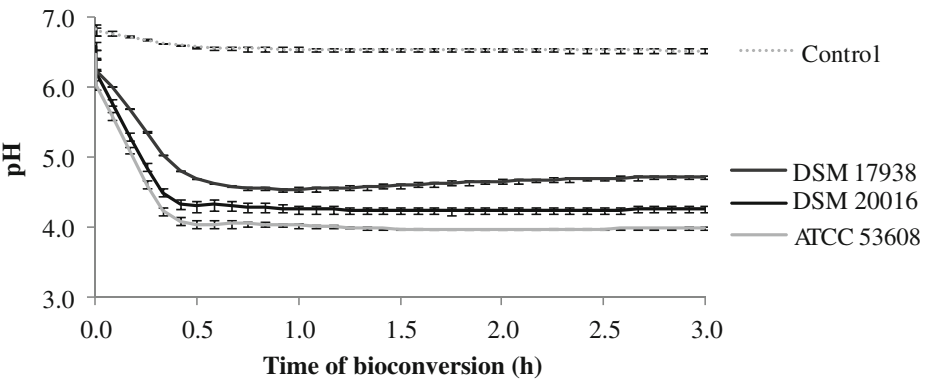


**Fig. 2** Acidification kinetics of the three *L. reuteri* strains during glycerol bioconversion at 37 °C in 200 mM of glycerol in batch system: pH evolution as a function of bioconversion time. Reported results are the means ± standard deviations of three acidification kinetics for each strain. The control corresponds to the mean ± standard deviation of the control sample (i.e., cells in distilled water) of each strain

change in temperature. Indeed, the suspensions of cold cells from washing step needed time to reach back the incubation temperature (37 °C).

The time required to reach a minimal pH value (i.e., 4.5, 4.2, and 4.0 for the DSM 17938, DSM 20016, and ATCC 53608 strains, respectively) from an initial pH of 6.5 slightly differed between the three strains: 30 min for the two latest and 45 min for the DSM 17938 (Fig. 2). A ranking based on the acidification performance of these strains under the performed bioconversion conditions can thus be established: the DSM 20016 and ATCC 53608 strains were the fastest, while the DSM 17938 strain was slower. In addition, the ATCC 53608 strain was the best acid producer, followed by the DSM 20016, while the DSM 17938 strain produced less organic acid. Compared to the negative control, the observed pH changes were only due to the presence of glycerol in the medium and to its bioconversion into organic acid through bacterial cell activity.

## Comparison of Glycerol Bioconversion into 3-HP Between the Three *L. reuteri* Strains

Figure 3 shows the evolution of glycerol, 3-HPA, 3-HP, and 1,3-PDO concentrations along the bioconversion process. In bottles without any glycerol added, neither 3-HPA nor 1,3-PDO and 3-HP were produced (data not shown). As displayed in Fig. 3, the glycerol initially present was not entirely consumed as 10.8, 6.6, and 4.4 $g.L^{-1}$ remained in the medium for the DSM 20016, ATCC 53608, and DSM 17938 strains, respectively. Glycerol consumption occurred during 1 to 1.5 h for all of the three strains. The highest 3-HP accumulation (i.e., 3.1 $g.L^{-1}$ after 2 h) was observed for the ATCC 53608 strain, whereas it reached 2.4 $g.L^{-1}$ for the DSM 20016 after 1 h and 2.3 $g.L^{-1}$ for the DSM 17938 strain after 1.5 h. These results are consistent with the previous observations made with the CinAc system (Fig. 2), especially the ranking of the kinetic parameters was the same for the three strains. The maximal 3-HPA concentration (5.0,

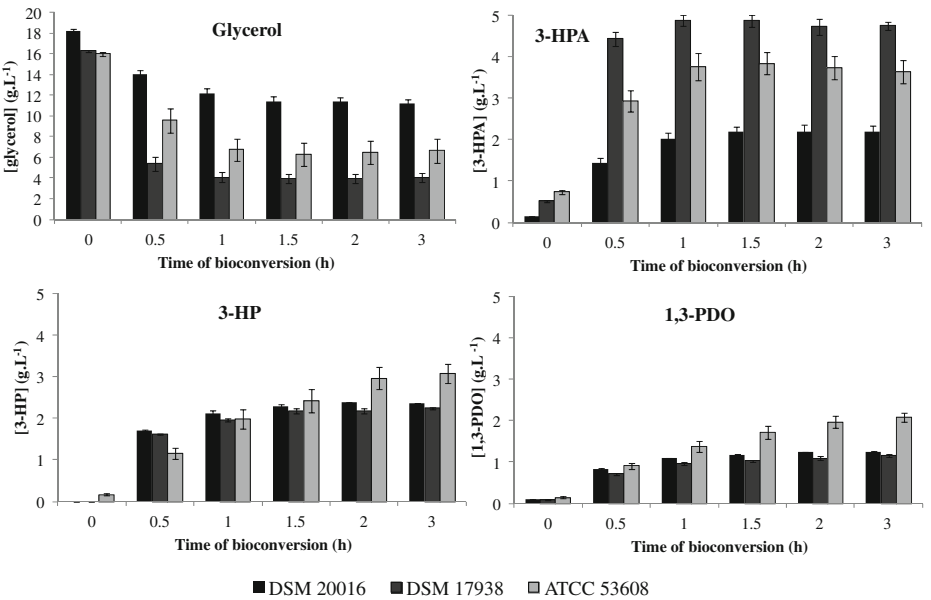


**Fig. 3** Evolution of the glycerol, 3-HPA, 3-HP, and 1,3-PDO concentrations determined by HPLC analyses of the medium during bioconversion performed by the three *L. reuteri* strains in bottles

3.4, and 2.1 $g.L^{-1}$ for the DSM 17938, ATCC 53608, and DSM 20016 strains, respectively) was achieved after 1.5 h, which was concomitant with the end of the glycerol uptake (Fig. 3). Concerning the 1,3-PDO production, results were similar to those obtained for 3-HP: the highest accumulation (2.1 $g.L^{-1}$ after 2 h) was reached by the ATCC 53608 strain, whereas it was lower for the two other strains (1.3 and 1.2 $g.L^{-1}$ after 1 to 1.5 h for the DSM 20016 and DSM 17938 strains, respectively).

The calculation of the 3-HP, 1,3-PDO, and 3-HPA production yields, determined after 3 h of bioconversion, allowed establishing metabolic variations between the three strains (Table 1). The yield of 3-HPA production differed between strains as the DSM 20016 and the ATCC 53608 were characterized by the lowest values (0.35 and 0.37 mol/mol, respectively), whereas the DSM 17938 strain showed the highest value (0.45 mol/mol, respectively). Concerning the 3-HP production, the best yield was obtained with the DSM 20016 strain (0.33 mol/mol) whereas the worst was achieved by the DSM 17938 (0.17 mol/mol). In the case of 1,3-PDO, the DSM 20016 and ATCC 53608 strains exhibited the highest yields of conversion (0.21 and 0.22 mol/mol), while the DSM 17938 strain displayed the lowest (0.10 mol/mol).

The ratio of 3-HP on 1,3-PDO concentrations reached at the end of the glycerol bioconversions (Table 1) provides information on the balance between the two enzymatic reactions starting from 3-HPA (Fig. 1): for the three strains, 3-HPA was preferentially transformed in 3-HP (ratios >1) and the highest ratio was obtained with the DSM 17938 strain (ratio = 1.65), immediately followed by the DSM 20016 strain (ratio = 1.59).

The molar sum of products (3-HP, 1,3-PDO, and 3-HPA) should theoretically equal the amount of glycerol used. Table 1 displays for each strain the molar balance (i.e., ratio of molar concentrations of produced 3-HP, 1,3-PDO, and 3-HPA on consumed glycerol, expressed in %). In the three cases, this molar balance was lower than 100 % (73, 86, and 88 % for the DSM 17938, ATCC 53608, and DSM 20016 strains, respectively, Table 1). In order to explain these results, $^{13}C$ NMR analyses were performed during bioconversion of $^{13}C$-labelled glycerol by *L. reuteri* DSM 20016 and DSM 17938, these strains displaying respectively the best and the worst molar balance. After 3 h of bioconversion in $D_2O$ (Fig. 4), the analysis showed that glycerol, 3-HPA (in the form of monomers and dimers), 3-HP, and 1,3-PDO were the only components of the bioconversion medium for both strains. No difference was highlighted between these two strains, indicating that the degradation products of glycerol were the same in both cases.

**Table 1** Characterization of the glycerol consumption and metabolite production during bioconversions performed by the three *L. reuteri* strains

| | DSM 17938 | DSM 20016 | ATCC 53608 |
|---|---|---|---|
| Glycerol consumed ($g.L^{-1}$) | 13.82 ± 0.06 | 7.39 ± 0.31 | 11.55 ± 1.19 |
| (% of the initial concentration) | 75.95 ± 0.30 | 40.60 ± 1.69 | 63.44 ± 6.54 |
| $Y_{3\text{-HPA/glycerol}}$ (mol/mol) | 0.45 ± 0.01 | 0.35 ± 0.01 | 0.37 ± 0.04 |
| $Y_{3\text{-HP/glycerol}}$ (mol/mol) | 0.17 ± 0.01 | 0.33 ± 0.01 | 0.27 ± 0.01 |
| $Y_{1,3\text{-PDO/glycerol}}$ (mol/mol) | 0.10 ± 0.01 | 0.21 ± 0.01 | 0.22 ± 0.01 |
| [3-HP]/[1,3-PDO] (mol/mol) | 1.65 ± 0.09 | 1.59 ± 0.03 | 1.25 ± 0.03 |
| Molar balance (%) | 73 ± 1 | 88 ± 4 | 86 ± 5 |

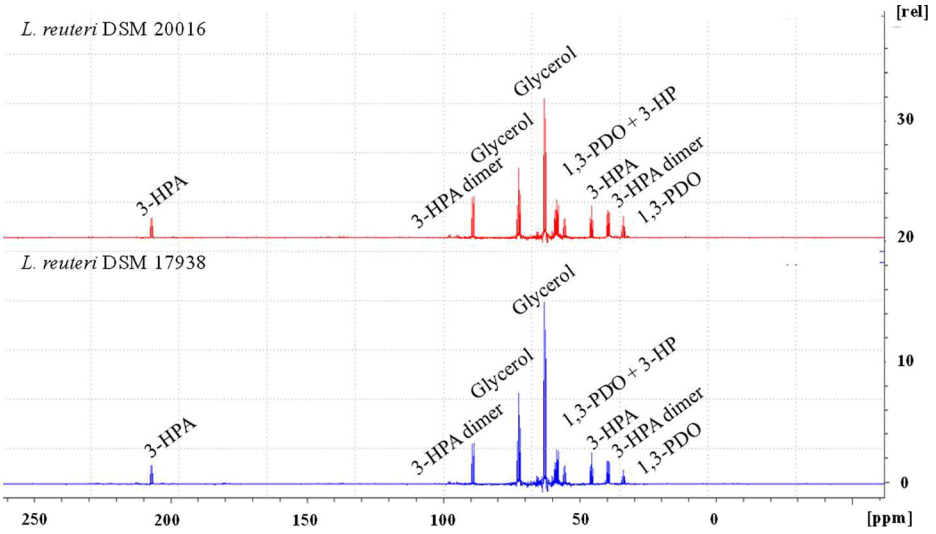


**Fig. 4** $^{13}C$ NMR spectra after 3 h of glycerol bioconversion performed by the DSM 20016 and the DSM 17938 strains

## Glycerol Bioconversion in Bioreactor Using the DSM 17938 Strain

Figure 5 displays the glycerol consumption and metabolite production by the *L. reuteri* DSM 17938 strain in bioreactor at 37 °C and pH regulated at pH = 6. The bioconversion quickly stopped (after 1 h, Fig. 5) despite pH regulation. Glycerol was less consumed than in bottles (7.9 vs. 13.8 $g.L^{-1}$) and 3-HP and 1,3-PDO were less produced (0.9 and 0.3 $g.L^{-1}$ vs. 2.3 and 1.2 $g.L^{-1}$, respectively). Moreover, the 3-HP production yield also decreased (0.11 vs. 0.17 mol/mol). Nevertheless, the ratio of 3-HP on 1,3-PDO increased (2.53 vs. 1.65 mol/

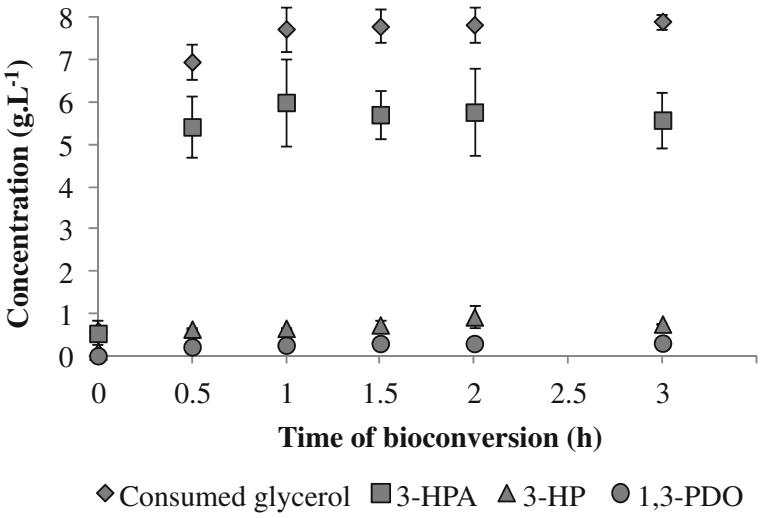


**Fig. 5** Evolution of the glycerol, 3-HPA, 3-HP, and 1,3-PDO concentrations determined by HPLC analyses of the medium during bioconversion performed by the *L. reuteri* DSM 17938 strain in bioreactor at pH 6

mol). Concerning 3-HPA, the final production (5.6 $g.L^{-1}$, Fig. 5) was slightly higher than the one obtained in Schott bottles (5.0 $g.L^{-1}$, Fig. 3) and the production yield almost doubled (0.86 vs. 0.45 mol/mol).

## Toxicity of the Produced Metabolites on *L. reuteri* Strains

As shown in Fig. 6, cell cultivability drastically dropped for the three strains after 1 h of glycerol bioconversion, with no more colony detected on agar plates after 2 h for the DSM 17938 and ATCC 53608 strains and after 3 h for the DSM 20016 strain (Fig. 6a). These results were consistent with the 3-HPA and 3-HP production kinetics determined by HPLC analyses (Fig. 3). In fact, the DSM 20016 strain was the lowest producer of 3-HPA and 3-HP which may explain that it is less exposed to their toxic effect. The study of the impact of pH and 3-HP and 3-HPA concentrations on cell cultivability during glycerol bioconversion showed a concomitant inhibition effect of these three parameters on *L. reuteri* but highlighted differences in the impact of each parameter on the three studied strains (Fig. 6b–d). The DSM 17938 strain seemed to be the most sensible to pH (Fig. 6b) and 3-HP (Fig. 6c) but the most resistant to 3-HPA (Fig. 6d).

Given these results and in order to better understand the role played by each parameter, *L. reuteri* DSM 17938 strain was selected and its physiological state investigated by flow cytometry analyses (Fig. 7). The bacterial cell concentration and enzymatic activity (more precisely, the esterase activity) was assessed during glycerol bioconversion (Fig. 7a, b). Total cell concentration did not change during the storage in distilled water (i.e., control samples; Fig. 7a) while the concentration of bacteria displaying an enzymatic activity and considered as

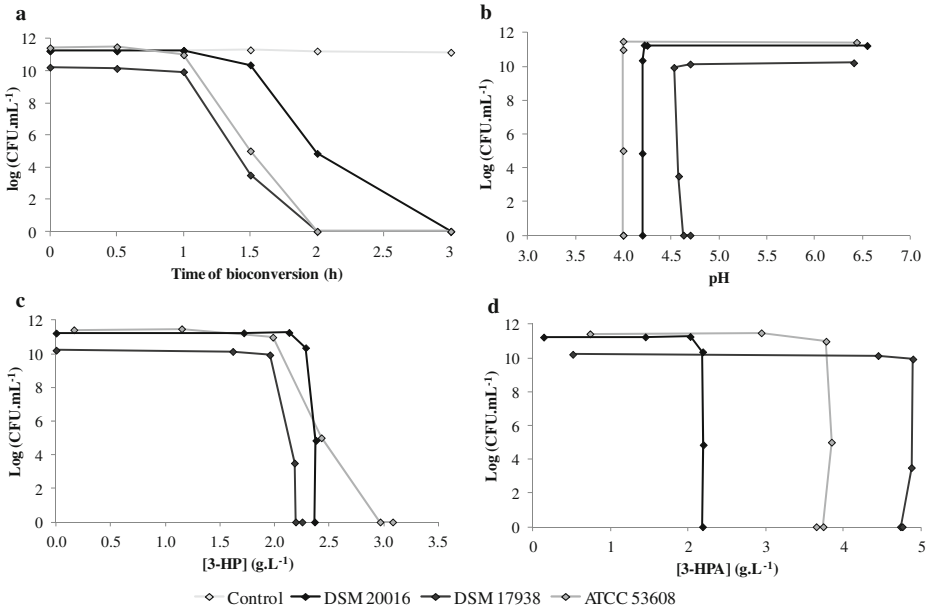


**Fig. 6** Impact of glycerol bioconversion on *L. reuteri* cultivability. **a** Evolution of the cultivability of the three strains during glycerol bioconversion and impact of **b** pH, **c** concentration of produced 3-HP, and **d** concentration of produced 3-HPA on cell cultivability

viable remained stable around 90 % (Fig. 7b). Thus, there was no cell inactivation due to a long time in an aqueous medium without any energy source. On the contrary, glycerol bioconversion and therefore 3-HPA and 3-HP productions and linked pH decrease led to a decline in the percentage of cells displaying an enzymatic activity from 93.4 % of the total population to 58.3 % in 3 h and only 1.2 % after 24 h (Fig. 7b), whereas the total cell concentration did not vary (Fig. 7a). It thus demonstrated that pH, 3-HP, and/or 3-HPA produced by bacterial cells affected their physiological state and more precisely their enzymatic activity. The comparison between the flow cytometry (Fig. 7b) and the plate counting (Fig. 6) results gives complementary and interesting information, as the loss in cultivability was total after 2 h whereas the loss in enzymatic activity was only of 35 % after the same time.

To deepen the understanding of metabolite impact on *L. reuteri* physiological state and the contribution of each of them to the global inhibition phenomenon, cells of the DSM 17938 strain were suspended in aqueous solutions of 3-HP (2.5 $g.L^{-1}$), 3-HPA (5 $g.L^{-1}$), and 1,3-PDO (2.5 $g.L^{-1}$) during 30 min and 3 h, before evaluation of their enzymatic activity and membrane integrity by flow cytometry. These concentrations were chosen because they correspond to those reached during the glycerol bioconversion by this strain. In the case of 3-HP, two different pH were tested (the pH of the solution at 2.5 $g.L^{-1}$, namely pH 3 and a pH adjusted at 5 with KOH 5 N) in order to investigate the role played by the pH in 3-HP toxicity. Contrary to the 1,3-PDO showing no more deleterious effect than to the control (i.e., cells in

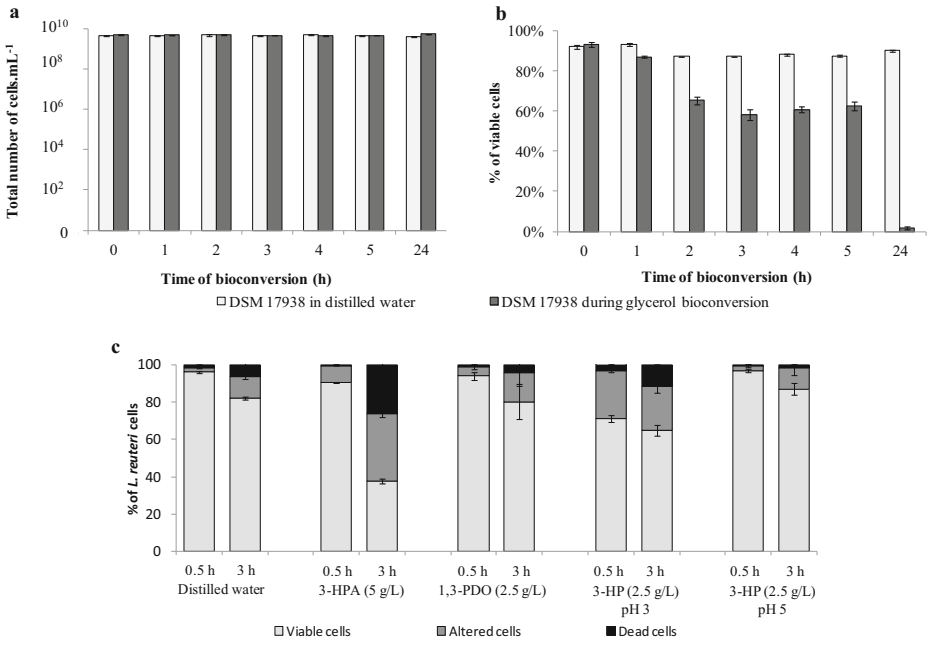


**Fig. 7** Evaluation of the physiological state of *L. reuteri* DSM 17938 performed by flow cytometry during the glycerol bioconversion compared to cells in distilled water (control). **a** Evolution of the total cell concentration (expressed in cells.$mL^{-1}$) as a function of time, **b** evolution of the percentage of cells displaying an enzymatic activity (i.e., stained by carboxyfluorescein diacetate). **c** Comparison of the bacterial physiological state after a 30-min and a 3-h exposure to 3-hydroxypropionaldehyde (5 $g.L^{-1}$), 1,3-propanediol (2.5 $g.L^{-1}$), and 3-hydroxypropionic acid (2.5 $g.L^{-1}$) at the free pH of the solution (i.e. pH 3) and at a fixed pH (i.e., pH 5) compared to cells suspended in distilled water (control)

distilled water), results revealed a major impact of 3-HPA on bacterial physiological state, particularly after 3 h of contact with cells, (Fig. 7c). The impact of 3-HP at free pH (i.e., pH 3) was lower than that of 3-HPA but already noticeable after 30 min of contact. It is worth to note that, when the pH of the 3-HP solution was fixed at 5, no alteration of the bacterial physiological state was detected (Fig. 7c), underlying the significant role played by the pH in 3-HP toxicity at the tested concentration.

## Discussion

Our results show that *L. reuteri* strains vary in their ability to convert glycerol into 3-HP. To get a better understanding of the dynamics of glycerol bioconversion by *L. reuteri* and to compare the performance of the three strains, metabolite concentration and cell viability were evaluated over time during incubation of bacterial cells in glycerol solution. It appears that the three studied strains exhibited different behaviors in bottles in terms of glycerol consumption and 3-HP production: the ATCC 53608 strain was the most productive (3.1 $g.L^{-1}$/34.2 mM, Fig. 3), but its production yield was intermediate (0.27 mol/mol, Table 1) and its 3-HP/1,3-PDO ratio was the lowest (1.25 mol/mol). The DSM 20016 strain was less productive (2.4 $g.L^{-1}$/26.3 mM) and consumed very little glycerol (41 % of the initial quantity), but its production yield was the highest (0.33 mol/mol) and its ratio 3-HP on 1,3-PDO was significant (1.59 mol/mol). Lastly, the DSM 17938 strain displayed an intermediate behavior characterized by the lowest production yield (0.17 mol/mol) and a similar 3-HP/1,3-PDO ratio (1.65 mol/mol) and 3-HP production (2.3 $g.L^{-1}$/25.7 mM) to the one produced by DSM 20016 strain, but the highest glycerol consumption (76 % of the initial quantity).

NMR analyses helped in explaining the quite low molar balance obtained for each strain during glycerol bioconversion. Based on the NMR results, we suspected that one or several molecules were probably not detected by HPLC detectors used in our experimental conditions and could be one or several forms of the 3-HPA system (including 3-HPA hydrate or 3-HPA oligomers). For example, the DSM 17938 strain produced more 3-HPA than the two others (5.0 $g.L^{-1}$ against 3.4 and 2.1 $g.L^{-1}$ for the ATCC 53608 and DSM 20016 strains, respectively) and its molar balance was the worst (73 %). As the form of 3-HPA (monomer or dimer) is directly linked to its concentration [28, 39], we supposed that the dimeric fraction could not be detected by the RI or UV detectors that may explain the molar unbalance observed for this strain. Conversely, for the DSM 20016 strain, less 3-HPA was produced and probably less dimeric form of 3-HPA was present. The molar balance (88 %) was better because the major part of the 3-HPA was under monomeric form and therefore more correctly quantified by our HPLC method. The results reported by Dishisha et al. [15] support our hypothesis, as the molar balance calculated from their data was close to 100 %, with a very low amount of produced 3-HPA (less than 1.5 $g.L^{-1}$), due to their implemented fed-batch strategy. A better understanding of the 3-HPA system and particularly the setting of an analytical method allowing the detection and quantification of the different forms of this complex system would be necessary..

For all the three stains, the 3-HP production starting from glycerol was higher than the 1,3-PDO one, that was surprising compared to previous study reporting an equimolar ratio between these two products using *L. reuteri* DSM 20016 strain [15]. Given that the production of 1 mol of 3-HP generates also 1 mol of ATP (Fig. 1), this could be more advantageous for *L. reuteri* cells to produce 3-HP rather than 1,3-PDO, if $NAD^+$ can be provided by another way

than the synthesis of 1,3-PDO. As the production of 3-HP yields 1 mol of NADH, $H^+$ per mole of product and the production of 1,3-PDO regenerates the $NAD^+$ both from 3-HPA (Fig. 1), the ratio between 3-HP and 1,3-PDO is expected to be 1. The excess in 3-HP observed here could be the result of the activity of a NADH oxidase (EC number, 1.6.99.3) whose sequence has been detected in the genomes of the three *L. reuteri* strains (Online Resources 2, 3, and 4). This could explain the higher molar concentration of 3-HP than that of 1,3-PDO by oxidation of NADH, $H^+$ into $NAD^+$ in the presence of $O_2$. In scaling up the glycerol bioconversion with the DSM 17938 strain to a 5-L bioreactor, where the headspace was bigger (4.1 L vs. 60 mL in bottles), the 3-HP/1,3-PDO ratio increased to 2.53 mol/mol (vs. 1.65 mol/mol). This result would be consistent with the hypothesis of an active NADH oxidase. However, the bioconversion performances in bioreactor with a controlled pH were not better than in bottles. In bioreactor, the ratio 3-HPA/biomass was significantly higher than in bottles (1.05 and 0.64 g/g, respectively), which could explain the quick inhibition of the producing cells and the consequent low efficiency of the bioconversion.

The differences between strains in terms of glycerol metabolism and 3-HP production capabilities are partly interrelated to differences of toxicity observed on the producing strains. Concerning this inhibition, it is well known that 3-HPA is toxic for the producing cells when present at a certain threshold concentration (between 10 and 30 mM, [28, 40]) which is a limiting factor for high 3-HP production. In addition, and even if its deleterious effect has been very little studied, 3-HP is toxic as other organic acids, at least because of the decrease in pH induced by its production. As shown in our study, glycerol consumption, 3-HPA, 3-HP, and 1,3-PDO productions stopped after 1 to 1.5 h of incubation depending on the strain (Fig. 3), which can be related to the inhibition of bacterial cells due to 3-HPA and 3-HP accumulation. The observed loss in cultivability (more than 10 logs of $CFU.mL^{-1}$ for the three strains, Fig. 6) and in enzymatic activity (about 40 % after 3 h and 99 % after 24 h for the DSM 17938 strain used as an example, Fig. 7b) may be due to the oxidative stress caused by 3-HPA on bacterial cells and/or to the decrease in pH linked to 3-HP production. In fact, 3-HPA acts via interaction of its aldehyde group with thiol groups of cell components, particularly proteins and enzymes [31]. Vollenweider et al. [41] completed this hypothesis and proposed an antimicrobial mechanism for 3-HPA, whereby its activity leads to depletion of free SH groups in cell proteins causing an imbalance of the cellular redox status, ultimately resulting in cell death.

As other organic acids and particularly as its positional isomer the 2-HP (or lactic acid), the 3-HP is deleterious to cell survival and to its own production notably by causing a decrease in extracellular and intracellular pH affecting enzyme and cellular protein activity or because of the expense of energy required to export organic acids out of the cell, particularly at low extracellular pH [42]. Our results showed a significant deleterious effect on *L. reuteri* cultivability when the pH decreased below 4.7 (Fig. 6b), when close to the $pK_a$ (i.e., 4.51). Flow cytometry analyses on the DSM 17938 strain corroborated this hypothesis, as the impact of 3-HP on cell physiological state was noticeable at pH 3 but not at pH 5 (Fig. 7c), confirming the importance of pH in 3-HP toxic effect. The ATP generation along the organic acid formation pathways, as for 3-HP production, from glycerol, by *L. reuteri* (Fig. 1), can also have a significant impact on product export and therefore on intracellular pH [42]. In addition to the pH-based inhibition linked to intra- and extracellular 3-HP, a specific interference of its anionic form with the cellular metabolism was reported by Warnecke et al. [43], who observed that the 3-HP toxicity in *E. coli* involved inhibition of the chorismate and threonine superpathways. A study on *K. pneumoniae* showed that when 3-HP was added to the culture medium, the rates of cell growth, glycerol consumption, and 3-HP production gradually decreased as the 3-HP

concentration increased between 120 and 790 mM (e.g., about four times less 3-HP production compared to culture performed without 3-HP) [17]. Moreover, the MIC for 3-HP has been estimated to 15 $g.L^{-1}$ in the case of *E. coli* [33]. Nevertheless, the effects of acid stress on lactic acid bacteria physiology [44] and particularly the impact of 3-HP on *L. reuteri* are not known in details. Furthermore, this impact would certainly be visible at lower 3-HP concentrations in the case of glycerol bioconversion by *L. reuteri* due to the joined inhibitory impact of 3-HPA.

In order to explain the differences between the three *L. reuteri* strains in terms of glycerol bioconversion, data mining analyses were performed. As the genomes of the strains have been sequenced and annotated, it is therefore possible to compare the nucleotide and amino acid sequences of the two main enzymes involved in the glycerol bioconversion pathway (i.e., the glycerol dehydratase, GDH, and the propionaldehyde dehydrogenase, PduP). This analysis revealed that the nucleotide sequences of each enzyme are highly conserved among the three strains (between 92.4 and 100 % identity for these GDH and PduP nucleotide sequences). It was the same for the comparison of amino acid sequences of the GDH (99.6 % identity between the DSM 17938 and the ATCC 53608 strains and 97.6 % identity between the former and the DSM 20016 strain) and the PduP (100 % identity between the DSM 17938 and the ATCC 53608 strains and 97.2 % identity between the former and the DSM 20016 strain), which showed that the differences in bioconversion performances observed between the three strains might partially be explained by this assumption. Other hypotheses could be proposed, such as a difference in the expression of genes encoding for the enzyme promoters, for the vitamin B12 synthesis (starting from glutamate) into bacterial cell, or for the glycerol transport through the membrane. Morita et al. [27] identified the PduF protein as the glycerol carrier in *L. reuteri* DSM 20016 cells. However, no difference in the amino acid sequences of this carrier was observed between the three strains after the BLASTP performed in our study (data not shown). Moreover, the comparison of the protein sequences of both GDH and PduP enzymes, and especially their number of cysteins which could bind 3-HPA via its aldehyde group (i.e., 11 cysteins for the GDH and 6 for the PduP), did not allow explaining the observed differences between the studied strains. The differences in terms of resistance to product toxicity and ability to produce 3-HP could therefore be due to 3-HP and/or 3-HPA tolerance, $NAD^+$ regeneration capacity, ability to produce vitamin B12, or differences in enzyme expression.

## Conclusion

With a productivity in non-optimal conditions around 2 $g.L^{-1}.h^{-1}$ of 3-HP and 4 $g.L^{-1}.h^{-1}$of 3-HPA depending on the strain, this study confirmed the potential of *L. reuteri* for the biotechnological production of 3-HP using a two-step process involving biomass production followed by glycerol bioconversion into 3-HP. Surprisingly, the mass balance analyses evidenced a higher 3-HP molar production than the one of 1,3-PDO for the three studied strains (molar ratios 3-HP/1,3-PDO > 1). This fact was confirmed in bioreactor for the DSM 17938 strain. Moreover, this scaling up should be improved to understand the role played by environmental conditions (pH, aeration, redox status) on bioconversion performances. As the toxicity of the products is critical, several directions can be considered to solve this problem: (i) the understanding of the mechanisms underlying the inhibitory effects of 3-HP and 3-HPA on the producing bacteria, (ii) the use of an integrated process coupling bioconversion and in situ extraction of 3-HP to optimize the production performances, and (iii) the use of a fed-batch mode for glycerol supply to avoid 3-HPA accumulation.

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**Conflict of Interest** The authors declare that they have no competing interests.

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