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Enantioselective lactic acid production by an *Enterococcus faecium* strain showing potential in agro-industrial waste bioconversion: physiological and proteomic studies

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Abstract

The growing demand of biodegradable plastic polymers is increasing the industrial need of enantiospecific L-lactic acid (L-LA), the building block to produce polylactides. The most suitable industrial strategy to obtain high amounts of LA is the microbial fermentation of fruit and vegetable wastes by lactic acid bacteria (LAB). In this paper seven LAB strains from our laboratory collection, were screened for their ability to produce the highest amount of pure L-LA. A strain of *Enterococcus faecium* (LLAA-1) was selected and retained for further investigations. *E. faecium* LLAA-1 was grown in different culture media supplemented with the most abundant sugars present in agricultural wastes (i.e., glucose, fructose, cellobiose and xylose) and its ability to metabolize them to L-LA was evaluated. All tested sugars proved to be good carbon sources for the selected strain, except for xylose, which resulted in unsatisfactory biomass and LA production. Growth under aerobic conditions further stimulated L-LA production in fructose supplemented cultures with respect to anoxic-grown cultures. Proteomic profiles of *E. faecium* LLAA-1 grown in aerobiosis and anoxia were compared by means of two-dimensional electrophoresis followed by MALDI-TOF mass spectrometry. Seventeen proteins belonging to three main functional groups were differentially expressed: the biosynthesis of 6 proteins was up-regulated in aerobic-grown cultures while 11 proteins were biosynthesized in higher amounts in anoxia. The *de novo* biosynthesis of the f-subunit of alkyl hydroperoxide reductase involved in the re-oxidation of NADH seems the key element of the global re-arragement of *E. faecium* LLAA-1 metabolism under aerobic conditions. An improved oxidative catabolism of proteinaceous substrates (i.e., protein hydrolisates) seems the main phenomenon allowing both higher biomass growth and improved LA production under these conditions.

Keywords:

Lactic acid bacteria, L-lactic acid, fructose, oxygen, proteomics.

1. Introduction

The need to replace various petro-chemical derived materials with polymers produced from renewable sources has strongly stimulated research on bio-based degradable plastic polymers such as polylactides (PLA). This has led to a growing demand of lactic acid (LA), the monomeric unit, resulting in a rapid increase of its production, that was about 150000 metric tons per year in 2003 (Hester, 2000a; Bizzarri and Kishi 2003) and has been estimated to grow yearly at 5–8% (Yadav et al., 2011). The annual world market demand for LA was expected to reach 259,000 metric tons by the year 2012 (Martinez et al., 2013), and is forecasted to reach 367,300 metric tons by the year 2017. PLA production capacity was evaluated as high as of 450,000 metric tons per year in 2008, however, it is still dwarfed by the 200 million metric tons of total plastics which are produced every year (Okano et al., 2010). The high cost associated with the production of LA by fermentation is among the main factors limiting PLA to compete with fossil-fuel-based plastics in extensive application (Okano et al., 2010). The primary costs of LA production by fermentation include expensive nitrogen sources and pure sugars required for cell growth along with LA downstream recovery and purification process (Abdel-Rahman et al., 2013).

The promising application of polylactides as biodegradable plastics (Hester, 2000b) requires the availability of high amounts of optically pure LA. LA can exist in two optical isomers: L-LA and D-LA. Relevant technological characteristics of PLA strongly depend on precise LA enantiomer mixture. Generally, poly-L-LA is more appreciated, since polymers obtained with D-LA are generally amorphous and not so requested by the market. On the contrary, L-LA polymers display crystalline structure and have different applications such as re-absorbable plates and screws, surgical suture thread (Lasprilla et al., 2012), disposable dishes, waste plastic bags and mulch (Jamshidian et al., 2010).

LA can be obtained by chemical synthesis (mostly lacto-nitrile route) or by microbial fermentation of carbohydrates and agro-industrial feedstocks (Wee and Ryu, 2009). LA-producing microorganisms reduce pyruvic acid, generally generated through glycolysis or by the phosphoketolase route, into LA as a mean to re-oxidize NADH generated by these metabolic pathways. Fermentative production displays several advantages as compared to the chemical synthesis: lower substrate costs, lower working temperature and reduced energy consumption during the process

(Djukic-Vukovic et al., 2012). Biotechnological production of LA shows further benefits since optically pure LA can be obtained by choosing suitable microbial strains. Currently, about 90% of the LA produced worldwide derives from fermentation processes, including that commercialized by the largest companies such as Cargill Dow (USA), Purac (The Netherlands), Galactic (Belgium) and Musashino Chemical Laboratory Ltd (Japan) (Datta and Henry, 2006).

In this scenario, lactic acid bacteria (LAB) are among the most suitable microorganisms, since LA is generally their major metabolic end product. LAB show homo-lactic, or hetero-lactic, or mixed-acid fermentative phenotypes, depending on the considered strain and the used growth conditions. Homofermentative LAB produce LA as the main end-product through the Embden-Meyerhof pathway. Heterofermentative and mixed-acid fermenting LAB produce additional end-products such as ethanol, acetic acid, formic acid and carbon dioxide through either the Embden-Meyerhoff or the pentose phosphate pathways and the biosynthesis of additional enzymes (i.e., pyruvate dehydrogenase and pyruvate-formiate lyase) which divert a part of pyruvic acid from its conversion to LA towards other end-products (Vink et al., 2003). Of course, the highest LA yields are obtained by homofermenters since theoretically 100% of the sugar carbon substrate can be converted to LA. Homofermenting LAB can produce L-LA and/or D-LA depending on their genetic repertoire: some LAB strains possess only the L-LA dehydrogenase (L-LDH) gene(s) or the D-LA dehydrogenase (D-LDH) gene(s) thus generating pure L-LA or D-LA, respectively (Garvie, 1980). Other species possess multiple genes encoding for both enzymes, and/or lactate racemase, that catalyzes D-LA / L-LA interconversion (Goffin et al., 2005), resulting in the production of LA racemic mixture. It is worth noting that genetic determinants for D-LDH, L-LDH and racemase can be easily acquired by horizontal gene transfer (plasmid conjugation), followed or not by transposition and stable localization on the chromosome.

Products at the highest purity are obtained if highly pure sugar is used as the fermentation substrate. Furthermore, this results in lower purification costs. However, taken as a whole, this process configuration is not economically viable, since pure sugars are expensive. Therefore, different biomasses such as molasses, starch, lignocellulose and wastes from agricultural and agro-industrial activities, featuring both low purchase price and renewability, have already been investigated as fermentative substrates for LAB. As the majority of LAB strains is not able to directly use starchy and lignocellulosic materials, these substrates must be pre-treated by physical-chemical and/or enzymatic methods prior to LAB fermentation (Okano et al., 2010). Relatively few natural LAB strains, e.g., *Lactobacillus amylophilus*, *Lactobacillus amylovorus*, *Lactobacillus amylolyticus* and some strains of *Lactobacillus plantarum*, which have starch-degrading properties (i.e. ALAB), have been isolated so far (Giraud et al., 1994; Guyot et al., 2000; Narita et al., 2004). However, most of them has shown either low LA yield or low enantioselectivity (John et al., 2007; Okano

et al., 2010). Production of LA from cellobiose and cellotriose by *Lactobacillus delbrueckii* was described by Adsul and co-workers (Adsul et al. 2007). However, as far as we know, no natural cellulolytic LAB has been isolated to date.

Several attempts have been performed to produce LA at both high productivity and final concentration (Wee et al., 2006; Zhang et al., 2007; Okano et al., 2010; Zhao et al., 2010a; Zhao et al., 2010b; Moon et al., 2012). In the present study, our laboratory collection of LA bacteria has been screened to select a strain able to produce high amounts of optically pure L-LA in complex media. Afterwards, the ability of the selected *Enterococcus faecium* LLAA-1 to produce optically pure LA from different sugars present in fruit and other vegetable waste biomasses (glucose, fructose, cellobiose and xylose) was tested. Cellobiose is the disaccharidic repetitive unit of cellulose and consists of two glucose moieties linked by β(1-4) glycosidic bonds (Kumar et al., 2008); it may originate from cellulose hydrolysis, by cellobiohydrolase activity, and it is present in vegetable wastes such as cereals, carrots, fruits, mainly apples, and bran. Xylose is a pentose sugar and is the main component of hemicellulose (e.g., arabinoglucuronoxylan) present mainly in oat, rye, barley and wheat bran (Bercier et al., 2007). Fructose is the most abundant sugar present in several fruits and vegetables both as free fructose and linked to glucose in the form of sucrose. The fruits containing the highest fructose amount are apples, bananas, pears and grape (Park and Yetley, 1993). A proteomic analysis on the strain grown in a fructose-supplemented medium in aerobiosis and anoxia was then performed to better elucidate the molecular mechanisms underlying the different amounts of LA which are produced in these two growth conditions.

2. Materials and Methods

2.1 Bacterial strains

Seven strains of LA bacteria belonging to the culture collection of the Department of Life Sciences and Systems Biology of Torino (Italy) were used in the present study: *Lactococcus lactis* 89A, *Lactococcus lactis lactis* 92A, *Lactococcus lactis* NCDO 2118, *Lactococcus lactis cremoris* 88 A, *Lactococcus lactis lactis* 150 A, *Lactobacillus plantarum* 31 A and *Enterococcus faecium* LLAA-1. All the strains were maintained at -24°C in aliquots of 0.5 mL of exponential phase cultures and 0.5 mL 40% v/v glycerol.

2.2 Culture conditions in vials and bottles

For the preliminary screening all the strains were cultured in 30 mL screw cap-glass vials at 37°C without agitation in standard commercial culture medium, i.e., M17 (Fluka) for cocci and MRS (Difco) for *L. plantarum* 31 A, respectively.

The selected *E. faecium* LLAA-1 was then cultured in 250 mL screw-cap glass bottles at 37°C without agitation in both modified M17 (29 g/L tryptone casein, 16 g/L Soy peptone, 10 g/L Yeast extract, 0.5 g/L ascorbic acid, 0.25 g/L Magnesium sulphate, 2.5 g/L hydrogen potassium phosphate, 19 g/L sodium glycerophosphate) and Chemically Defined Medium (CDM) (Petry et al., 2000). Bacterial cultures were performed in either one or the other of these media after supplementation with 111 mM glucose, or 111 mM fructose, or 56 mM cellobiose or 111 mM xylose. Replicates of these cultures were perfomed by additional supplementation of 0.1 M sodium acetate. Bacterial growth was monitored by measurement of optical density at 600 nm (Pharmacia Biotech, Spectrophotometer Ultrospec 2000).

All the described cultures were performed with inocula from pre-cultures grown in the same media in order to have an initial OD_{600} of about 0.1.

2.3 Culture conditions in fermenter

A Diaferm Basic fermenter (DiaChrom Sa) was used to compare *E. faecium* LLAA-1 growth and LA production under aerobic and anoxic conditions. Experiments were performed in 3.5 L of modified M17 medium, as described above, fortified with 111 mM fructose. The fermentations were performed at 37°C, under 200 rpm shaking with an initial pH of 6.9 that was not subsequently adjusted. All the fermentations were performed with inocula from pre-cultures grown in the same medium in order to have an initial OD_{600} of about 0.1.

The aerobic condition was maintained by blowing air into the medium throughout bacterial growth. The anoxic condition was achieved by nitrogen bubbling into the medium; to ensure the latter condition butyl rubber tubings with low permeability to gases were used.

2.4 LA quantification

Two mL of stationary phase cultures for all the tested strains were collected and biomass were separated from supernatant by centrifugation (12000xg, 10 min, 4°C). Since LAB extrude LA in the external environment to gain energy by a membrane transport system (Konings 2002), the analyses were performed on the supernatants. The concentration of the two LA enantiomers was determined by the K-DLATE enzymatic kit (Megazyme).

2.5 HPLC analysis

An Agilent 1200 Series Technologies HPLC system equipped with a multichannel diode array detector (DAD) and a refractive index detector (RID) was used. Separation of metabolites in the extracellular growth media was performed by an Aminex HPX-87H 300 x 7.8 mm column equipped with a thermostat and a guard column. Isocratic elution was obtained by 0.5 mL/min of 5 mM H_2SO_4 at 40°C.

Standards of L-LA, fructose, acetic acid and ethanol were prepared at different concentrations ranging from 0.5 to 30 g/L. Eight-hundred µL of fermentation broths were subjected to precipitation with 200 µL zinc sulfate 0.3 M and 200 µL barium hydroxide 0.3 M to remove free proteins and peptides.

Chromatogram record and analysis, both peak integration and analysis of the purity of peaks, were performed with the ChemStation software (Agilent Technologies).

2.6 Proteomic analysis

2.6.1 Protein sample preparation

Fifty mg of cells were treated in each protein preparation. The cells were collected in the late exponential growth phase by centrifugation (4000xg, 20 min, 4°C) and washed three times with NaCl 0.85% (w/v). The obtained pellets were re-suspended in 3 mL 50 mM Tris-HCl pH 7.3, 1 mM EDTA and disrupted by sonication twice as previously described (Pessione et al., 2009), to recover the highest amount of proteins. After clarification (4000xg, 20 min, 4°C), supernatants were supplemented with 10 µL/mL Nuclease mix (GE Healthcare) and after 30 min incubation at room temperature they were centrifuged (100000xg, 1h, 4°C) in a Beckman L8-60M ultra-centrifuge (Type 60 rotor). The obtained supernatants were then dialyzed against four volumes of ddH_2O and precipitated with methanol/chloroform according to Wessel and Flugge (Wessel and Flugge, 1984). The obtained pellets were then dissolved in rehydration solution [6.5 M urea, 2.2 M thiourea, 4% (w/v) CHAPS, 5 mM Tris-HCl pH 8.8, 0.5% IPG buffer 4-7 (GE-Healthchare), 100 mM DTT]. The protein concentrations were evaluated by the 2-D Quant-Kit (GE Healthcare).

2.6.2 Two-dimensional electrophoresis experiments

Isoelectrofocusing was performed using 13 cm IPG strips (GE Healthcare) with a linear gradient ranging from 4 to 7. Three-hundred µg proteins were loaded by in-gel rehydration and IEF was performed using IPGphor (Amersham Biosciences) at 20°C with 59000 VHrs. After IEF, the strips were equilibrated for 15 minutes in 6.0 M urea, 30% v/v glycerol, 2% w/v SDS, 5 mM Tris HCl pH 8.6 fortified with 2% w/v DTT and then for 15 minutes with the same buffer containing 4.5 w/v IAA instead of DTT. SDS-PAGE was performed on vertical, homogeneous, 1.0 mm thin 11.5% T and 3.3% C acrylamide (Biorad) gels. Molecular weight markers were from GE Healthcare (LMW-SDS marker). The running buffer was 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3. Running conditions were 11°C,

600 V constant voltage, 20 mA per gel, 60 W for 15 min and 11°C, 600 V constant voltage, 40 mA per gel, 80 W for approximately 2 h. Gels were automatically stained with Colloidal Coomassie blue using the Processor Plus device (Amersham Biosciences, GE Healthcare) following the manufacturer's instructions.

2.6.3 Image analysis

The stained 2-DE gels were digitized with the Personal Densitometer SI (GE Healthcare). Image analysis was performed using the Progenesis PG220 software (Nonlinear Dynamics): spot detection was automatically performed by using the algorithm named "2005 detection". After the establishment of some user seeds, matching was automatically performed and manually verified.

2.6.4 Statistical analysis

For both the growth conditions (i.e., aerobiosis and anoxia) two biological and three analytical replicates were performed. Spot intensities were measured *via* both absolute and normalized volumes and were statistically analyzed by means of the *t-test*: means were considered significantly different when $p<0.05$ and the expression difference was higher than 1.5 fold.

2.6.5 Protein identification

Protein spots were excised from the dried gels and rehydrated with MilliQ water. They were destained in 50% ACN in 5 mM NH_4CO_3, dried in 100% ACN and then digested overnight with 5 µL trypsin solution (0.1 mg/mL trypsin in 5 mM NH_4CO_3). A MALDI TOF (MALDI micro MX, Waters) mass spectrometer with a delayed extraction unit was used. Peptide spectra were obtained in reflectron mode in the range 800-3000 Da. Database searching was performed by using the 25 most intense measured peptide masses against the NCBI database *via* the free search program MASCOT (www.matrixscience.com). The following parameters were considered in the search: taxa Firmicutes, trypsin digestion, one missed cleavage, carbamidomethylation of cysteine as fixed modification, methionine oxidation as variable modification and maximum allowed error 100 ppm. Only protein identifications with significant Mascot score ($p<0.05$) were taken into account.

3. Results and Discussion

3.1 Selection of LAB strains producing high amounts of optically pure L-LA

Seven LAB present in our laboratory collection (see Table 1) were submitted to a preliminary screening to quantify the amount of produced LA on complex commercial media, i.e., MRS for bacilli and M17 for cocci, respectively. Both L- and D-LA production was evaluated at the end of the bacterial growth. As shown in table 1, *L. plantarum*

31A produced the highest amount of LA (35.50±1.51 mM). However, L-LA was just the 67.92% of the whole produced LA, while the rest was D-LA. Since for PLA production pure L-LA enantiomer is preferable (Vink et al. 2003), *L. plantarum* 31A was discarded from further investigations. Among the other strains tested, the most interesting was *E. faecium* LLAA-1 which was able to produce the highest amount of LA (28.26±0.49 mM) with the highest L-LA enantioselectivity (98.70%). The other strains were able to produce L-LA but at lower amount, ranging from 17.72±0.42 mM to 27.17±1.00 mM and with slightly lower L-LA enantioselectivity, ranging from 96.65% and 98.45%, than *E. faecium* LLAA-1.

On the basis of these results *E. faecium* LLAA-1 was chosen for further investigations.

3.2 Biomass yield and L-LA production by *E. faecium* LLAA-1 on different soluble sugars

Because of industrial demand of LA-producing bioprocesses converting low-cost fermentable substrates, the ability of *E. faecium* LLAA-1 to efficiently ferment sugars present in fruit and other vegetable waste biomasses (glucose, fructose, cellobiose and xylose), was tested. LA production by *E. faecium* LLAA-1 was analysed in both Chemically Defined Medium (CDM) and modified M17 medium supplemented with glucose, fructose, cellobiose or xylose.

As expected, M17 medium supported higher growth rates than CDM medium: final OD_{600} ranged from 5.0 to 6.7 in M17-grown cultures while values were comprised between 2.0 and 3.0 in CDM-grown cultures (data not shown). Indipendently from the used culture medium, *E. faecium* LLAA-1 was able to ferment all the tested mono-/di-saccharides, although resulting in different final biomass. Glucose, fructose and cellobiose were readily consumed by *E. faecium* LLAA-1 in both CDM and M17 media. On the contrary, in xylose supplemented cultures the lag phase was longer and the final biomass was lower than with the other sugars, suggesting the difficulty of the strain to gain energy just from xylose metabolism (data not shown). As expected, glucose or fructose supported the highest growth rates.

Supernatants of stationary phase cultures were collected in order to quantify both L- and D-LA isomers by enzymatic assay. No detectable amount of D-LA was found, suggesting that under the tested growth conditions the whole LA produced is consistent with the L-isomer. As shown in figure 1, the highest amount of L-LA was produced by modified M17-grown cultures as compared to the CDM-grown cultures, for all the tested sugars. Furthermore, a higher production of L-LA was obtained by using glucose or fructose as the sugar substrate: the conversion yields in M17-grown cultures were about 90% of the maximum theoretical value for glucose and fructose, about 70% for cellobiose and only about 22% for xylose.

In order to improve L-LA production further studies were performed to test the effect of sodium acetate supplementation and of culture oxygenation on LA production from the different sugars.

3.3 Effect of sodium acetate supplementation on L-LA production by *E. faecium* LLAA-1

Previous studies reported that addition of sodium acetate can improve both LA yield and enantioselectivity in LAB (Iino et al., 2001, 2002). Sodium acetate has been supposed to improve the carbon flux through either the glycolytic pathway or the pentose phosphate route (Iino et al., 2001, 2002) resulting in a higher production of pyruvic acid and hence of LA. It has also been hypothesized that sodium acetate may stimulate either L-LA dehydrogenase biosynthesis or its catalytic activity (Iino et al., 2001, 2002). In the present investigation, the addition of sodium acetate to the culture medium did not modify the growth kinetics of *E. faecium* LLAA-1 under any of the tested conditions. Furthermore, sodium acetate had no effect on L-LA production by *E. faecium* during growth on fructose- or xylose-supplemented media and even inhibited LA production in glucose- and cellobiose-fortified cultures (Fig. 2). Therefore, sodium acetate effect on L-LA production by *E. faecium* LLAA-1 was not consistent with the data referred in the literature for other LAB strains.

3.4 Effect of culture oxygenation on L-LA production by *E. faecium* LLAA-1

To evaluate the effect of oxygen on L-LA production, different cultures were set up in fermenter in modified M17 medium under both aerobic and anoxic conditions. Since fructose is abundant in several fruit and vegetable scraps, it was chosen as the sugar substrate for the experiments in fermenter. Data obtained by the present study could therefore be useful for future utilization of *E. faecium* LLAA-1 to produce L-LA by bioconversion of waste feedstocks containing this sugar.

The experiments in fermenter revealed that the overall bacterial growth rate (μ) was very similar in aerobic and anoxic environments (a μ of about 0.98 h^{-1} and 1.01 h^{-1} were measured in aerobic and anoxic cultures, respectively); the same growth rate was maintained all along growth in anoxia, while a faster growth ($\mu = 1.39\ h^{-1}$) occurred in the early exponential growth of aerobic cultures followed by a slower growth ($\mu = 0.20\ h^{-1}$) between 3 h and 4.5 h after inoculum (Fig. 3). Actually, higher final biomass under aerobic conditions (final OD_{600} around 7.7) than under anoxic conditions (final OD_{600} around 7.1) was observed. This observation is partially in contrast with several literature data indicating that oxygen can have an inhibitory effect on the growth of microaerophylic LA bacteria. Nevertheless, in the literature it has been demonstrated that *Lactobacillus sake* NCFB 2813 was not inhibited by oxygen (Amanatidou et al., 2001a, 2001b). A time-course investigation on fructose consumption and L-LA production during growth under both aerobic and anoxic conditions was performed by HPLC. As shown in figure 3, aerobic conditions

promoted the metabolization of a higher amount of fructose (about 132 mM as respect to about 82 mM in anoxic cultures) and the production of a greater total LA quantity which was almost 3 fold higher (377.90±8.32 mM) than in anoxia (129.99±10.12 mM). However, decay of extracellular medium pH was almost identical under the two growth conditions: as shown in Figure 3, pH values diminished of about 2.2 units during the whole fermentation under either aerobic or anoxic conditions. Since final LA concentration was much higher in aerobic cultures, we can hypothesize that under the former conditions either unidentified acid molecules were consumed or alkaline molecules were produced concomitantly, so that the final pH was similar under both growth conditions (Figure 3). At the end of the anoxic fermentation, the amount of LA accumulated in the growth medium corresponded to about 79% of LA which could be theoretically obtained by the homolactic metabolization of 82 mM fructose (maximum theoretical yield= 2 LA per fructose), which is lower than apparent fructose-LA conversion yields calculated for microaerophylic cultures (Fig. 1). On the contrary, in aerobic cultures, final concentration of accumulated LA corresponded to 143% of maximum LA that could be obtained by homofermentative conversion of 132 mM fructose. In more detail, about 280 mM L-LA was produced during the early exponential phase of aerobic-grown cultures while just 50 mM fructose was consumed; furthermore, 100 mM L-LA was produced in the stationary phase of aerobic-grown cultures concomitantly with the consumption of only 20 mM fructose. These results clearly indicate that other alternative metabolic pathways leading to the production of L-LA, probably using alternative substrates, were activated under aerobic growth conditions.

Several metabolic pathways leading to LA production exist in nature, for instance L-LA can be generated by LAB through malo-lactic fermentation. Intracellular malic acid decarboxylation to L-LA is part of a biochemical system which also includes electrogenic LA/malic acid antiporters (Pessione et al., 2010). The whole system provides both alkalinization of the environment (by converting a dicarboxylic acid, such as malic acid, into a monocarboxylic acid, LA) and metabolic energy for the cells, since malic acid (from out to in)/ LA (from in to out) exchange generates proton motive force across the cellular membrane (Pessione et al., 2010). However, such strategy depends on the availability of high amounts of malic acid in the growth medium in order to explain for the high amounts of LA which are accumulated in aerobic-grown cultures of *E. faecium*. Since M17 medium composition should not contain high concentration of malic acid, this hypothesis seems unlike. However, M17 is a complex medium rich in protein hydrolysates (see materials and methods). It is therefore possible that aerobic conditions stimulate the activation of routes converting the so-called “gluconeogenetic” amino acids (e.g., alanine, aspartate) into pyruvate which can then be readily converted by LA dehydrogenase to LA.

In order to get a more in depth comprehension of the overall picture of *E. faecium* metabolism under both anoxic and aerobic growth conditions, and possibly explain why L-LA is produced in higher amounts in aerobiosis than in anoxia, comparative proteomic analyses were performed as described below.

3.5 Comparative proteomic analysis of fructose-grown cultures of *E. faecium* LLAA-1 under aerobic-anoxic conditions.

For both aerobic and anoxic growth conditions, cells were collected at the beginning of the stationary phase. The total cell proteome ("*in toto*" proteome) in the 4-7 pI range was analyzed (figure 4). Twenty-three spots showed significantly different intensities between the two tested conditions: 9 were up-regulated and 14 down-regulated under aerobic conditions. These spots were all identified by MALDI TOF mass spectrometry, exception made for spots 21, 22 and 23 which did not give a significant protein identification. Considering that some spots were different isoforms of the same protein, a total of 17 proteins were found to be differentially expressed between the two tested conditions: 6 proteins were expressed to a higher level in aerobiosis and 11 proteins were up-regulated under the anoxic condition. All these proteins are listed in table 2 and may be divided into two main classes according to their function: i. energy metabolism proteins; ii. stress response proteins. All the other proteins were grouped together as "iii. other proteins".

Among proteins involved in the energy metabolism, dihydrolipoamide dehydrogenase (PDH E3, spots 1 and 2), phosphate acetyltransferase (PTA, spot 3), pyruvate dehydrogenase (PDH E1, spots 4 and 5, present in aerobic-grown cells only), ketopantoate reductase ApbA/PanE (spot 6) and f-subunit of alkyl hydroperoxide reductase (Nox, spot 7, present in aerobic-grown cells only) were up-regulated in aerobiosis; on the contrary 6-phosphogluconate dehydrogenase (6PGODH, spot 10), glucose-1-dehydrogenase (G1DH, spot 11), 2-dehydropantoate 2-reductase (spot 12, present in anoxic-grown cells only) and aldo/keto reductase (spot 13) were up-regulated in anoxia.

Dihydrolipoamide dehydrogenases are homodimeric flavoproteins that catalyze the NAD^+-dependent re-oxidation of dihydrolipoamide in several multienzymatic complexes such as the pyruvate dehydrogenase (PDH) complex, the 2-oxoglutarate dehydrogenase complex and the branched-chain 2-oxo-acid dehydrogenase complex (Perham et al., 1987; de Kok et al., 1998). These three complexes share a common architecture composed of multiple copies of three components: E1, a thiamine pyrophosphate-dependent 2-oxo-acid dehydrogenase, E2, a dihydrolipoamide acyltransferase and E3, a FAD-containing dihydrolipoamide dehydrogenase. Since in aerobiosis also two isoforms of the pyruvate dehydrogenase (corresponding to the E1 component, spots 4 and 5) were up-regulated (actually, spots 4 and 5 are detectable in aerobic-grown cultures only), it is reasonable to assume that all these proteins (spots 1, 2, 4, 5)

are part of the same PDH complex. The induction of PDH during growth in aerobiosis has already been reported for other enterococci and LAB (Snoep et al., 1992; Jensen et al., 2001). PDH competes with other enzymes such as lactate dehydrogenase (LDH) for intracellular pyruvate. It is reasonable to assume that in aerobic-grown cells pyruvate will be partly diverted towards acetyl-CoA biosynthesis instead of LA production. Theresfore, this result seems in contrast to the fact that, under this culture condition, *E. faecium* LLAA-1 accumulates larger amounts of LA than in anoxic environment (see above). Once more, this result indicates that a relevant part of LA accumulated under aerobic conditions was obtained by the conversion of other substrates than fructose (see below).

It is worth noting that enterococci generally lack a complete Krebs cycle. In these microorganisms acetyl-CoA can be used for: i) citrate biosynthesis (the only step of Krebs cycle present in this genus); ii) acetate production via acetyl phosphate; iii) production of ethanol, by either direct reduction of acetyl-CoA or through the formation of acetyl phosphate intermediate (Fig. 5) (Hofvendahl and Hahn-Hägerdal, 2000). Acetate production pathway also involves ATP synthesis through substrate-level phosphorylation catalyzed by acetate kinase (AK) (Fig. 5). This route is very common especially in those bacteria such as LAB lacking a functional respiratory chain, whose energy metabolism is chiefly based upon substrate level phosphorylation. Since our proteomic analyses also detected a strong up-regulation of phosphate acetyltransferase (PTA, spot 3) in aerobic-grown cultures, it is likely that, under this condition, a significant amount of pyruvate is diverted through acetyl-CoA and acetyl phosphate. However no up-regulation of either AK or Aldehyde / Alcohol dehydrogenase (AlDH/ ADH, respectively) was detected in aerobic-grown cultures by proteomic analyses. Moreover, neither acetate nor ethanol accumulation was detected in these cultures (data not shown). These data suggest, that the activation of this pathway i) does not involve a massive re-routing of pyruvate towards acetate or ethanol; or ii) should be used for other, yet unidentified, purposes, such as the synthesis of other cellular intermediates or catabolites.

A partial explanation of the fact that the amount of LA accumulated under anoxic conditions is lower than in aerobic-grown cultures is given by the finding that glucose 1-dehydrogenase (G1DH, spot 11) and 6-phosphogluconate dehydrogenase (6PGODH, spot 10) are up-regulated in anoxia. These data support the hypothesis that a significant part of fructose is fermented through heterolactic pathways in absence of oxygen (Fig. 5).

A recent study by Chambellon et al. (2009) brought evidences that a number of enzymes annotated as ketopantoate reductase on the basis of primary sequence homologies found in LAB, could belong to a new family of proteins, known as 2-hydroxyacid dehydrogenases, involved in amino acid catabolism. Notably, in LAB the first step of amino acid catabolism is generally catalyzed by aminotransferases, which generate 2-oxo acids (Chambellon et al., 2009). 2-

oxo acids can then be either oxidized by 2-oxo acid dehydrogenases, or reduced by 2-hydroxyacid dehydrogenases (Fig. 5) (Yvon and Rijnen, 2001). In the case of pyruvate (which can be obtained by deamination of aminoacids such as alanine or aspartate), LDH catalyzes such reduction, while incorrectly annotated ketopantoate reductase from *Lactococcus lactis* is involved in the catabolism of branched-chain amino acids. It is therefore possible that ketopantoate reductase (ApbA/PanE, spot 6) identified in the present study rather functions as a 2-hydroxyacid dehydrogenases. Therefore, the up-regulation of this enzyme in aerobic-grown cultures of *E. faecium* would be related to an improved amino acid catabolism. It is worth noting that spot 12 was identified as a homologue of a 2-dehydropantoate 2-reductase, i.e., an alternative name for ketopantoate reductase (E.C. 1.1.1.169). Since protein corresponding to spot 12 was detectable in anoxic-grown cells only, it is likely that its function differs from that of the enzyme contained in spot 6 and possibly really corresponds to ketopantoate reductase.

Among the proteins biosynthesized in detectable amounts in aerobic-grown *E. faecium* only, the f-subunit of alkyl hydroperoxide reductase (Nox, spot 7) was identified. This enzyme is a dimeric protein complex involved in the scavenging of radical oxygen species (Costa Seaver and Imlay, 2001). The f subunit (AhpF) is an homologous of the NADH oxidase (Nox-1) catalyzing the reduction of oxygen to H_2O_2 and the parallel re-oxidation of NADH to NAD^+; the c subunit (AhpC) is involved in the following conversion of H_2O_2 to H_2O (Poole et al., 2000; Diaz et al. 2004). Although a AhpC homologue was not identified as among the proteins overexpressed in aerobic-grown cultures by the present study, nonetheless, the over expression of AhpF indicates that *E. faecium* LLAA-1 is able to re-oxidize NADH through NADH oxidase-like reaction when O_2 is present. This metabolic pathway is exploited by several LAB as a mean to re-oxidize excess NADH produced by catabolic reactions (Guo et al., 2012). Therefore, NADH oxidase activation offers the opportunity to increase oxidative catabolism, constituted mainly by glycolysis and/or other catabolic pathways, since a further mechanism, not available under anoxic conditions, useful to drain reducing equivalent excess can be exploited. Taken together, experimental findings described above strongly suggest that overproduction of LA detected in aerobic-grown cultures of *E. faecium* is partly the result of improved fructose catabolism through glycolysis (a higher amount of fructose is consumed in aerobic cultures, see above) and partly the effect of activation of amino acid catabolism (Fig. 5). It is worth reminding that amino groups transferred by transamines to 2-oxo acid acceptors, such as 2-oxo glutarate, are finally discarded as ammonia in oxidative reactions catalyzed by amino acid dehydrogenases, such as glutamate dehydrogenase, which also produce NADH (Fig. 5). Therefore, amino acid catabolism is only possible under conditions allowing improved NADH re-oxidation, like aerobic conditions, enhancing NADH oxidase activity. The activation of amino acid oxidative catabolism provides

the cells with at least two metabolic advantages: i) new substrates for LA biosynthesis; ii) production of ammonia. In LAB, lactate biosynthesis is coupled to electrogenic efflux of lactate by 2 H^+/lactate symporters (Konings, 2002), that is used for proton motive force generation. It was calculated that energy gain corresponds to 0.5-0.66 ATP per each lactate molecule. As described above, in aerobic-grown cultures of *E. faecium* final biomass is higher than in anoxic cultures, which confirms that under this condition a higher amount of energy is available for cell growth. Production of ammonia compensates for stronger acidification connected to such increased LA production: this would therefore explain why the extent of medium acidification measured in aerobic cultures is identical to those of anoxic cultures.

Regarding the stress proteins identified in this study, the ATP-binding subunit clpL of the ATP-dependent Clp protease, the chaperon protein dnaK and the DNA-binding protein DPS were more expressed in anoxia. Both clpL and dnaK are described as heat shock proteins in *Streptococcus pneumoniae* (Kwon et al., 2003); their over-expression under anoxic conditions seems to highlight aspecific stress counteracting actions in the tested *E. faecium* LLAA-1 strain. The DNA-binding protein DPS belongs to the ferritin superfamily and is involved in DNA protection. The first protein of this family was discovered in *E. coli* (Almiron et al., 1992) in which it is expressed during the stationary phase, when all nutrients are consumed, and it non-specifically binds the chromosome forming a dps-DNA co-crystal able to protect DNA from several damages (Wolf et al., 1999). The up-regulation of these three stress proteins under anoxic condition seems to indicate that aerobiosis represents a beneficial condition for *E. faecium* LLAA-1 since it could attenuate general metabolic stresses such as stationary phase starvation and pH stress, typical of the harvesting time (early stationary phase) for proteomic analyses.

The other differentially expressed proteins that were identified are involved in protein synthesis, such as the translation elongation factor Tu, which was up-regulated under aerobic conditions and the arginyl-tRNA synthetase which was up-regulated under anoxic condition. Other three spots up-regulated in anoxia were identified as conserved hypothetical proteins whose metabolic function has not been determined yet.

4. Conclusions

Many lactic acid bacteria can be used for industrial LA production, nevertheless, the enantioselective reaction leading to the more appreciated L-LA isomer is characteristic of only few natural strains. In the present study the selected *E. faecium* LLAA-1 proved to produce high amounts of optically pure (about 99%) L-LA from a number of soluble sugars (e.g., fructose and cellobiose) which are important components of agro-industrial waste biomasses. Growth conditions possibly modulating L-LA production (i.e., addition of sodium acetate and growth under aerobic/anoxic

conditions) were tested. In contrast to previous reports, sodium acetate supplementation did not improve LA production in any of the tested conditions. However, growth under aerobic conditions almost tripled LA amounts accumulated by *E. faecium* LLAA-1 during growth on fructose-supplemented M17 medium, although final biomass was only slightly higher than in anoxic cultures. Proteomic analyses helped to clarify the reasons of such improved LA production. Induction of the biosynthesis of a NADH oxidase homologue, catalyzing re-oxidation of NADH produced by oxidative catabolic reactions by means of O_2, seems to be a key factor of the global re-arrangement of *E. faecium* LLAA-1 metabolism under aerobic conditions. Improved fructose consumption through the Embden-Meyerhof pathway (with concomitant down-regulation of heterolactic routes) only partly explains accumulation of higher amounts of LA. Up-regulation of the biosynthesis of PDH, PTA and 2-oxo acid dehydrogenases suggests that also amino acid catabolism is improved under aerobic conditions, likely owing to the high content of protein hydrolysates contained in M17 medium (Fig. 5). Activation of amino acid oxidative catabolism contemporarily provides the cells with higher metabolic energy, since LA flux outside of the cells is used by LAB for proton motive force generation, and compensates improved acidification linked to the accumulation of higher amount of LA with production of ammonia. Although this hypothesis will be confirmed only by fermentations in chemically defined medium (allowing precise quantification of amino acid consumption), the findings obtained by the present study have highlighted the potential of *E. faecium* LLAA-1 to be used for the production of pure L-LA not only from polysaccharidic biomass, but also from other agro-industrial waste with high proteinaceous content (e.g. milk whey, by-products of meat, poultry and fish processing industries) (Jayathilakan et al., 2012). The combined approach of comparative proteomics with metabolic analyses proved to be a strategy with high potential to get key information on newly selected microbial strains for biotechnological applications, such as the industrial production of optically pure L-LA by fermentation.

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Figures

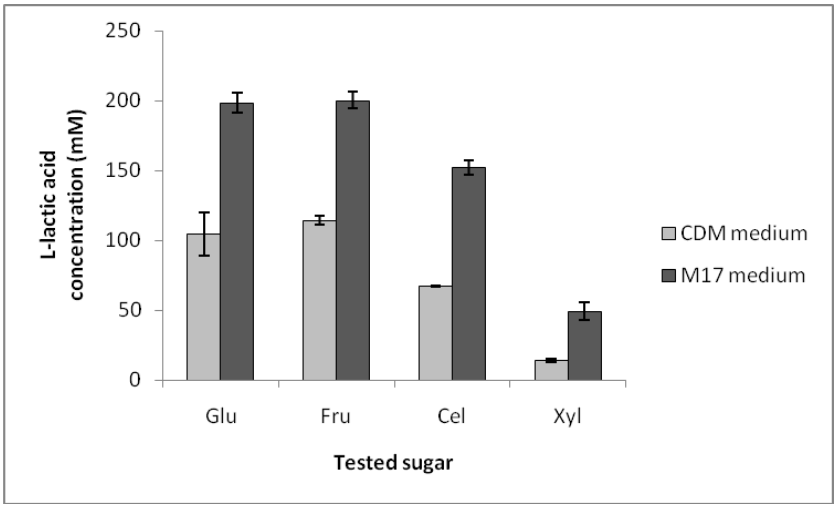

Fig1 L-LA production by *Enterococcus faecium* LLAA-1 grown in CDM and M17 media fortified with glucose (glu), fructose (fru), cellobiose (cel) and xylose (xyl) one by one.

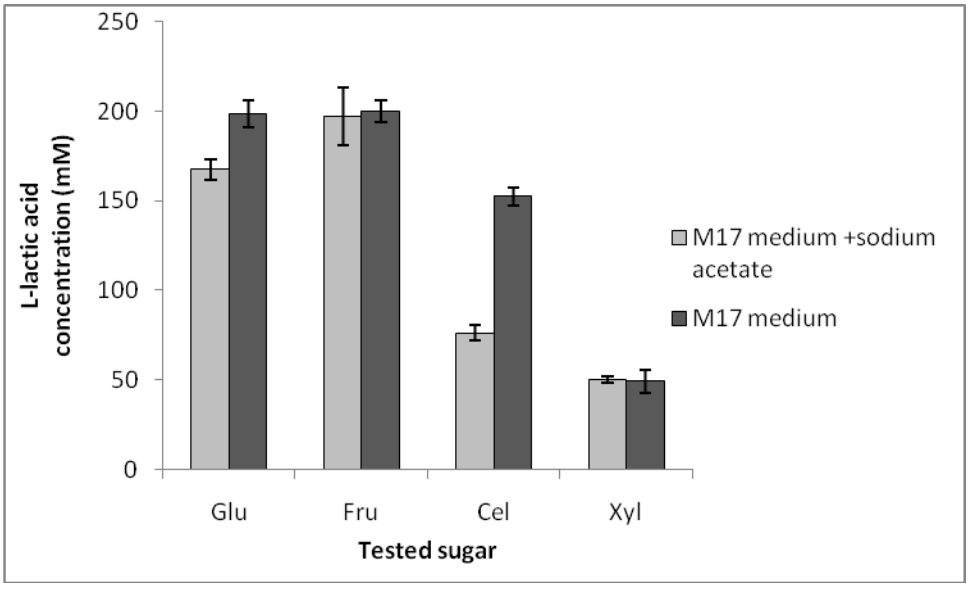

Fig2 Effect of sodium acetate on L-LA production by *Enterococcus faecium* LLAA-1 grown in M17 medium fortified with glucose (glu), fructose (fru), cellobiose (cel) and xylose (xyl) one by one.

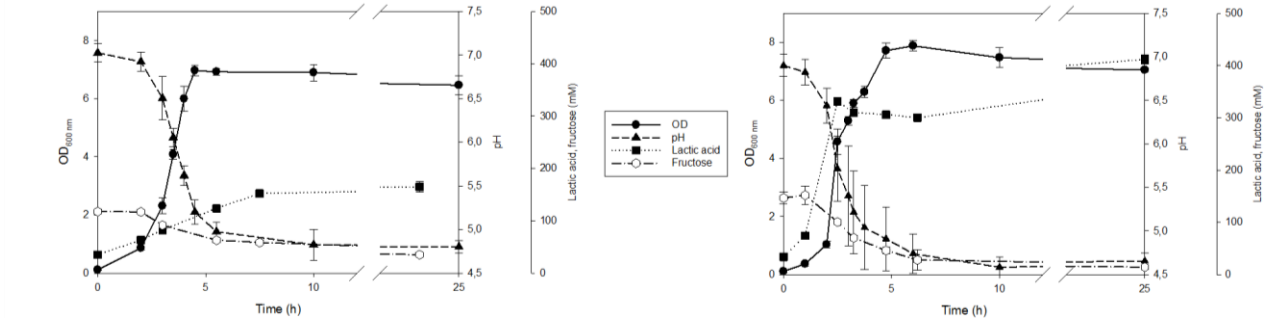

Fig3 Growth kinetics of *Enterococcus faecium* LLAA-1 in fructose-supplemented M17 medium under aerobic (A) and anoxic (B) conditions. Fructose consumption, L-LA production and pH trend are reported.

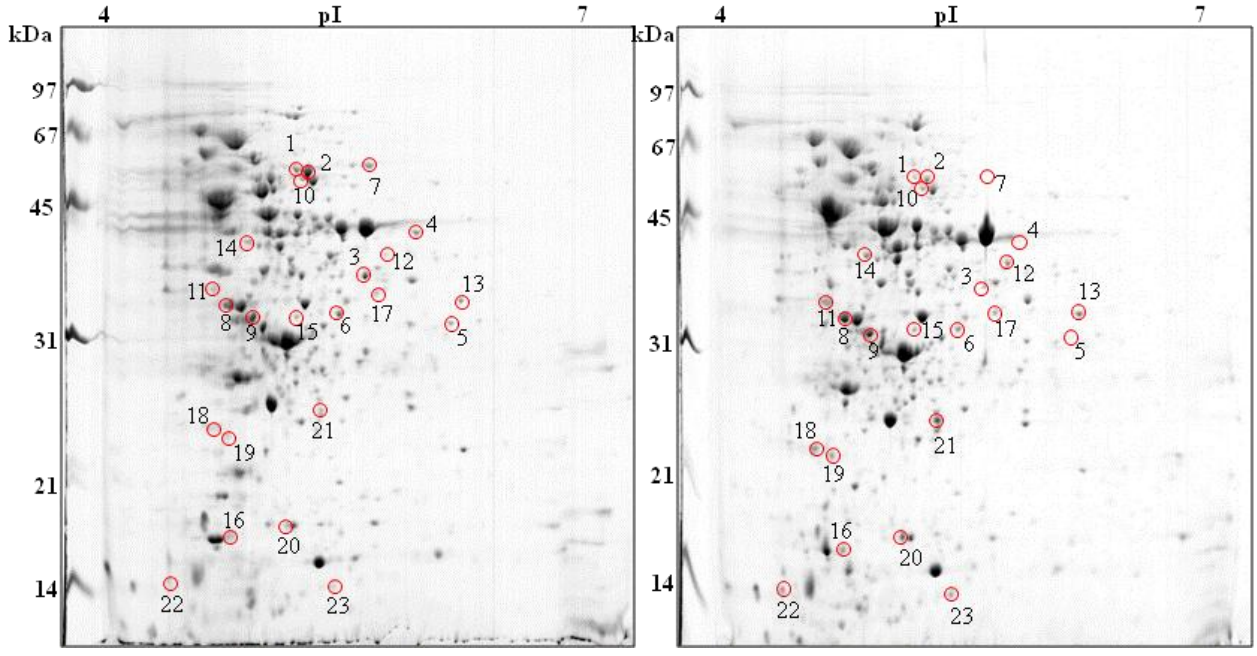

Fig4 2-DE maps of *in toto* proteins extracted from *E. faecium* LLAA-1 grown under aerobic (A) and anoxic (B) conditions. Differentially expressed spots are highlighted.

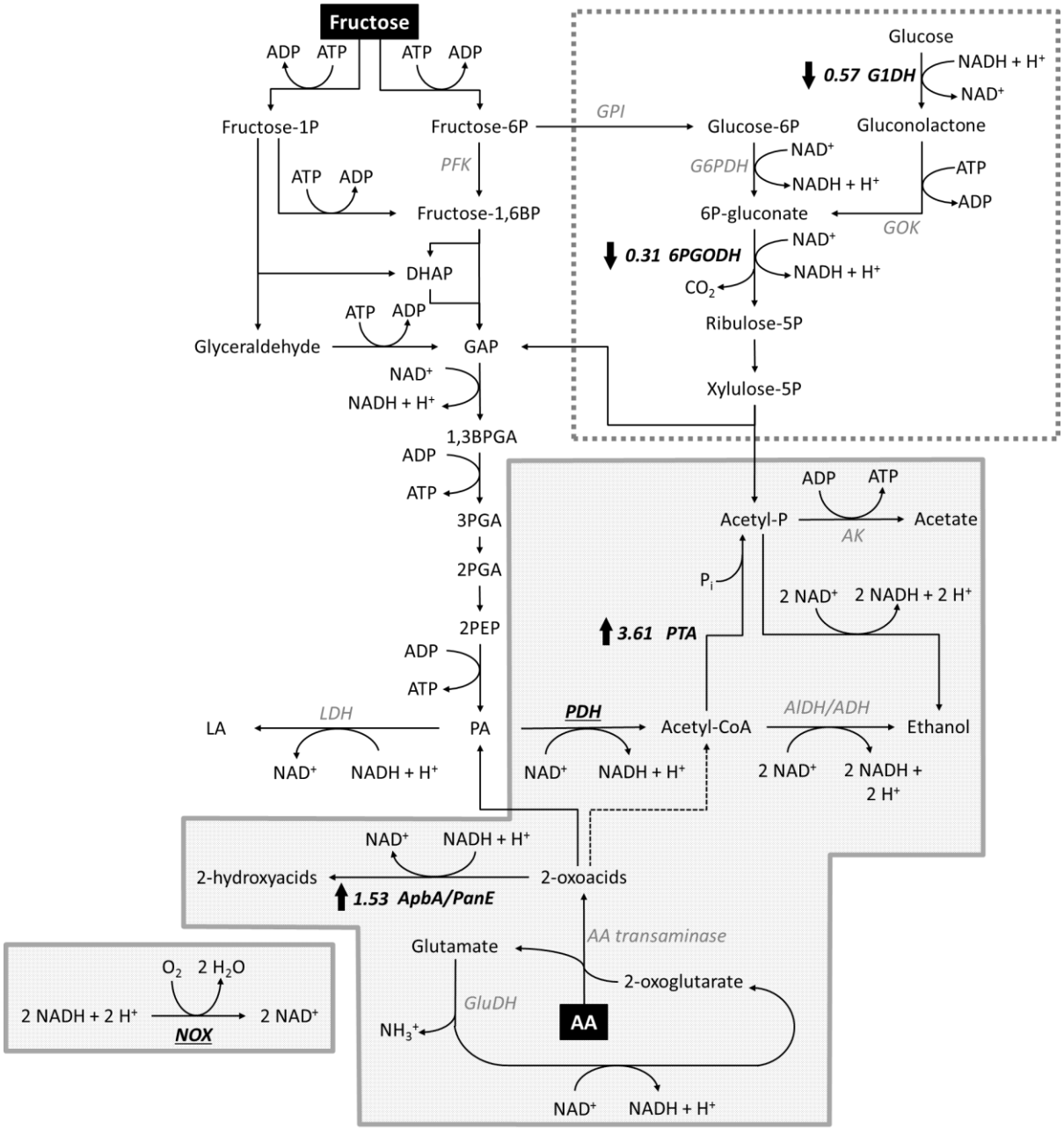

Fig5 Proposed picture of *E. faecium* LLAA-1 central catabolic pathways during growth in fructose-supplemented M17 medium. Enzymes which have been identified in this study are indicated in bold and include: proteins which are detectable in aerobic-grown cells only (underlined), enzymes whose biosynthesis is up-regulated in aerobic-grown cultures (arrow up, followed by aerobiosis/anoxia expression ratio), enzymes which are down-regulated under the same conditions (arrow down, followed by aerobiosis/anoxia expression ratio). Solid light grey and dotted boxes highlight metabolic networks which are likely activated and repressed, respectively, under aerobic conditions. 1,3BPGA, 1,3-bisphosphoglyceric acid; 2PEP, 2-phosphoenolpyruvic acid; 2PGA, 2-phosphoglyeric acid; 3PGA, 3-phosphoglyceric acid; 6PGODH, 6-phosphogluconate dehydrogenase; AA, amino acid; ADH, alcohol

dehydrogenase; AK, acetate kinase; AlDH, aldehyde dehydrogenase; ApbA/PanE, potential 2-hydroxyacid dehydrogenase (incorrectly annotated as ketopantoate reductase); DHAP, dihydroxy acetone phosphate; GAP, glyceraldehyde 3-phosphate; G1DH, glucose 1-dehydrogenase; GluDH, glutamate dehydrogenase; GOK, gluconate kinase; GPI, glucose phosphate isomerase; LA, lactic acid; LDH, lactate dehydrogenase; NOX, NADH oxidase; PA, pyruvic acid; PFK, phosphofructokinase; PDH, pyruvate dehydrogenase; PTA, phosphate acetyltransferase.