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*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1575278> since 2017-07-25T14:43:57Z

*Published version:*

DOI:10.3389/fmicb.2016.01050

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## GABA production in *Lactococcus lactis* is enhanced by arginine and co-addition of malate

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*Submitted to Journal:*  
Frontiers in Microbiology

*Specialty Section:*  
Microbial Physiology and Metabolism

*ISSN:*  
1664-302X

*Article type:*  
Original Research Article

*Received on:*  
18 Apr 2016

*Accepted on:*  
22 Jun 2016

*Provisional PDF published on:*  
22 Jun 2016

*Frontiers website link:*  
[www.frontiersin.org](http://www.frontiersin.org)

*Citation:*  
Laroute V, Yasaro C, Narin W, Mazzoli R, Pessione E, Coccagn-bousquet M and Loubière P(2016) GABA production in *Lactococcus lactis* is enhanced by arginine and co-addition of malate. *Front. Microbiol.* 7:1050. doi:10.3389/fmicb.2016.01050

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Provisional

1 **GABA production in *Lactococcus lactis* is enhanced by arginine and**  
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16

17 **Key words:** GABA, arginine, glutamate decarboxylase, ADI, Malo-lactic fermentation.

1 **ABSTRACT**

2 *Lactococcus lactis* NCDO 2118 was previously selected for its ability to decarboxylate glutamate to  
3  $\gamma$ -aminobutyric acid (GABA), an interesting nutritional supplement able to improve mood and  
4 relaxation. Amino acid decarboxylation is generally considered as among the biochemical systems  
5 allowing lactic acid bacteria to counteracting acidic stress and obtaining metabolic energy. These  
6 strategies also include arginine deiminase pathway and malolactic fermentation but little is known  
7 about their possible interactions of with GABA production. In the present study, the effects of  
8 glutamate, arginine and malate (*i.e.*, the substrates of these acid-resistance pathways) on *L. lactis*  
9 NCDO 2118 growth and GABA production performances were analyzed. Both malate and arginine  
10 supplementation resulted in an efficient reduction of acidity and improvement of bacterial biomass  
11 compared to glutamate supplementation. Glutamate decarboxylation was limited to narrow  
12 environmental conditions (pH < 5.1) and physiological state (stationary phase). However, some  
13 conditions were able to improve GABA production or activate glutamate decarboxylation system  
14 even outside of this compass. Arginine clearly stimulated glutamate decarboxylation: the highest  
15 GABA production (8.6 mM) was observed in cultures supplemented with both arginine and  
16 glutamate. The simultaneous addition of arginine, malate and glutamate enabled earlier GABA  
17 production (*i.e.*, during exponential growth) at relatively high pH (6.5). As far as we know, no  
18 previous study has reported GABA production in such conditions. Although further studies are  
19 needed to understand the molecular basis of these phenomena, these results represent important  
20 keys suitable of application in GABA production processes.

21

## 1 1. INTRODUCTION

2 Lactic acid bacteria (LAB) are gram-positive microaerophilic microorganisms extensively used in  
3 the agro-food industry because of their high lactic acid production and consequent food  
4 acidification. This is an appreciated feature for both prolongation of food shelf-life and biocontrol  
5 of food born infections, since most spoilage and pathogenic bacteria are acid-sensitive (Trias et al.,  
6 2008). The acid-resistance of LAB is based upon different, either constitutive or inducible,  
7 mechanisms which include: i) cytoplasm alkalization by H<sup>+</sup> consumption through decarboxylation  
8 mechanisms, or arginine deiminase (ADI) pathway or urease reaction; ii) changes in the  
9 composition of the cell envelope; iii) production of general shock proteins (chaperones); iv)  
10 changes in cell density (for a review, see Cotter and Hill, 2003). Most of these strategies involve the  
11 expression of genes which improve cell resistance to adverse conditions. In lactococci the main  
12 metabolic mechanisms involved in pH homeostasis are ADI, malolactic fermentation (MLF) and  
13 glutamate decarboxylase (GAD) systems (Figure 1). Nowadays, little is known about possible  
14 interactions between these metabolic systems in *L. lactis*.

15 ADI pathway consists of three reactions converting arginine to ornithine, NH<sub>3</sub>, CO<sub>2</sub> and  
16 ATP. This route is catalyzed by three enzymes: ADI (converting arginine to citrulline and NH<sub>3</sub>),  
17 ornithine transcarbamylase (converting citrulline to ornithine and carbamoyl phosphate) and  
18 carbamate kinase (converting carbamoyl phosphate to NH<sub>3</sub>, CO<sub>2</sub> and ATP), encoded by the genes  
19 *arcA*, *arcB*, and *arcC*, respectively (Figure 1). These enzymes appear to be acid resistant (Casiano-  
20 Colón and Marquis, 1988). The efficiency of the overall pathway is increased by arginine  
21 supplementation (Poolman et al., 1987). The ADI pathway provides both cytoplasm alkalization,  
22 through release of NH<sub>3</sub>, and metabolic energy production, since ATP is generated by substrate level  
23 phosphorylation in the reaction catalyzed by carbamate kinase. The ADI systems of *Lactobacillus*  
24 *sakei* (Zuniga et al., 1998), *Enterococcus faecalis* (Simon et al., 1982) and LAB strains associated  
25 with cheese fermentations (Crow and Thomas, 1982) or colonizing oral cavity (Dong et al., 2002;  
26 Marquis et al., 1987) are subject to catabolite repression by glucose. However, in *Lactobacillus*  
27 *sanfranciscensis* (De Angelis et al., 2002), *Oenococcus oeni*, and other wine LAB (Liu et al., 1996)  
28 glucose and arginine can be concomitantly catabolized.

29 MLF is the conversion of dicarboxylic malic acid to monocarboxylic L-lactic acid by  
30 malolactic enzyme (Figure 1). L-lactate is excreted via electrogenic transporters, *i.e.*, by either  
31 lactate-malate antiporter (*Lactococcus lactis*) or lactate uniport (*O. oeni* and *Lactobacillus*  
32 *plantarum*) (Konings et al., 1997) depending on the strain, which allow production of proton motive  
33 force. Furthermore, due to the pK<sub>a</sub> difference of the carboxylic groups of malate and lactate,  
34 replacement of malate by lactate results in alkalization of the extracellular medium. A further  
35 relevant malate dissimilation pathway which can contribute to acid resistance of LAB is oxidative

1 decarboxylation of malate to pyruvic acid (catalyzed by malic enzyme) (Landete et al., 2013). It is  
2 generally accepted that malate utilization through MLF cannot sustain growth *per se*, (since lactate  
3 cannot be used as a carbon source by most LAB), while its metabolism via malic enzyme enables  
4 LAB to use it as the only carbon source. Distribution of malic enzyme currently seems restrained to  
5 fewer strains (including *L. lactis* NCDO 2118) with respect to MLF (Landete et al., 2013; Oliveira  
6 et al., 2014). However, malic enzyme has been studied at much lesser extent than MLF and,  
7 currently, contribution of malic enzyme pathway to LAB metabolism cannot be precisely evaluated.

8 Like other amino acid decarboxylations, glutamate conversion to  $\gamma$ -aminobutyric acid  
9 (GABA) is an important strategy to counteract excess of acidity (Schelp et al., 2001; Van De  
10 Guchte et al., 2002; Pessione 2012) since the reaction itself is proton consuming and results in  
11 alkalization of the cytoplasmic compartment (Small and Waterman, 1998). Glutamate  
12 decarboxylase system has been reported in both gram-positive and gram-negative bacteria (Small  
13 and Waterman, 1998; Cotter et al., 2005; Bhagwat and Bhagwat 2004; Tramonti et al., 2006) and  
14 includes proton-consuming decarboxylation of glutamate by glutamate decarboxylase (GAD) in the  
15 cytoplasm and cell membrane-located electrogenic glutamate/GABA antiporters which generate  
16 proton motive force (Molenaar et al., 1993; Higuchi et al., 1997; Lu et al., 2013; Tsai et al., 2013)  
17 (Figure 1). The expression of GAD in *L. lactis* is increased by low pH and glutamate  
18 supplementation (Sanders et al., 1998). Recent studies have identified a further acid-resistance  
19 mechanism in *Lactobacillus reuteri* and *E. coli* which is based on glutamine and can be interpreted  
20 as an “extension” of the GAD system (Lu et al., 2013; Teixeira et al., 2014). Both glutaminase,  
21 which catalyzes glutamine deamidation (producing ammonia and glutamate), and GAD are present  
22 in the cytoplasm of these strains and contribute to intracellular alkalization. These studies  
23 indicated that GadC, which had been previously identified as a glutamate/GABA antiporter, is also  
24 able to mediate uptake of glutamine or extrusion of glutamate. A gene encoding a putative  
25 glutamine/GABA antiporter has been identified in the genome of *O. oeni* PSU-1 also (Mills et al.,  
26 2005).

27 *L. lactis* NCDO 2118 is able to biosynthesize GABA by glutamate decarboxylation. A  
28 previous transcriptomic and proteomic study demonstrated that ADI pathway genes (*arcA*, *arcDI*,  
29 *arcB* and *arcC2*) are down-regulated in glutamate-supplemented/GABA-producing conditions, thus  
30 suggesting that glutamate decarboxylation and arginine deimination are competing routes in this  
31 strain (Mazzoli et al., 2010). The present investigation aimed to better establish the relative  
32 contribution of GAD, ADI and MLF pathways in energy metabolism and acid resistance of *L. lactis*  
33 NCDO 2118 and possible reciprocal interactions of these metabolic systems.

34

## 35 2. MATERIAL AND METHODS

## 1 **2.1. Bacterial strain**

2 *Lactococcus lactis* subsp. *lactis* NCDO 2118 from vegetable origin was used throughout this study.  
3 This strain was selected during preliminary studies as the only one able to biosynthesize detectable  
4 amounts of GABA among the *L. lactis* strains available in the laboratory microbial collection  
5 (LISBP of INSA-Toulouse, France).

## 6 7 **2.2. Culture conditions**

### 8 2.2.1. Cultures in tubes

9 Cultures were grown in the chemically defined medium (CDM) (Otto et al., 1983; Poolman and  
10 Konings, 1988), containing glucose (20 g.L<sup>-1</sup>) under anaerobic conditions, *i.e.*, in N<sub>2</sub> atmosphere, in  
11 butyl rubber-stoppered tubes at 30°C. The initial pH was 6.6. Furthermore, different concentrations  
12 of glutamate and/or arginine and/or malate were added into the medium depending on the study. All  
13 the experiments were performed in duplicate. Inoculation was with cells from precultures harvested  
14 during the exponential phase and concentrated in order to obtain an initial optical density at 580 nm  
15 (OD<sub>580</sub>) of 0.05 in the tubes. During incubation, 1 mL samples were taken every 30 min so as to  
16 measure the OD<sub>580</sub> with Spectronic 301 spectrophotometer (Milton Roy, Pont Saint Pierre, France).  
17 The maximum growth rate ( $\mu_{\max}$ ) was then determined. pH was also regularly measured with pH  
18 meter (Metrohm 744, Villebon Sur Yvette, France).

### 19 20 2.2.2. Cultures in fermenter

21 Bacterial cultures were performed in duplicate in 2 L Biostat B plus fermenter (Sartorius,  
22 Melsungen, Germany) filled with glucose (20 g.L<sup>-1</sup>) containing-CDM or the same medium  
23 supplemented with 5 g.L<sup>-1</sup> (34 mM) glutamate and/or 5 g.L<sup>-1</sup> (29 mM) arginine and/or 20 g.L<sup>-1</sup> (149  
24 mM) malate. Cultures were incubated at 30 °C in anaerobiosis, obtained by slight N<sub>2</sub> overpressure.  
25 pH was maintained at 6.6 by KOH addition until cultures reached OD<sub>580</sub> = 1, in order to reach  
26 enough biomass for further analytical procedures, and then pH was not regulated anymore.  
27 Bacterial growth was monitored by measurement of OD<sub>580</sub> (Libra S11, Biochom, 1 Unit of  
28 absorbance is equivalent to 0.3 g.L<sup>-1</sup>). Samples were collected every 30 min for HPLC  
29 determination of metabolite concentration in the growth medium.

## 30 31 **2.3. Metabolite determination**

32 Glucose, malate and metabolite (*i.e.*, lactate, acetate, formate and ethanol) concentrations  
33 were measured in culture supernatants by high performance liquid chromatography (Agilent  
34 Technologies 1200 Series, Waldbronn, Germany) using a HPX87H<sup>+</sup> Biorad column and the



1 following conditions: a temperature of 48 °C, eluent H<sub>2</sub>SO<sub>4</sub> (5 mM) at a flow rate of 0.5 mL.min<sup>-1</sup>,  
2 and dual detection (refractometer and UV).

3 Free amino acid and GABA concentration in culture supernatants was measured by HPLC  
4 system (Agilent Technologies 1200 Series, Waldbronn, Germany). Prior to HPLC determination,  
5 proteins in the samples were precipitated by adding four volumes of methanol followed by  
6 overnight incubation on ice. The mixture was centrifuged and the supernatant kept for HPLC  
7 analysis. Amino acids were automatically derived with OrthoPhtalic Aldehyde (OPA) and 9-  
8 fluorenylmethyl-chloroformiate (FMOC-C1). The derivatives were separated on Hypersil AA-ODS  
9 column (Agilent Technologies) at 40 °C by a linear gradient of acetate buffer (pH 7.2) with  
10 triethylamin (0.018 %), tetrahydrofuran (0.3 %) and acetonitrile. A diode array detector, at 338 nm  
11 for OPA derivatives and 262 nm for FMOC derivatives, was used.

## 12 13 **2.4. Statistical methods**

14 Student's t-test was applied to each parameter (Table 1) in order to detect significant  
15 differences between culture conditions (CDM or CDM supplemented with glutamate as the  
16 references). A *p*-value lower than 0.025 was considered as significant.

## 17 18 **3. RESULTS**

### 19 **3.1. Growth and metabolism of *L. lactis* NCDO 2118 in control conditions.**

20 Fermentation profiles of *L. lactis* NCDO 2118 in glucose-containing chemically defined medium  
21 (CDM), in unregulated pH conditions, were determined. Growth started immediately after inoculum  
22 at maximal growth rate ( $\mu_{\max} = 0.97 \text{ h}^{-1}$ ), and stopped after 5 h, at a biomass concentration of about  
23 1 g.L<sup>-1</sup> (Figure 2, Table 1). At that time, the pH was about 4.9-5.0, and it further gradually  
24 decreased during the stationary phase until 4.2 (13 h after inoculum). *L. lactis* NCDO 2118 showed  
25 high maximal glucose consumption rate (27.7 mmol.g<sup>-1</sup>.h<sup>-1</sup>) and exhibited homolactic metabolism  
26 all along the growth phases, leading to accumulation of 65.6 mM lactate (Table 1). Growth stopped  
27 before glucose depletion. We have performed cultures in the same medium but at regulated pH (6.6)  
28 during all the fermentation. In these conditions, growth continued until glucose exhaustion,  
29 demonstrating that acidic pH is responsible for the growth arrest in our control conditions.

30 The growth medium used in this study did contain no glutamate while low concentrations of  
31 arginine and glutamine (0.6 and 3.0 mM, respectively) were present. Arginine was quickly  
32 exhausted ( $q_{\max} = 1.44 \text{ mmol.g}^{-1}.\text{h}^{-1}$ ) and stoichiometrically converted to ornithine and citrulline.  
33 About half of the initial glutamine was consumed ( $q_{\max} = 1.22 \text{ mmol.g}^{-1}.\text{h}^{-1}$ ) in 48 h, leading to  
34 accumulation of 0.5 mM of glutamate and 0.4 mM of GABA in the growth medium (Table 1).

## 1 **3.2. Effect of glutamate or arginine or malate supplementation on growth and metabolism of** 2 ***L. lactis* NCDO 2118**

3 In order to study the effect of glutamate or arginine or malate on metabolic profiles of *L.*  
4 *lactis* NCDO 2118 cultures were performed in glucose-CDM medium supplemented with each of  
5 these single compounds at regulated and unregulated pH as described below. GABA was never  
6 produced in regulated pH conditions (pH = 6.6). It was detected at unregulated pH and only cultures  
7 performed in these conditions are described below.

### 8 **3.2.1. Effect of glutamate**

9 The effect of different glutamate concentrations, ranging from 0 to 20 g.L<sup>-1</sup> (0-136 mM) was  
10 tested in tube cultures. Neither specific growth rate nor final biomass was affected by the different  
11 glutamate concentrations used (Figure 3A). However, slight variations in final pH and, more  
12 importantly, changes in amounts of accumulated GABA were observed among the different  
13 glutamate-supplemented cultures. The higher was glutamate supplementation, the higher was the  
14 final pH and the amount of GABA which was accumulated (pH = 4.1, 0.3 mM of GABA in cultures  
15 without glutamate supplementation; pH = 4.5, 3.8 mM GABA in cultures supplemented with 136  
16 mM glutamate). It is worth noting that glutamate/GABA conversion yield was not the same in each  
17 condition since it was higher in cultures supplemented with lower glutamate concentration (*i.e.*, 35  
18 % in cultures containing 3.4 mM glutamate) and progressively decreased at higher glutamate  
19 supplementation (*i.e.*, 3% in cultures containing 136 mM glutamate) (Figure 4).

20 The medium containing 5 g.L<sup>-1</sup> glutamate (34 mM) retained our attention since it displayed  
21 high production of GABA with intermediary glutamate/GABA conversion yield. This condition  
22 was also previously used for transcriptome-proteome analysis (Mazzoli et al., 2010). Cultures were  
23 performed in fermenter in order to provide detailed metabolic parameters. As determined for tube-  
24 cultures (see above), glutamate supplementation did not affect  $\mu_{\max}$  and final biomass with respect  
25 to control cultures. Glutamate supplementation did not have any effect on glucose consumption  
26 rate, although a slightly higher glucose amount was consumed in these condition leading to  
27 accumulation of higher amounts of lactic acid (Table 1). Both final pH and GABA accumulation at  
28 48 h were higher in glutamate-supplemented culture, thus confirming results obtained in tube  
29 cultures. It is worth noting that the amount of glutamine consumed was similar to control cultures,  
30 although maximal glutamine consumption rate was significantly lower, and that 5.5 mM of  
31 glutamate was consumed leading to accumulation of 3.12 mM GABA (Table 1). Finally, glutamate  
32 supplementation did not display any significant influence on arginine consumption rate (Table 1).

### 33 **3.2.2. Effect of arginine**

34 Cultures of *L. lactis* NCDO 2118 were performed in tubes containing glucose-CDM  
35 medium supplemented with arginine concentrations ranging from 0 to 25 g.L<sup>-1</sup> (0-144 mM).

1 Increasing amounts of supplemented arginine progressively caused: i) a slight decrease of the  
2 maximal growth rate (from  $1 \text{ h}^{-1}$  to  $0.8 \text{ h}^{-1}$ ); ii) a strong increase of both the final biomass and iii)  
3 final pH (from  $\text{pH} = 4.1$  to  $\text{pH} = 6.6$ ) (Figure 3B). Arginine was depleted 6 h after inoculum in  
4 every tested culture and progressive alkalization of final pH (proportional to increasing initial  
5 concentration of arginine) was observed. This was likely related to production of higher amounts of  
6  $\text{NH}_3$  by arginine metabolization through the ADI route. Interestingly, arginine addition up to  $10 \text{ g.L}^{-1}$   
7 ( $57 \text{ mM}$ ) progressively enhanced GABA production up to  $1.9 \text{ mM}$  (Figure 3B). Since the medium  
8 did not contain glutamate, this was likely the result of increased bioconversion of glutamine.  
9 However, for higher initial arginine concentrations, GABA accumulation was markedly decreased  
10 to levels similar to those of control cultures. This was probably caused by excessive medium  
11 alkalization and consequent inhibition of glutamate decarboxylation system.

12 Arginine at  $29 \text{ mM}$  ( $5 \text{ g.L}^{-1}$ ) was chosen for further experiments in fermenter, since this  
13 culture condition was characterized by the highest specific GABA production (i.e. amount of  
14 GABA/ final biomass ratio). Such arginine supplementation did not significantly increase the  $\mu_{max}$   
15 but doubled final biomass with respect to control conditions (Table 1). Although arginine was  
16 completely exhausted at the growth arrest at 6 h, the extracellular pH at this point, was not much  
17 more alkaline than in control conditions. However, in arginine-supplemented cultures a two-fold  
18 higher glucose amount was consumed leading to a final lactate concentration of about  $123.3 \text{ mM}$   
19 which likely had a neutralizing effect on the  $\text{NH}_3$  released via the ADI pathway (Table 1).  
20 Consistently, ornithine and citrulline accumulated in the culture broth and levels were proportional  
21 to arginine consumption. No putrescine was detected.

22 As observed in tube-cultures, arginine supplementation also enhanced GABA production.  
23 Accumulation of increased amounts of GABA cannot be explained only by the higher biomass  
24 achieved in arginine-supplemented cultures, since biomass was increased by two-fold whereas  
25 GABA accumulation was enhanced by four-fold at 48 h. On the other hand, specific glutamine  
26 consumption rate was similar to values measured in control conditions. These observations suggest  
27 that arginine directs a higher proportion of glutamine metabolic flux towards GABA production  
28 diverting it from other pathways.

### 29 3.2.3. Effect of malate

30 Cultures of *L. lactis* NCDO 2118 in tubes containing glucose-CDM medium supplemented  
31 with malate concentration ranging from 0 to  $50 \text{ g.L}^{-1}$  ( $0\text{-}373 \text{ mM}$ ) were performed (Figure 3C).  
32 With increasing malate supplementation, final pH and  $\mu_{max}$  were affected in somehow similar ways  
33 as what observed in arginine fortified cultures, i.e. final pH progressively raised, while  $\mu_{max}$  was  
34 lower for higher malate supplementation. Taking into account that supplemented concentrations of  
35 malate were more than two-fold higher than for arginine, malate supplementation had more limited

1 effects on acid neutralization (final pH with 50 g.L<sup>-1</sup> malate supplementation was about 7). On the  
2 contrary, such high malate concentration negatively affected  $\mu_{max}$  (*i.e.*, it was reduced from 1 h<sup>-1</sup> to  
3 0.4 h<sup>-1</sup>). A moderate final biomass increase (up to 1.4 g.L<sup>-1</sup>) was observed up to malate  
4 concentration of 40 g.L<sup>-1</sup> (298 mM). Higher malate supplementation caused reduction of final  
5 biomass to levels lower than control conditions.

6 Cultures of *L. lactis* NCDO 2118 in glucose-CDM medium containing 20 g.L<sup>-1</sup> (149 mM)  
7 malate were performed in fermenter also. This condition was chosen since the GABA produced was  
8 significantly increased compared to the reference condition while the growth rate was similar.  
9 Maximal specific growth rate, final biomass and final extracellular pH were coherent with results  
10 obtained in tube cultures with the same malate concentration. This condition was the one  
11 stimulating the consumption of the highest amount of glucose compared to reference conditions or  
12 supplementations with glutamate or arginine at 48 h (Table 1). Actually, glucose was almost  
13 depleted after 12 h of culture, although specific glucose consumption rate was similar the other  
14 growth conditions tested. However, malate supplementation did not stimulate any further GABA  
15 accumulation with respect to control conditions (Table 1). Curiously, malate seems to increase  
16 arginine consumption rate (2.69 mmol.g<sup>-1</sup>.h<sup>-1</sup>) with respect to cultures on CDM.

17

### 18 **3.3. Cultures supplemented with glutamate plus arginine or glutamate plus malate**

#### 19 ***3.3.1. Effect of simultaneous arginine and glutamate supplementation***

20 The simultaneous addition of arginine and glutamate to the glucose-CDM medium was studied in  
21 fermenter. The concentrations of arginine (5 g.L<sup>-1</sup>, 29 mM) and glutamate (5 g.L<sup>-1</sup>, 34 mM) were  
22 chosen according to the individual fermenter conditions previously tested. A slightly decreased  
23 maximal growth rate ( $\mu_{max} = 0.89$  h<sup>-1</sup>) was observed while final pH was similar to cultures  
24 supplemented with glutamate only (Table 1). Final biomass at the growth arrest was also  
25 significantly higher like cultures supplemented with arginine only. However, GABA production  
26 was strongly enhanced since 8.6 mM were accumulated in the medium, that is almost three-fold  
27 higher than in cultures supplemented with glutamate only. This was the highest amount of GABA  
28 accumulation observed in this study and was likely the result of both higher substrate availability  
29 (*i.e.*, glutamate supplementation) and stimulation of GABA production by arginine, confirming  
30 results obtained in cultures supplemented with arginine only.

31

#### 32 ***3.3.2. Effect of simultaneous malate and glutamate supplementation***

33 In a similar way, the effect of simultaneous malate and glutamate supplementation was  
34 tested in fermenter. This condition noticeably reduced  $\mu_{max}$  (0.81 h<sup>-1</sup>) with respect to control  
35 conditions as observed in cultures supplemented with glutamate only (Table 1). However, the

1 specific glucose consumption rate was similar (Table 1). Final biomass and extracellular pH at the  
2 growth arrest, as well as glucose consumption and lactate production at 48 h were higher than the  
3 reference but similar to that measured in cultures fortified with malate only. Hence, contribution of  
4 glutamate to energy metabolism seems negligible. This is confirmed by the fact that in this  
5 condition only 2.54 mM of GABA was accumulated at 48 h. This value is not significantly different  
6 from GABA amounts observed in culture supplemented with glutamate only. These data, taken  
7 together with the fact that in malate-plus-glutamate-supplemented cultures biomass production was  
8 higher than in culture supplemented with glutamate only, suggest that malate somehow repressed  
9 GABA production pathway(s). On the other hand, specific malate consumption rate was slightly  
10 lower than in cultures supplemented with malate only (Table 1).

#### 11 12 **3.4. Cultures supplemented with glutamate plus arginine plus malate**

13 Cultures of *L. lactis* NCDO 2118 were performed in CDM supplemented with 5 g.L<sup>-1</sup> arginine (29  
14 mM), 20 g.L<sup>-1</sup> malate (149 mM) and 5 g.L<sup>-1</sup> glutamate (34 mM). Since usual glucose concentration  
15 (20 g.L<sup>-1</sup>, 110 mM) was exhausted after 10 h of growth (in agreement with the high sugar  
16 consumption rate observed in other malate-supplemented cultures) initial glucose concentration was  
17 increased to 45 g.L<sup>-1</sup> (250 mM) so as to avoid growth limitation by sugar depletion.

18 As for other malate supplemented cultures, growth was at slightly lower  $\mu_{\max}$  (0.85 h<sup>-1</sup>) with respect  
19 to control cultures supplemented with glutamate. Simultaneous supplementation of glutamate,  
20 malate and arginine had additive effects on i) attenuating extracellular acidity caused by lactic acid  
21 accumulation (353.3 mM was produced 48 h after inoculum), since pH at both time of growth arrest  
22 and after 48 h was the highest observed among the growth conditions tested in this study and ii)  
23 increasing biomass formation up to 2.2 g.L<sup>-1</sup>. Growth arrest occurred 9 h after inoculum. At that  
24 time, while malate had already been depleted, only 71.1 mM of glucose had been consumed,  
25 leading to accumulation of 228.05 mM lactic acid. The high amount of lactic acid observed in these  
26 conditions could contribute to growth arrest for reasons independent from acidification.

27 The specific consumption rates for glucose and glutamine were of the same order of magnitude of  
28 values measured in control conditions (Table 1). Specific malate consumption rate was slightly  
29 lower than in the other malate-supplemented cultures described above, while arginine was  
30 consumed at a highest specific rate (14.58 mmol.g<sup>-1</sup>.h<sup>-1</sup>, Table 1).

31 Finally, GABA was produced until a maximal concentration of 5.22 mM at 48 h, which is  
32 higher than amounts accumulated in glutamate- and glutamate-plus-malate-supplemented cultures  
33 but lower than those accumulated in glutamate-plus-arginine supplemented cultures. This data  
34 confirmed that arginine enhances while malate decreases GABA accumulation. Interestingly,  
35 simultaneous glutamate-malate-arginine supplementation triggered an earlier production of GABA

1 with respect to all the other tested conditions: a concentration of 2.86 mM was already detected 12  
2 h after inoculum and it reached 4.28 mM at 24 h. This earlier production of GABA can be observed  
3 in Figure 5 which combines the kinetics of the different experiments. When glutamate, arginine and  
4 malate were contemporarily supplemented to *L. lactis* NCDO 2118 cultures, GABA production  
5 started at the beginning of the exponential growth phase and at a pH as high as 6.6 while only  
6 tardive GABA production in acidic conditions (pH<5.1) was observed in all other tested conditions.  
7

## 8 **DISCUSSION**

9 Several systems can be activated or enhanced by LAB to attenuate acidic environments  
10 and/or to improve metabolic energy. If the most obvious strategy involves F<sub>0</sub>F<sub>1</sub>-ATPase, other  
11 mechanisms such as the ADI pathway or the decarboxylation of malate and amino acids can be  
12 used to neutralize or reduce acidity (Budin-Verneuil et al., 2004). ADI pathway and malate  
13 decarboxylation by MLF are often present in LAB which live in wine ecological niche in which  
14 both malate and arginine are abundant. In LAB, biogenic amine production (including GABA  
15 biosynthesis) through amino acid decarboxylation usually occurs in response to adverse conditions,  
16 *e.g.*, as a mean to counteract acidic environments (Van De Guchte et al., 2002) and to obtain  
17 metabolic energy when the primary substrates (*e.g.*, glucose) are exhausted (Molenaar et al., 1993;  
18 Pessione et al., 2010). However, little is known about the relative role of the considered metabolic  
19 pathways in pH homeostasis, their possible synergistic/antagonist effects and consequences on  
20 global metabolism and growth of LAB. In the present study, the contribution of energy  
21 supplying/alkalinizing routes (*i.e.* ADI pathway, malate fermentation and production of GABA), to  
22 growth and metabolism of *L. lactis* NCDO 2118 and their reciprocal relationships was investigated.  
23 Effects of supplementation of different amounts of substrates (arginine, malate and glutamate) or  
24 their mixtures on growth (specific growth rate, final biomass), substrate consumption and GABA  
25 production have been quantitatively determined.

### 26 *Growth Parameters*

27 In all tested conditions, the growth profile was characteristic of *L. lactis* cultures, in which  
28 the growth rate was maximal in the early phase and then progressively decreased. Growth arrest  
29 was likely caused by low pH and/or lactic acid accumulation in every condition considered here.  
30 The increase in final biomass seems inversely correlated to the pH decrease: the higher the initial  
31 concentration of arginine or malate, the higher was likely the activation of corresponding acid  
32 resistance mechanisms (ADI pathway and MLF, respectively) resulting in weaker and slower pH  
33 decrease, hence the growth could be supported for longer time periods. It is worth noting that while  
34 in arginine-supplemented cultures the pH acidification caused by lactic acid accumulation by  
35 glycolysis is neutralized by release of ammonia, pH increase by MLF leads to additional lactate

1 production. It can be speculated that huge lactic acid accumulation is likely the main growth  
2 inhibiting factor in malate-supplemented cultures, for reasons independent from medium  
3 acidification. For instance, this could explain why in cultures supplemented with 149 mM malate  
4 cell growth stopped at lower final biomass, *i.e.* 1.86 g.L<sup>-1</sup>, and higher pH, *i.e.* pH = 5.76, with  
5 respect to cultures supplemented with arginine. However, we cannot exclude that arginine and/or  
6 malate stimulate biomass production by additional mechanisms (other than homeostasis), such as  
7 production of biosynthetic intermediates, for instance through malate conversion by ME (Landete et  
8 al., 2013) or arginine conversion to pyrimidine precursors.

9 The maximal glucose consumption rate was similar in all tested conditions, and the central  
10 metabolism remained homolactic. From this macro-kinetic analysis, no evidence that glycolytic flux  
11 was affected by alternative acid-resistance related pathways could be inferred. This is in agreement  
12 with the observation that the expression of two enzymes of the central metabolism, namely  
13 phosphoglucomutase and pyruvate dehydrogenase, was unaffected by arginine or malate in  
14 *Lactobacillus hilgardii* (Lamberti et al., 2011). The efficiency of arginine, glutamate and malate  
15 dissimilation pathways of *L. lactis* NCDO 2118 was very diverse, as demonstrated by specific  
16 substrate consumption rates. Malate was consumed at the highest rate (maximal  $q_{\text{malate}}$  was  
17 comprised between 21.5 and of 29.5 mmol.g<sup>-1</sup>.h<sup>-1</sup>), followed by the arginine (maximal  $q_{\text{Arg}}$   
18 comprised between 1.2 and of 14.6 mmol.g<sup>-1</sup>.h<sup>-1</sup>), and finally by glutamate (maximal  $q_{\text{Glu}} \leq 0.2$   
19 mmol.g<sup>-1</sup>.h<sup>-1</sup>). These data suggest that MLF is the most rapid system for neutralizing acidity in this  
20 strain. In fact, the slowest acidification was observed in cultures supplemented with malate. Malate  
21 consumption rate seems negatively affected by both glutamate and arginine supplementation,  
22 suggesting that in these conditions MLF is inhibited. It has been previously reported that malate  
23 consumption was not affected by histidine in the culture medium in *L. hilgardii* ISE5211 (Mazzoli  
24 et al., 2009; Lamberti et al., 2011). Similarly ornithine-putrescine conversion does not affect MLF  
25 in *Oenococcus oeni* (Mangani et al., 2005). Hence amino acid and malate decarboxylation can be  
26 activated in parallel without reciprocal interference in these two wine-isolated strains. This is not  
27 the case in *L. lactis* NCDO 2118 in which also glutamate decarboxylation is negatively affected by  
28 malate suggesting that these routes are competing. One possible explanation for this different  
29 behaviour is that *L. lactis* NCDO 2118 has been isolated from another ecological niche (frozen  
30 peas) and can be encountered in milk fermentation. Arginine consumption rate is strongly  
31 influenced by environmental conditions. Notably, both arginine and malate supplementation  
32 increases maximal specific consumption rate of arginine (Table 1). These data suggest that both  
33 arginine and malate may activate ADI pathway. The activation of ADI by arginine has been  
34 previously described for many LAB species (Manca de Nadra et al., 1986; Tonon et al., 2001; De  
35 Angelis et al., 2002; Lamberti et al., 2011). However, positive regulation of ADI by malate has

1 never been reported so far. Proteomic analyses showed that the expression of ADI pathway  
2 enzymes is not affected by malate in *L. hilgardii* (Lamberti et al., 2011). Furthermore, malate  
3 inhibited arginine consumption in some lactobacilli and pediococci isolated from wine (Araque et  
4 al., 2011). However, Rallu and co-workers (1996) previously suggested that lactic acid can activate  
5 arginine metabolism, including ADI pathway, in *L. lactis*. We can hypothesize that malate is able to  
6 enhance ADI pathway in *L. lactis* NCDO 2118 through additional lactate production by MLF  
7 although a direct activation of this pathway by malate cannot be excluded to be specific to the strain  
8 considered in this study.

### 9 *GABA production*

10 GABA production was clearly dependent on the environmental conditions. In absence of  
11 glutamate, only low amounts of GABA were produced likely as a consequence of the conversion of  
12 glutamine (which is a CDM component) into glutamate. The higher glutamate supplementation, the  
13 higher the final concentration of GABA (Figure 3A). However, relationship between the amount of  
14 supplemented glutamate and produced GABA is not linear but rather looks like a Michaelis-Menten  
15 plot. Since glutamate amount did not affect significantly final biomass and growth rate, these data  
16 suggest that the same amount of enzymes involved in glutamate/GABA conversion (*i.e.*, glutamate  
17 decarboxylase and/or glutamate/GABA antiporters) were present in all these conditions, *i.e.*  
18 glutamate did not improve their biosynthesis. In these conditions, the system is semi-saturated for  
19 glutamate concentrations of about 7 mM. The present data therefore confirm previous results  
20 obtained on *L. lactis* NCDO2118 which indicated that glutamate supplementation did not induce  
21 overexpression of GAD (Mazzoli *et al.*, 2010).

22 Arginine supplementation significantly enhanced GABA production in both absence or  
23 presence of glutamate fortification. The latter condition corresponds with the production of the  
24 highest GABA amount observed in this study (8.6 mM). It is possible to hypothesize that arginine  
25 can replace glutamine/glutamate in some metabolic function, allowing a higher proportion of these  
26 compounds to be directed towards GABA production. For instance, it is well known that in *L. lactis*  
27 glutamine can be converted to carbamoyl phosphate, the building block for pyrimidine biosynthesis,  
28 by carbamoyl phosphate synthase (EC 6.3.5.5) (Martinussen and Hammer, 1998). Since also  
29 arginine can generate carbamoyl phosphate through the ADI pathway, it could replace glutamine  
30 allowing it to be used for GABA biosynthesis. Similarly, arginine could replace glutamate as amino  
31 group donor allowing higher glutamate conversion to GABA.

32 An analysis of GABA production as a function of the pH of the culture confirmed the strong  
33 dependency of this metabolic system on pH (Figure 5). Significant GABA production was observed  
34 only at pH lower than 5.1 during stationary phase (Figure 5), thus confirming previous observations  
35 on the same strain (Mazzoli et al., 2010). These data also agree with recent studies reporting that



1 acidic pH is necessary for activating glutamate/GABA antiport by GadC (Lu et al., 2013; Tsai et al.,  
2 2013). However, when glutamate, arginine and malate were contemporarily supplemented to *L.*  
3 *lactis* NCDO 2118 cultures, GABA production started at the beginning of the exponential growth  
4 phase and at a pH as high as 6.6 (Figure 5). At pH 6.6, the intracellular pH is estimated to be close  
5 to the neutrality (Even et al., 2002) and is not compatible with the GAD activity that was found to  
6 be highly inhibited above pH 5.4 in our bacteria (results not shown). This suggests that another  
7 enzyme probably ensures decarboxylation of glutamate in neutral conditions. As far as we know,  
8 this is the first evidence of significant GABA production during the exponential growth phase and  
9 at nearly neutral pH. The biomass profile could not explain this production since it was similar to  
10 cultures supplemented with arginine plus glutamate, where early activation did not occur. Since this  
11 effect was not observed when each of these three metabolites was supplemented individually or as  
12 binary mixtures (glutamate plus arginine, or glutamate plus malate) it can be speculated that the  
13 simultaneous activation of ADI pathway and malolactic fermentation is susceptible to activate  
14 GABA production, even at higher pH and during exponential growth. Although more work is  
15 needed to understand the biochemical basis of this phenomenon, this observation could have  
16 significant impact on industrial GABA production process.

## 18 CONCLUSION

19 Acidic environments constitute a major stress for LAB which developed several acid-  
20 counteracting systems which include ADI pathway, MLF and amino acid decarboxylation.  
21 Regulation and reciprocal interactions of these pathways seem to vary among microbial species.  
22 The present study indicated that glutamate decarboxylation plays minor roles in *L. lactis* NCDO  
23 2118 physiology with respect to malate and arginine dissimilation. In fact, glutamate  
24 supplementation had very limited effect in neutralizing acidity and in stimulating biomass  
25 production in contrast to results obtained through malate and arginine supplementation.  
26 Furthermore, GABA production was restrained to narrower environmental conditions than malate  
27 or arginine dissimilation, since both acidic pH (pH < 5.1) and stationary phase were generally  
28 required for its activation. However, this study indicated some strategies which enabled activation  
29 of glutamate decarboxylation system outside of this compass. Notably, arginine was able to strongly  
30 stimulate GABA production, while simultaneous addition of arginine and malate was able to trigger  
31 glutamate decarboxylation in earlier growth phase (i.e., exponential phase) at near neutral pH. Even  
32 if understanding the molecular basis of these phenomena will require further studies, these results  
33 are valuable tracks for developing more performant industrial processes for enhanced and earlier  
34 GABA production through fermentation.

35

1 **AUTHOR CONTRIBUTIONS**

2 VL: Contribution to acquisition, analysis and interpretation of the data, contribution in drafting the  
3 article. CY, WN: contribution to experimental and chemical analysis. RM, EP, MCM: contribution  
4 to interpretation of data and in drafting the article. PL: design of the work, analysis and  
5 interpretation of data, writing of the manuscript.

6  
7 **ACKNOWLEDGEMENTS**

8 The authors wish to thank Sophie Mondeil for technical assistance. Grants for Chonthicha Yasaro  
9 and Waranya Narin were provided by PHC Thailand N°16595UC and DUO-Thailand program.

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4

Provisional

1 **Figure legends**

2

3 **Figure 1** Schematic representation of metabolic pathways potentially contributing to acid resistance  
4 in *Lactococcus lactis*. *ADI*, arginine deiminase; Arg, arginine; *CK*, carbamate kinase; GABA,  $\gamma$ -  
5 aminobutyric acid; *GAD*, glutamate decarboxylase; Gln, glutamine; Glnase, glutaminase; Glu,  
6 glutamate; *ME*, malic enzyme; *MLE*, malolactic enzyme; Orn, ornithine; *OTC*, ornithine  
7 transcabamylase.

8

9 **Figure 2** Evolution of biomass ( $\text{g.L}^{-1}$ ) (square), specific growth rate ( $\text{h}^{-1}$ ) (triangle) and pH (circle)  
10 during growth of *L. lactis* NCDO 2118 in CDM.

11

12 **Figure 3** Specific growth rate ( $\text{h}^{-1}$ ) ( $\blacklozenge$ ) of *L. lactis* subsp. *lactis* NCDO 2118, biomass ( $\text{g.L}^{-1}$ ) ( $\blacktriangle$ ),  
13 pH ( $\square$ ) and GABA production (mM) ( $\bullet$ ) in CDM containing various concentrations of glutamate  
14 (A), arginine (B) or malate (C) at 48 h of culture. (---) trend curve.

15

16 **Figure 4** GABA production (mM) (grey) and percentage of glutamate converted to GABA (black)  
17 according to various initial glutamate concentrations in CDM, at 48 h of culture.

18

19 **Figure 5** GABA production (mM) as a function of the pH of the medium in all conditions tested at  
20 48 h of culture : CDM ( $\blacksquare$ ), CDM with glutamate ( $\blacklozenge$ ), with arginine ( $\bullet$ ), with malate ( $\blacktriangle$ ), with  
21 arginine and glutamate ( $\circ$ ), with malate and glutamate ( $\Delta$ ) and with arginine, malate and glutamate  
22 (\*).

23

1

2 **Table 1** Maximal specific rates; maximal biomass, time and pH at the growth arrest; glucose  
 3 consumption, lactate and GABA production, and pH at 48 h; during growth of *L. lactis* NCDO 2118  
 4 on seven different synthetic media.  $v_{\text{lactate}}$  is calculated only with lactate coming from glucose, and  
 5 not from malate. Arg, arginine; Glu, glutamate.

6

7

Parameter	CDM	CDM <sup>1</sup> + Glu	CDM <sup>1</sup> + Arg	CDM <sup>1</sup> +Malate	CDM <sup>2</sup> +Arg+Glu	CDM <sup>2</sup> +Glu+Malate	CDM <sup>2</sup> +Arg+Glu+Malate
<b>Maximal specific rates</b>							
$\mu_{\text{max}}$ (h <sup>-1</sup> )	0.97 ± 0.10	0.97 ± 0.04	1.01 ± 0.11	0.90 ± 0.02	0.89* ± 0.01	0.81* ± 0.01	0.85* ± 0.00
$q_{\text{glucose}}$ (mmol.g <sup>-1</sup> .h <sup>-1</sup> )	27.7 ± 3.4	26.9 ± 5.3	24.6 ± 2.1	26.6 ± 4.4	27.0 ± 3.6	32.0 ± 3.1	28.8 ± 7.9
$v_{\text{lactate}}$ (mmol.g <sup>-1</sup> .h <sup>-1</sup> )	46.5 ± 3.0	49.9 ± 6.8	43.8 ± 3.6	42.1 ± 8.1	41.6* ± 4.8	56.2** ± 2.2	56.2 ± 13.9
$q_{\text{malate}}$ (mmol.g <sup>-1</sup> .h <sup>-1</sup> )				29.5 ± 0.2		24.8 ± 0.1	21.5 ± 2.4
$q_{\text{glutamine}}$ (mmol.g <sup>-1</sup> .h <sup>-1</sup> )	1.22 ± 0.27	0.72 ± 0.22	0.97 ± 0.25	1.69 ± 0.67	0.51 ± 0.14	1.39** ± 0.31	1.20** ± 0.15
$q_{\text{arginine}}$ (mmol.g <sup>-1</sup> .h <sup>-1</sup> )	1.44 ± 0.26	1.39 ± 0.58	9.80** ± 2.26	2.69** ± 0.44	7.91** ± 1.18	3.3** ± 0.35	14.58** ± 0.83
<b>At growth arrest:</b>							
Biomass (g.L <sup>-1</sup> )	0.98 ± 0.00	1.00 ± 0.11	1.96** ± 0.06	1.86* ± 0.17	1.98** ± 0.20	1.67** ± 0.02	2.20** ± 0.01
time (h)	5	6	6	7	6	9	9
pH	4.94 ± 0.09	5.14 ± 0.18	5.07 ± 0.05	5.76* ± 0.13	5.31 ± 0.30	5.78** ± 0.06	6.22** ± 0.00
glucose cons. (mM)	34.4 ± 4.0	43.4 ± 5.9	70.7* ± 5.1	85.7** ± 5.0	63.4** ± 3.8	80.4** ± 0.9	71.1** ± 0.07
lactate produced (mM)	65.6 ± 0.5	78.6 ± 8.4	123.3** ± 6.8	275.2* ± 4.7	112.5 ± 7.1	267.7** ± 6.9	228.05** ± 3.1
GABA (mM)	0	0.08 ± 0.01	0.32 ± 0.88	0.14 ± 0.61	0.17 ± 0.20	1.89** ± 0.14	1.71** ± 0.13
<b>At 48h</b>							
glucose cons. (mM)	51.31 ± 1.7	68.93 ± 17.9	89.9** ± 8.7	105.5** ± 0.7	89.4 ± 12.6	106.8** ± 3.7	121.2** ± 2.7
lactate produced (mM)	102.2 ± 4.7	130.3 ± 24.8	153.3** ± 7.1	318.5** ± 10.6	166.2 ± 13.6	325.3** ± 8.0	353.3** ± 4.0
GABA (mM)	0.44 ± 0.02	3.12* ± 0.85	1.88** ± 0.25	0.38 ± 0.13	8.60* ± 2.0	2.54 ± 0.03	5.22 ± 0.33
pH	4.20 ± 0.02	4.52* ± 0.09	4.47** ± 0.03	4.95** ± 0.08	4.50 ± 0.21	4.90** ± 0.01	4.97** ± 0.00
Ornithine (mM)	0.42 ± 0.03	0.58 ± 0.37	34.90** ± 4.40	0.47 ± 0.18	36.95** ± 2.41	0.07 ± 0.01	25.81** ± 1.04
Citrulline (mM)	0	0	2.07** ± 0.46	0	1.14** ± 0.07	0	3.63** ± 0.23

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10 Notes:

11 Values are means ± standard deviation.

12 Parameters were compared to (1) CDM or (2) CDM+Glu condition with Student t-test. The statistical significance of the test  
 13 was represented with (\*) or (\*\*) according to  $p$ -value threshold of 0.025 and 0.01, respectively.

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Figure 1

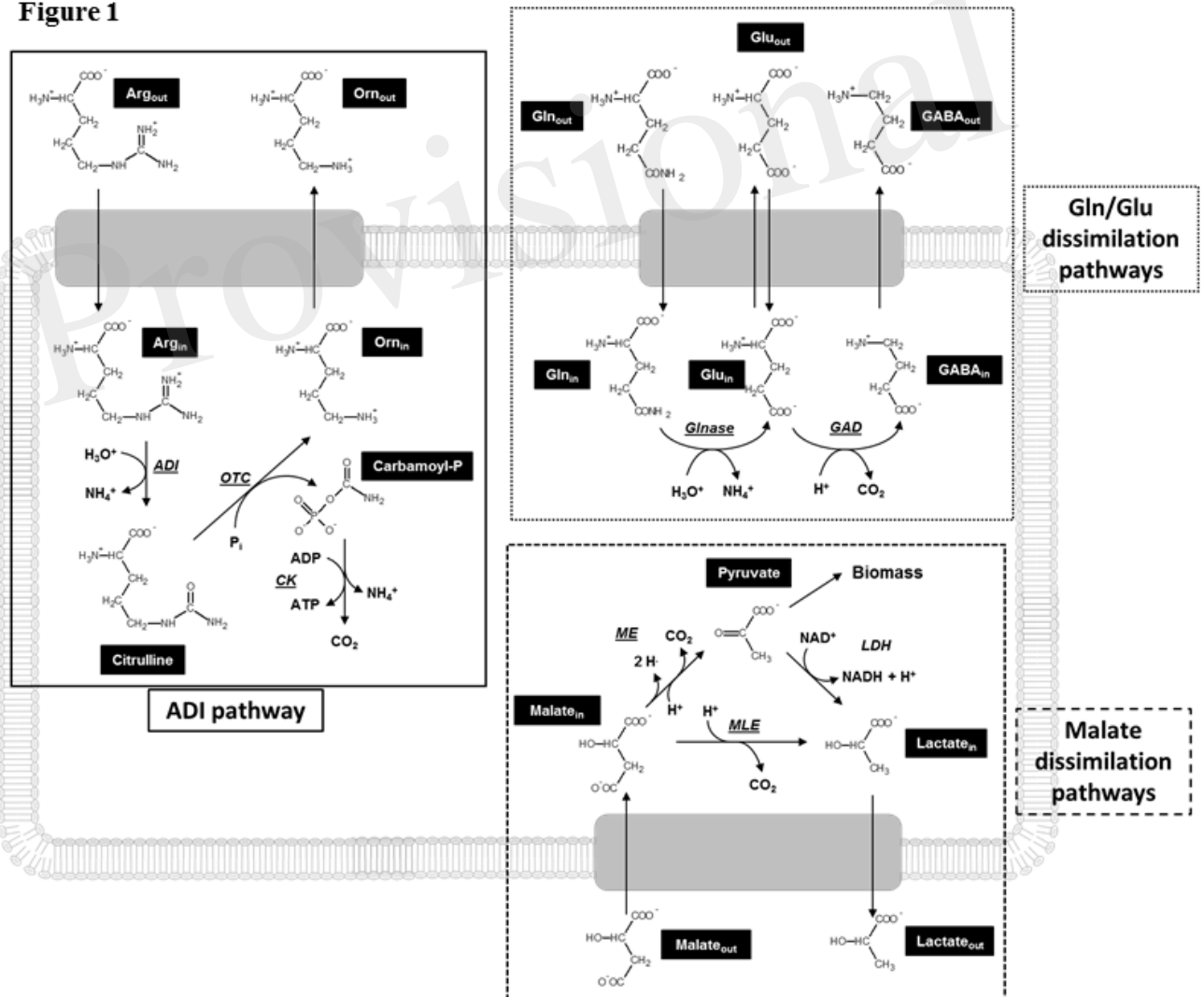


Figure 2

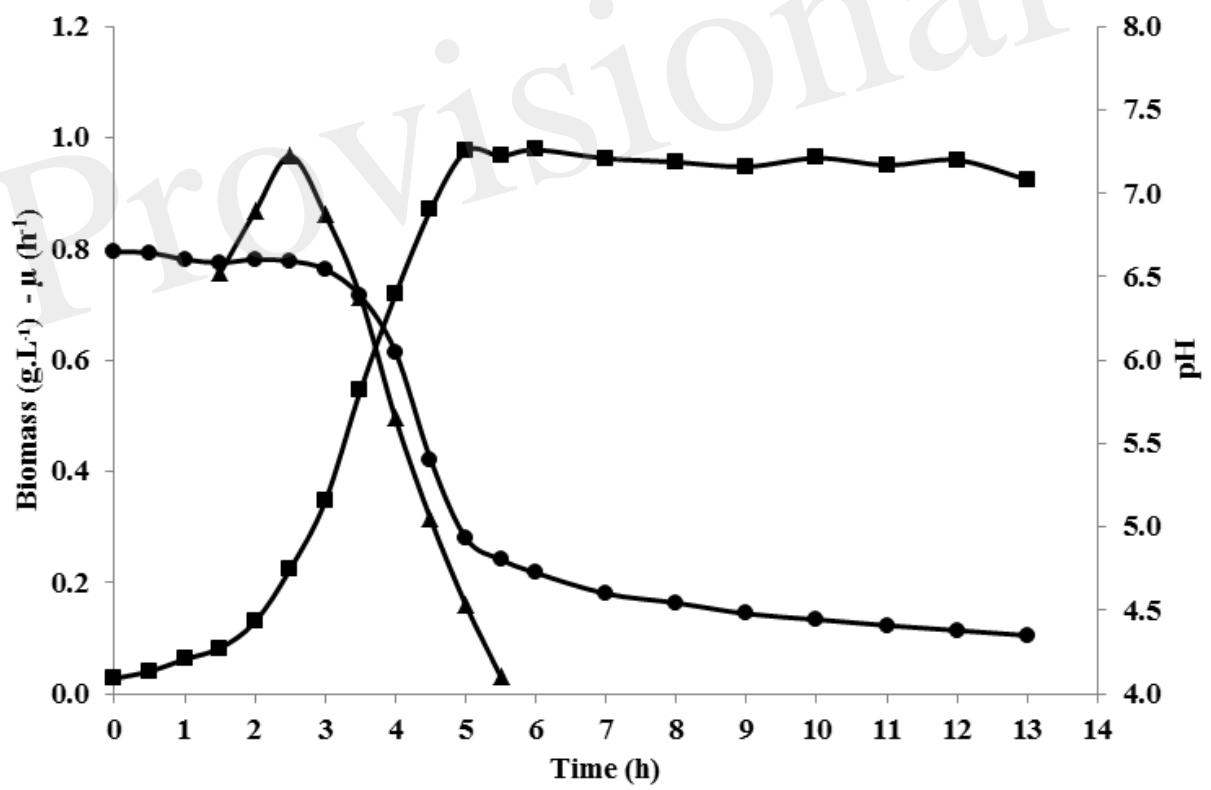
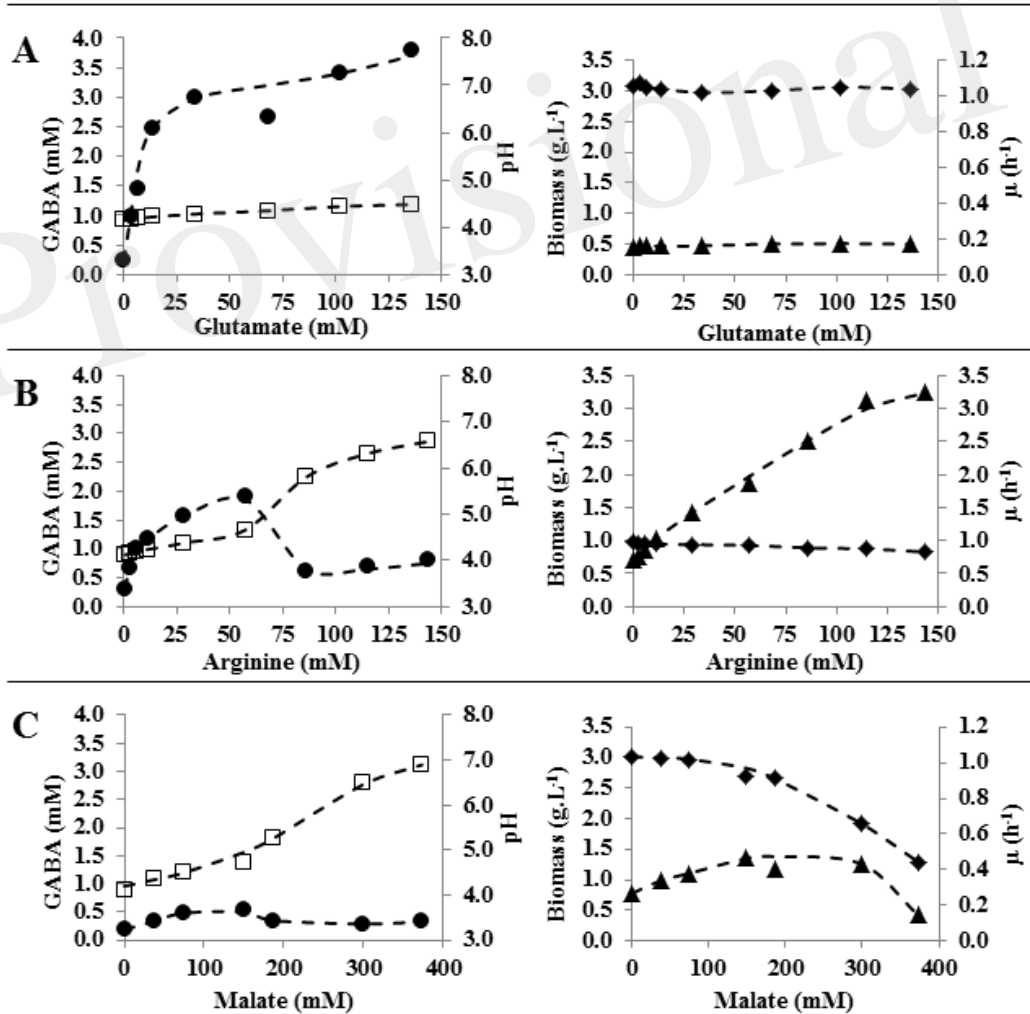


Figure 3



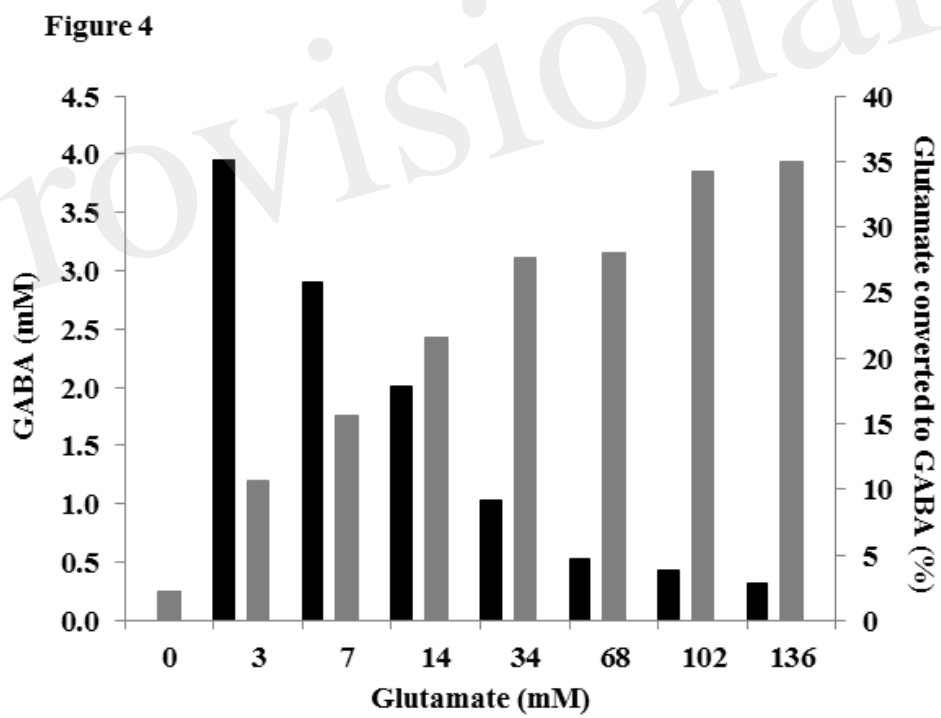


Figure 5

