SELECTION AND OPTIMIZATION OF ACETIC ACID BACTERIA FOR D-GLUCONIC ACID PRODUCTION

Florencia Sainz Perez

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Selection and optimization of acetic acid bacteria for D-gluconic acid production

DOCTORAL THESIS

Directed by Dr. Albert Mas and Dr. María Jesús Torija

Department of Biochemistry and Biotechnology – Universitat Rovira i Virgili

UNIVERSITAT
ROVIRA i VIRGILI

Tarragona 2016

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WE STATE,

That the present study, entitled **"Selection and optimization of acetic acid bacteria for D-gluconic acid production"**, presented by **Florencia Sainz Perez** for the award of the degree of Doctor with International Mention, has been carried out under our supervision at the Department of Biochemistry and Biotechnology of this University

Tarragona, 29th August 2016

Dr. Albert Mas Baron

Dr. María Jesús Torija Martínez

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AGRADECIMIENTOS

No hace mucho tiempo que empecé a ser consciente de la gran importancia que tiene la palabra AGRADECER. Es necesario decir Gracias todos los días por esas pequeñas cosas que nos rodean y hacen con que nuestra vida sea grande y especial. Es momento de cerrar un circulo y no puedo hacerlo sin agradecer a cada persona que se ha cruzado en mi camino.

Gracias al Dr. Albert Mas por haber confiado en mí sin siquiera conocerme. Gracias Albert, por la gran oportunidad que me has dado de trabajar junto a ti y al grupo de Biotecnología Enológica. Gracias. Siempre le digo que es mi madre acética, y ella lo es. Gracias, Dr. María Jesús Torija por haber confiado en mí, por contagiarme el cariño que le tienes a la investigación, por enseñarme y transmitirme todo lo que sabes. Por haberme tirado de las orejas cuando era necesario y haber reído y festejado los logros que obtuvimos. Gracias por tus palabras de ánimo para que no desistiera y viera que no todo estaba perdido. ¡Millones de gracias por haber estado en cada momento, eres grande y te lo agradeceré siempre!

A la Dr. María José Valera, más conocida por Majo o Majito, muchísimas gracias por haberme acompañado durante estos años en el 122. Gracias por compartir conmigo frustraciones y alegrías, por haberme enseñado donde estaba cada cosa y por nuestras discusiones hipotéticas para intentar entender el porqué de muchas locuras de éstas bichas. Gracias. A mis ex compañeros acéticos: Dr. Estibaliz Mateo y Dr. Claudio Hidalgo, gracias por haber estado siempre. Gracias por cada palabra de ánimo y fuerza.

Para mi eran personas lejanas con apellidos impronunciables, pero que se volvieron con los años cada vez más cercanos. Los cracks en relación a bacterias acéticas, enzimas y ácido glucónico. Soy de pensar que el NO siempre existe y que no se pierde nada con intentar, por haberlo intentado y no desistir tuve la oportunidad de vivir una de las experiencias más intensas de mi vida. Gracias al Dr. Kazunobu Matsushita y a su equipo por recibirme de brazos abiertos, por haberme enseñado su trabajo y compartir conmigo sus conocimientos. Gracias a mis compañeros por haber hecho un esfuerzo enorme en ayudarme, por su tiempo y por enseñarme su cultura y costumbres. Arigato Gozaimasu Japanese people!

Gracias también a mis compañeros de Levaduras, Lácticas y de Tecnología Enológica. Gracias por la ayuda que siempre he recibido de todos, por compartir preocupaciones y sobretodo alegrías. Para los que aún les queda camino por delante, no desistan, confíen en ustedes y en su trabajo, todo saldrá bien.

Braulio, Rosa, Santiago, Isa, muchísimas gracias por haberme salvado más de una vez. Por ayudarme siempre con todo lo que necesitaba. Gracias. A las chicas de la secretaria también muchísimas gracias por la ayuda de siempre y por esos emails con recordatorios importantes. A conserjería, gracias por recibir y entregar todos los paquetes y por los buenos días de cada mañana. A los de seguridad por abrirme la puerta los fines de semana con alegría y una palabra de ánimo. Gracias.

Nuria, aprendí de ti que nunca es tarde para volver a empezar y que es necesario re-inventarse siempre para poder seguir adelante. Gracias. Tarde o temprano el universo

siempre recompensa. Bea, gracias por haber estado a mi lado todos estos años, por compartir conmigo cada guerra y cada batalla ganada. Gracias por sacarme una sonrisa siempre, por los dulces de Dulce Mente y las delicias de Miguel. Espero poder seguir acompañándote por muchos años más.

Paloma, Myrna, Xavi, Omar, Isa, Gabino, Miguel y Maria gracias infinitas por ser parte de mi vida Tarraconina, gracias por ser fieles amigos, por estar en todas horas para lo bueno y para lo malo. Gracias por cuidarme y preocuparse por mí. Todas las cervezas o fiestas que me he recusado a asistir en estos últimos meses, os lo juro que las recuperaremos. Gracias infinitas, los quiero.

Tengo fieles mosqueteras del otro lado del charco, que en éstos años poco nos hemos visto, pero han estado siempre a mí lado. Agus, Valeria, Miche, Valentina´s (Martin, Tasende, Pocha), Emi, Laura, Taís, Karine, Aline y Rosi. Gracias por ser las mejores, por acompañarme y vibrar conmigo en cada conquista. Las quiero.

Andrea, Jesús, Tusky y Kai, muchísimas gracias por ser los mejores primos del mundo mundial, por estar presentes, por cuidarme y preocuparse. Gracias. A mis padrinos que siempre han estado a mi lado. Isabel, Jose, Rocio y Yoel, gracias familia por estar.

Jaime, a ti un monumento porque el cielo después de esto ya lo tienes ganado. GRACIAS. Gracias por tu cariño, por tu compañía, fuerza, energía positiva y por entender lo inentendible. Han sido meses difíciles de lágrimas, pataletas y sonrisas. Gracias por haber estado en cada momento, sujetándome y dándome ánimo para seguir adelante. Te amo.

A mis padres, que desde siempre han estado a mi lado, los grandes responsables por ser quien soy el día de hoy. Sin ustedes nunca hubiera llegado hasta aquí, gracias por haberlo dado todo siempre, por estar en cada momento, por empujarme, por confiar en mí, por el sudor y lágrimas que cuesta la distancia. Gracias por enseñarme a nunca bajar los brazos, a no desistir, a vivir cada momento. GRACIAS INFINITAS. Ésta conquista también es de ustedes. Los amo infinitamente por siempre.

Gracias.

If you just feel happy for what you have, have an attitude of gratitude, and be grateful, then it will come true—you will be GREAT and you will be FULL. Yogi Bhajan.

A mis padres,

UNIVERSITAT ROVIRA I VIRGILI
SELECTION AND OPTIMIZATION OF ACETIC ACID BACTERIA FOR D-GLUCONIC ACID PRODUCTION
Florencia Sainz Perez

INDEX

JUSTIFICATION AND OBJECTIVES

UNIVERSITAT ROVIRA I VIRGILI
SELECTION AND OPTIMIZATION OF ACETIC ACID BACTERIA FOR D-GLUCONIC ACID PRODUCTION
Florencia Sainz Perez

The work that is the support of this thesis has been performed in the research group of Oenological Biotechnology at the Department of Biochemistry and Biotechnology of the Faculty of Oenology at Rovira i Virgili University (URV) between the years 2011 and 2016. During this period, I held a grant from URV for one year, a six-month predoctoral scholarship from Programa Martí Franquès and finally a FI Fellowship from AGAUR, Government of Catalunya.

The work that I carried was part of the project “Microbiological selection and control for transforming non-commercial strawberries into new beverages” funded by the Spanish Ministry of Science and Innovation (AGL2010-22152-C03-02). This project was the continuation of a previous project entitled “Elaboration of food seasonings from fruits with double fermentation: microbiological, chemical and sensory control and analysis” (AGL2007-66417-C02/ALI). This project had as main objective the development of seasonings using fruits of secondary quality (persimmon and strawberry), which are not suitable for its direct sale, being a way to take advantage of surplus and highly perishable raw material to produce new products. The results obtained were satisfactory for both fruits; however, the product from strawberry showed better features (aroma, taste and higher concentration of bioactive antioxidants) than the persimmon, resulting in a more attractive final product. Moreover, Spain is the main producer of strawberries (30%) in the EU, followed by Germany, Poland, United Kingdom and France (Figure 1, FAOSTAT) and one of the most important over the world. In fact, in 2007, Spain was the second larger producer of strawberries in the world and in 2012 became the fourth after United States, Mexico and Turkey (FAOSTAT, 2012). Therefore, this high production of strawberry, a very sensitive and perishable fruit, together with the previous results obtained in the elaboration of seasonings, made strawberry a good candidate for the development of new products, such as a new non-alcoholic beverage.

JUSTIFICATION AND OBJECTIVES

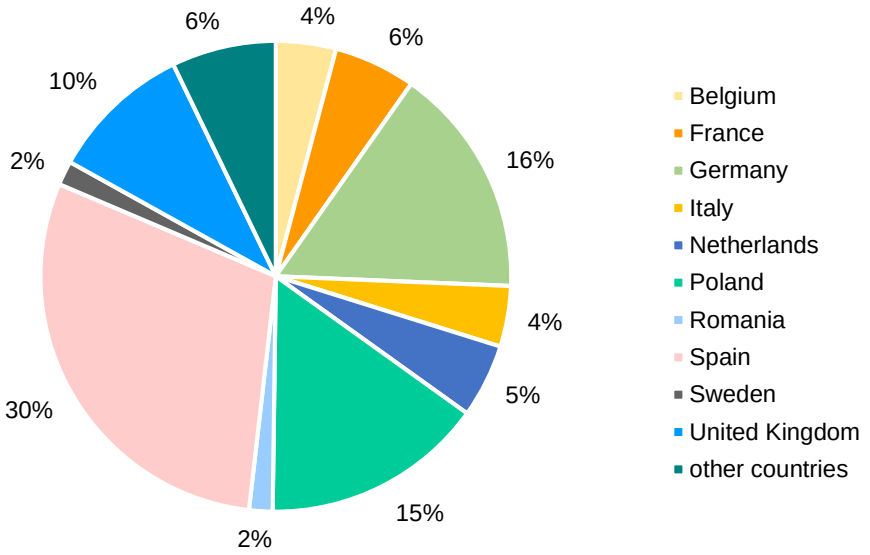

Figure 1. Strawberry production in EU (2012). Source: FAOSTAT http://faostat.fao.org/.

Thus, this soft beverage, produced with strawberry surplus, was the result of a mixture of two natural fermentations. The first product consisted of an alcoholic fermentation followed by an acetification ("strawberry vinegar") and the second one, the oxidation of D-glucose into D-gluconic acid without consuming D-fructose using acetic acid bacteria (AAB) ("Gluconated strawberry juice"). Therefore, the project proposed a selective fermentation of D-glucose, in which D-glucose was mainly metabolized to D-gluconic acid and acetic acid. The new beverage would have an acid and refreshing sour flavor, sweetened with the natural D-fructose of the fruit. As the strawberry vinegar was the object of study in the previous project, in this case, we focused on the elaboration of the gluconated strawberry juice. The project was done in collaboration with the Universities of Sevilla and Cordoba, being our group responsible for the microbiological aspects of the process.

Therefore, the working hypothesis of the present thesis was: **Appropriate starter cultures will be able to produce a new non-alcoholic fermented beverage with surplus strawberry by oxidizing D-glucose to D-gluconic acid.**

To test this hypothesis, our general aim was to make a selective fermentation carried out by AAB, in which D-glucose was transformed into D-gluconic acid without fermenting D-fructose, to maintain a part of the natural sweetness of strawberry that will increase the acceptability of the product. To achieve this general aim, four specific objectives were developed:

Objective 1: To select an acetic acid bacteria able to oxidize D-glucose to D-gluconic acid without consuming D-fructose.

Objective 2: To analyze the enzymatic activity of the membrane-bound dehydrogenases responsible for D-glucose oxidation in the selected acetic acid bacteria strains.

Objective 3: To determine the nitrogen requirements of the selected acetic acid bacteria strains.

Objective 4: To monitor the selected acetic acid bacteria strains during the process.

UNIVERSITAT ROVIRA I VIRGILI
SELECTION AND OPTIMIZATION OF ACETIC ACID BACTERIA FOR D-GLUCONIC ACID PRODUCTION
Florencia Sainz Perez

EXPERIMENTAL DESIGN

UNIVERSITAT ROVIRA I VIRGILI
SELECTION AND OPTIMIZATION OF ACETIC ACID BACTERIA FOR D-GLUCONIC ACID PRODUCTION
Florencia Sainz Perez

To achieve these objectives, the following experimental design was followed:

<u>Objective 1:</u> To select an acetic acid bacteria able to oxidize D-glucose to D-gluconic acid without consuming D-fructose.

CHAPTER 1: Comparison of D-gluconic acid production in selected strains of acetic acid bacteria. Results published in *International Journal of Food Microbiology*, 222, 40-47 (2016).

AAB has the ability to perform oxidative fermentation of a wide range of substrates, like D-glucose, ethanol, glycerol or D-sorbitol and to release to the medium the corresponding products. This characteristic makes AAB interesting for the biotechnology industry.

We studied the production of D-gluconic acid in different strains of *Gluconobacter* and *Acetobacter* genera. Two experiments were performed in the study. In the first experiment, AAB strains were grown in different media (minimal medium, synthetic must and 2x strawberry concentrate) and the best three strains were selected. The main selection criteria were: highest concentration of D-gluconic acid, total consumption of D-glucose and minimal oxidation of D-fructose. From this, the selected strains were tested in 3x strawberry concentrate with two different pH (3.3 and 4.8).

A part of this work was also used in the collaboration with the University of Sevilla and as a result an article in the journal *Food Chemistry* was published: Impact of gluconic fermentation of strawberry using acetic acid bacteria on amino acids and biogenic amines profile (Ordóñez et al., 2015)

Objective 2: To analyze the enzymatic activity of the membrane-bound dehydrogenases responsible for D-glucose oxidation in the selected acetic acid bacteria strains.

CHAPTER 2: Determination of dehydrogenase activities involved in D-glucose oxidation in *Gluconobacter* and *Acetobacter* strains. Results published in *Frontiers in Microbiology,* doi: 10.3389/fmicb.2016.01358

Most of the oxidative fermentations in AAB are catalyzed by membrane-bound dehydrogenase enzymes, which can be PQQ or flavin dependent. In D-glucose metabolism, the membrane-bound D-glucose dehydrogenase (mGDH) oxidize D-glucose to D-gluconic acid. Then, D-gluconic acid could be metabolize by D-gluconate dehydrogenase (GADH) or glycerol dehydrogenase (GLDH) to 2-keto-D-gluconic acid (2KGA) or 5-keto-D-gluconic acid (5KGA), respectively. Furthermore, 2KGA could be oxidize to 2,5-di-keto-D-gluconic acid by the membrane-bound 2-keto-D-gluconate dehydrogenase (2KGDH).

In six different strains of three AAB species, the enzymatic activities of the membrane-bound dehydrogenases responsible for D-glucose metabolism were compared. The mGDH and GLDH activities were measured by phenazine methosulfate (PMS) and 2,6-dichlorophenol indophenol (DCIP) as electron acceptors and for GADH and 2KGDH ferricyanide was used as electron acceptor. All these oxidative compounds produced during the experiment were analyzed by high performance liquid chromatography (HPLC). Finally, primers were designed for the partial amplification of the genes coding for mGDH, GLDH, GADH, 2KGDH and phylogenetic trees were constructed to compare the sequences of these genes in our strains with the sequences available in the Genbank Database.

<u>Objective 3:</u> To determine the nitrogen requirements of the selected acetic acid bacteria strains.

CHAPTER 3: Effect of nitrogen source on the growth of different acetic acid bacteria strains. This manuscript is being reviewed in the *International Journal of Food Microbiology*

AAB are considered fastidious microorganisms due to the difficulties for isolation and cultivation on solid media. AAB are strictly aerobic and their growth depends on available carbon sources and molecular oxygen. Different media have been designed for the isolation of AAB from different sources, but little is known about the needs of AAB for nitrogen sources. The aim of this study was to analyze the best nitrogen sources and the minimal concentration of nitrogen necessary for AAB growth.

Three different media (Synthetic medium (SM), minimal medium (M9) and Yeast nitrogen base (YNB)) were tested by adding a complete solution of amino acids and ammonium in a range of concentrations (from 25 to 1000 mg N/L). After the selection of the best medium and the best nitrogen concentration for each strain, the nitrogen source was added as a single amino acid or ammonium ion at the optimal concentration.

<u>Objective 4:</u> To monitor the selected acetic acid bacteria strains during the process.

To monitor the selected strains during the two processes involved in the elaboration of the fermented beverage, the research group has available specific TaqMan-MGB process and primers for the species *G.oxydans* and *A.malorum*, but not for *G.japonicus*. Due to difficulties to design a probe for *G.japonicus*, a study of the 16S-23S rDNA internal transcribed spacer (ITS) in different AAB species was done.

CHAPTER 4: Analysis of ribosomal internal transcribed spacers (16S-23S) in acetic acid bacteria (Manuscript in preparation)

Different molecular techniques based on PCR methods are used for AAB identification at different levels (genus, species or strains). The use of the 16S-23S rDNA internal transcribed spacer (ITS) presents higher discriminatory power than the use of 16S rDNA, since it shows differences in length and sequence between species, being a good solution for the identification of AAB at species level.

The 16S-23S rDNA ITS of a high number of AAB strains belonging to different genera and species was amplified in this work to do a variability study. As in some strains, more than one fragments were obtained, these amplicons were purified using a QIAquick Gel Extraction Kit (Qiagen, Netherlands) and sequenced. Furthermore, in some strains, the different ITS amplicons were cloned into pGEM-T Easy vector (Promega, MA, USA), and introduced in *E. coli* cells. Then, some transformed colonies from each strain were purified and the plasmid inserted was isolated and screened by digestion with the restriction endonuclease E*co*RI (Roche), to determine which ITS amplicon was inserted in each plasmid and what was the proportion between them.

CHAPTER 5: Draft genome sequence of *Acetobacter malorum* CECT 7742, a strain isolated from strawberry vinegar. *Genome Announcements* 4(3):e00620-16 (2016). (doi:10.1128/genomeA.00620-16).

CHAPTER 6: Draft genome sequences of *Gluconobacter cerinus* CECT 9110 and *Gluconobacter japonicus* CECT 8443, acetic acid bacteria isolated from grape must. *Genome Announcements* 4(3):e00621-16 (2016). (doi:10.1128/genomeA.00621-16).

The whole genome sequencing of different strains of AAB species allows to understand diverse aspects of diversity, evolution and above all, gives genomic information.

Technological advances and bioinformatics improvements have allowed the development of faster and more efficient sequencing methods. Nowadays, it is possible to use sequencing methods as a routine tool in research, due the reduction in the cost of the analysis.

The genome of three AAB strains belonging to different species and genera was sequenced. The DNA was extracted according to the cetyltrimethyl ammonium bromide (CTAB) method and the whole-genome was sequenced using the Genome Analyzer Ion Torrent PGM (Thermo Fisher Scientific, Madrid, Spain). The whole genome sequences were deposited in the GenBank Database.

UNIVERSITAT ROVIRA I VIRGILI
SELECTION AND OPTIMIZATION OF ACETIC ACID BACTERIA FOR D-GLUCONIC ACID PRODUCTION
Florencia Sainz Perez

INTRODUCTION

UNIVERSITAT ROVIRA I VIRGILI
SELECTION AND OPTIMIZATION OF ACETIC ACID BACTERIA FOR D-GLUCONIC ACID PRODUCTION
Florencia Sainz Perez

1. ACETIC ACID BACTERIA

1.1. AAB general characteristics

The acetic acid bacteria (AAB) are gram-negative or gram-variable bacteria, with ellipsoidal or rod-shape morphology. Their size is between 0.4 to 1 µm wide and 0.8 to 4.5 µm long; they are mobile due the presence of flagella that can be peritrichous or polar and are not able to form spores (Yamada and Yukphan, 2008). Microscopically, AAB can be seen as individual cells, in pairs or chains. However, depending on the environmental conditions and culture age, they could change their morphology, forming aggregates (Park et al., 2003; Trček et al., 2007), which is a problem for their isolation and quantification.

AAB are catalase positive and oxidase negative and have a strictly aerobic metabolism with oxygen as the terminal electron acceptor. According to Holt et al. (1994), AAB grow well between pH 5.5 and 6.3, although they are able to grow at pH lower than 4. Moreover, Du Toit and Pretorius (2002b) reported that AAB could be isolated at pH between 2 and 3 in culture media with acetate and aeration. The optimal range of temperature for growth is between 25ºC and 30ºC, while some species are able to grow among 30ºC to 40ºC (Ndoye et al., 2006; Saeki et al., 1997) and weakly at temperatures as low as 10ºC (Joyeux et al., 1984).

AAB are widespread in different niches in nature, usually they are found in substrates containing sugar and/or ethanol. They can occur in fruits, flowers or palm sap, but also in manufactured products like food and fermented beverages, such as fruit juices, wine, cider, beer, cocoa and vinegar (Nielsen et al., 2007; Yamada and Yukphan, 2008). In addition, they were found in the plant rhizosphere (Fuentes-Ramírez et al., 2001) and recently as symbiotic microorganism in insects (Crotti et al., 2010).

The ability to oxidize incompletely an extensive number of carbohydrates and alcohol and the quick release in the media of the corresponding products, like acetic acid, cellulose, sorbose, sorbitol, D-gluconic acid, made AAB important for industry and

biotechnology applications. People have benefited for many years from AAB, without knowing that they were responsible for many processes such as vinegar production. Nowadays, more about AAB and their benefits is known, thus many new fields can be exploited by using these microorganisms (Raspor and Goranovic, 2008).

1.2. AAB Taxonomy

AAB are classified in the *Acetobacteraceae* family, in α-class of Proteobacteria (De Ley et al., 1984: Sievers et al., 1994a, b) and their taxonomy has been updated many times in the last forty years mainly due to the improvements and developments of new technologies. If we do a brief review of the history of AAB, F.T. Kützing can be considered as the first who observed microscopically AAB in a naturally fermented vinegar in 1837, when he described that the thin film that covered the surface of the vinegar was made by “globulles” six times smaller than yeasts. However, the first systematic study of acetification was carried out by Louis Pasteur in 1868, who described that only with the presence of a mass of live microorganism, “vinegar mother”, was possible to produce acetic acid and obtain the final product. Ten years later, Hansen reported that the “vinegar mother” responsible for transforming alcohol into acetic acid was a mixture of several bacterial species and not just one.

Taxonomically, *Acetobacter* and *Gluconobacter* were the first genera proposed by Beijerinck in 1898 and Asai in 1935, respectively, using morphological and biochemical characteristics for this first classification (Cleenwerck and Vos, 2008). Later, Frateur, in 1950, suggested a classification based on five physiological criteria: (i) catalase activity; (ii) production of D-gluconic acid from D-glucose; (iii) oxidation of acetic acid to carbon dioxide and water; (vi) oxidation of lactic acid to carbon dioxide and water and (v) oxidation of glycerol to dihydroxyacetone. Thereby, the genus *Acetobacter* was subdivided in four groups: *peroxydans*, *oxydans*, *mexosydans*, and *suboxydans* (review by Barja et al., 2003). In 1974, in the eight edition of *Bergey´s Manual of Determinative Bacteriology* AAB had still only two genera *Acetobacter* and *Gluconobacter* according to

the ability to oxidize acetate and lactate to carbon dioxide and water and the flagellation pattern. Later, Yamada and Kondo (1985) presented a different classification based on chemotaxonomic methods, where differences on ubiquinone system were analyzed. The genus *Acetobacter* showed Q-9, as primary respiratory quinone, while genus *Gluconobacter* had Q-10. Due to this feature, the *Gluconacetobacter* genus was created (Yamada et al., 1997a) and some strains included hitherto in the genus *Acetobacter* (*A. xylinus*, *A. liquefaciens*, *A. hansenii*, *A. diazotrophicus* and *A. europaeus)* were reclassified into this new genus. Some years later, in 2012, Yamada et al. proposed the genus *Komagataeibacter* for some species included in *Gluconacetobacte*r genus, among them *Ga. europaeus*, *Ga. xylinus*, *Ga. hansenii*,...

Although morphological, biochemical and physiological criteria have been commonly used to differentiate and characterize AAB genera and species, these traditional tests are laborious, time consuming, not accurate and difficult to interpret, therefore, not enough reliable (Cleenwerck and De Vos, 2008; Gullo et al., 2012). These difficulties led, in recent decades, to study the AAB taxonomy by the combination of phenotypic, chemotaxonomic and genotypic data (Cleenwerck and De Vos, 2008; Mamlouk and Gullo, 2013). DNA-molecular methods are quicker and more reproducible for the differentiation and classification of new species/genera of AAB. Nowadays, diverse methods are used, such DNA-DNA hybridization, key to discriminate among closely species of bacteria (Cleenwerck and De Vos, 2008); analysis of 16S rDNA (Yamada et al., 2012a), this gene is a highly conserved gene among AAB and allows the differentiation of AAB species. However, in some cases, this gene may not be enough resolutive for taxonomic studies. Thus, additional genotypic tests must be done, like the analysis of the internal transcribe spacer (ITS) between the genes 16S and 23S rDNA. This region shows a higher discriminatory power than 16S rDNA gene, with more variation in sequence and length (González et al., 2006a; González and Mas, 2011; Ruiz et al., 2000; Trček and Barja, 2015). Different works has proven the usefulness of this method differentiating closely related species such as *Acetobacter malor*um and

Acetobacter cerevisiae (Valera et al., 2011). In the last years, new technologies for a rapid identification of bacteria and routine analysis, such as MALDI-TOF MS, have been developed. The use of the protein profiles obtained by this method has been successfully applied to the differentiation of AAB at different levels (genera, species and strains) (Andrés-Barrao et al., 2013; Wieme et al., 2014).

As we above mentioned, the AAB taxonomy has been updated many times since 1898, and these modifications and rearrangements are expected to continue in the coming years due to that AAB are present in many different habitats (Trček and Barja, 2015). To date, AAB are classified in nineteen genera (Table 1), being *Acetobacter*, the one with a higher number of described species (25 species), followed by *Gluconobacter* (15 species), *Komagataeibacter* (14 species), *Gluconacetobacter* (10 species), *Asaia* (8 species); the other AAB genera has only 1 or 2 species described (Trček and Barja, 2015). It is important to highlight that although the genera *Commensalibacter* and *Endobacter* belong to the *Acetobacteraceae* family and were included in the list of AAB by Trček and Barja (2015), they are not yet accepted as acetic acid bacteria.

Table 1. Genera of the acetic acid bacteria actually described, with the type species of the genus and the number of described species (edited from Trček and Barja, 2015).

Genus	Type species	Number of described species	Author(s)
Acetobacter	*A.aceti*	25	Beijerinck (1898)
Gluconobacter	*G.oxydans*	15	Asai (1935)
Acidomonas	*A.methanolica*	1	Urakami et al. (1989)
Gluconacetobacter	*G.liquefaciens*	10	Yamada et al. (1997b)
Asaia	*A.bogorensis*	8	Yamada et al. (2000)
Kozakia	*K.baliensis*	1	Lisdiyanti et al. (2002)
Saccharibacter	*S.floricola*	1	Jojima et al. (2004)
Swaminathania	*S.salitolerans*	1	Loganathan and Nair (2004)
Neoasaia	*N.chiangmaiensis*	1	Yukphan et al. (2005)
Granulibacter	*G.bethesdensis*	1	Greenberg et al. (2006)
Tanticharoenia	*T.sakaeratensis*	1	Yukphan et al. (2008)
Commensalibacter	*C.intestini*	1	Roh et al. (2008)
Ameyamaea	*A.changmaiensis*	1	Yukphan et al. (2009)
Neokomagataea	*N.thailandica*	2	Yukphan et al. (2011)
Komagataeibacter	*K.xylinus*	14	Yamada et al. (2012 a,b)
Endobacter	*E.medicaginis*	1	Ramírez-Bahena et al. (2013)
Swingsia	*S.samuiensis*	1	Malimas et al. (2013)
Nguyenibacter	*N.valangensis*	1	Thi Lan Vu et al. (2013)
Bombella	*B.intestini*	1	Li et al. (2015)

1.3. Metabolism of AAB

The AAB are strictly aerobic microorganisms, the availability of molecular oxygen is crucial for their growth, as it acts as terminal electron acceptor. This group of microorganisms is known for the ability to incompletely oxidize a wide range of sugars and alcohols, accumulating intermediate metabolites in the media, without relevant toxicity for the bacteria (De Ley et al., 1984). The carbon source is also an important factor in AAB growth and different strains or species could adapt to diverse carbon sources.

Two kinds of enzyme systems that differ on localization in the cell are able to oxidize sugar, alcohols and polyols. The first pathway involves dehydrogenase enzymes located in the plasma membrane and the final products from the oxidation are released to the external medium. The second system is located inside the cell and the enzymes are $NAD(P)^+$ dependent. These enzymes are believed to contribute to the production of biosynthetic precursors and to the cell maintenance in the stationary phase (Matsushita et al., 1994).

One of the main AAB characteristic and the best known is their ability to oxidize ethanol to acetic acid by two membrane-bound enzymes. This pathway has two consecutive stages: first, ethanol is oxidized to acetaldehyde by the membrane-bound alcohol dehydrogenase (ADH) and after, acetaldehyde is oxidized to acetic acid by the membrane-bound aldehyde dehydrogenase (ALDH). Both enzymes presented pyrroloquinoline (PQQ) cofactor and are independent of $NADP^+$. The enzyme ADH has an optimal pH of 4 with a restricted substrate specificity and ALDH showed an optimal pH among 4 and 5, however the oxidation could happen at lower pH (Adachi et al., 1980). The productivity of acetic acid is higher in *Acetobacter* species than in *Gluconobacter* species. This is because *Acetobacter's* ADH is more active and the resistance to acetic acid in this species is higher (Matsushita et al., 1994). It is important to consider that *Acetobacter* and *Gluconacetobacter* species are able to completely oxidize acetic acid

to carbon dioxide and water via tricarboxylic acid pathway. Ethanol is a potent inhibitor of this process. Instead, *Gluconobacter* species are incapable of completely oxidizing ethanol, because this genus does not present a functional tricarboxylic acid pathway.

AAB are able to oxidize different primary alcohols to their respective ketones, for example 2,3-Butanediol to acetoin or 1,2-Propanediol to hydroxyacetone. Also AAB are able to metabolize polyalcohols to sugars, like mannitol to D-fructose or sorbitol into sorbose. All these products will be accumulated in the medium. Moreover, organic acids such as citric, lactic, fumaric, pyruvic and succinic acids can also be used by AAB. In this case, these substrates are at intermediate degrees of oxidation, and therefore can be completely oxidized to carbon dioxide and water. As mentioned above, a complete oxidation occurred only in the species of AAB that present a functional tricarboxylic acid pathway.

Different carbohydrates, such as arabinose, D-fructose, galactose, ribose, sorbose, mannose and xylose, can be oxidized by AAB, although D-glucose is the preferred carbon source for most AAB (De Ley et al., 1984).

1.3.1. Oxidation of D-glucose by AAB

As we above mentioned, two different pathways (Figure 2) could be responsible for D-glucose oxidation (Gupta et al., 2001; Muynck et al., 2007; Velizarov and Beschkov, 1998) and, therefore, two different enzyme systems (Matsushita et al., 1994) that differ in cellular localization, function and substrate specificity are present in AAB (Deppenmeier et al., 2002; Muynck et al., 2007).

One enzyme system is located in the cytosol, thus, D-glucose must be taken up into the cell, through the cytoplasmic membrane, and then be oxidized intracellularly to D-gluconate by $NADP^+$-dependent D-glucose dehydrogenase or be metabolized by the pentose phosphate pathway (Muynck et al., 2007; Silberbach et al., 2003). After, this D-gluconate could be further oxidized to 2-keto-D-gluconic acid (2KGA) or 5-keto-D-gluconic acid (5KGA) by the cytoplasmic $NADP^+$-dependent enzymes, 2-keto-D-

gluconate reductase (2KGR) and 5-keto-D-gluconate reductase (5KGR), respectively. Moreover, these enzymes can catalyze the reverse reaction; reducing the keto-D-gluconic acids again to D-gluconate (Elfari et al., 2005; Klasen et al., 1995; Muynck et al., 2007; Pronk et al., 1989).

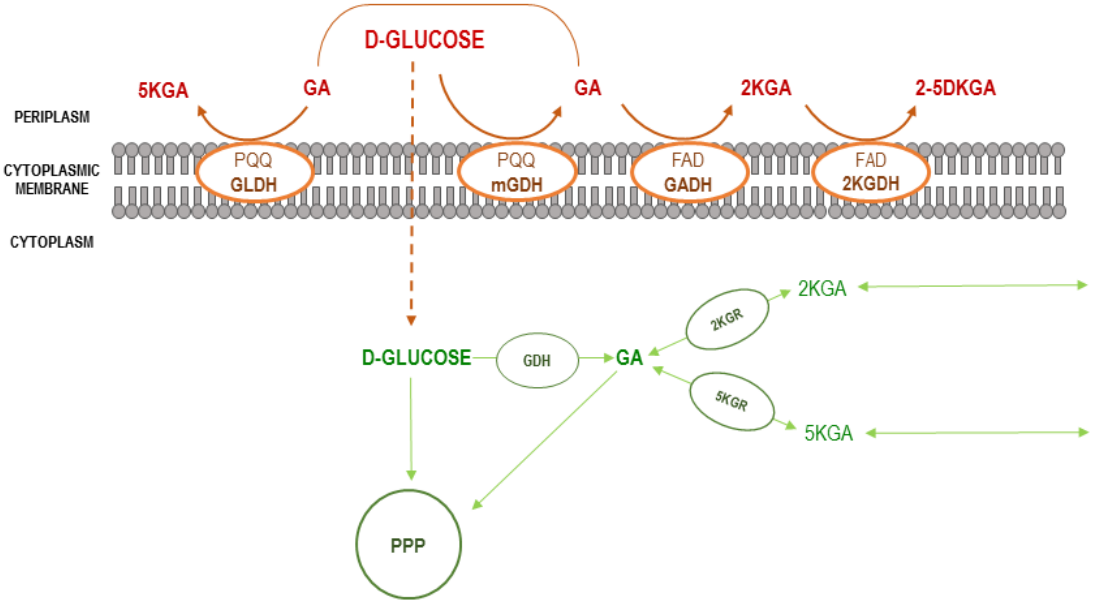

Figure 2. Enzymatic reactions for D-glucose oxidation by AAB.

According to Matsushita et al. (1994), these $NADP^+$-dependent enzymes are mainly involved in the synthesis of biosynthetic precursors and in the cell maintenance during the stationary phase, without much contribution to the oxidative fermentation.

The second and the most important pathway for D-glucose metabolism, known as “direct D-glucose oxidation” pathway, (Figure 2) take place in the periplasmic space. In this case, D-glucose is directly oxidized by the membrane-bound PQQ-dependent D-glucose dehydrogenase (mGDH) (Deppenmeier et al., 2002; Elfari et al., 2005; Gupta et al., 2001; Krajewski et al., 2010; Matsushita et al., 1994) to glucono-δ-lactone, and then is converted to D-gluconate by glucono-δ-lactonase or spontaneously (Matsushita et al., 1994; Shinagawa et al., 1999). D-glucose is almost quantitatively converted to D-gluconate and only when the concentration of D-glucose decreases below a certain value (30 mM), the keto-D-gluconic acid production starts (Weenk et al., 1984). The substrate

specificity of mGDH seems to be restricted to D-glucose (Adachi et al., 2007). The membrane-bound dehydrogenases are linked to the respiratory chain, which transfers electrons to ubiquinone and then to the terminal oxidase, resulting in the synthesis of ATP (Matsushita et al., 1994). mGDH is the key enzyme in the metabolism of D-glucose in AAB (Ameyama et al., 1981). Afterwards, D-gluconate can be oxidized to 2KGA by a FAD-dependent membrane-bound D-gluconate dehydrogenase (GADH). This enzyme is specific for D-gluconate oxidation (Adachi et al., 2007; Ameyama et al., 1981; Matsushita et al., 1994; Toyama et al., 2007). 2KGA may also be oxidized to 2,5-di-keto-D-gluconate (2-5DKGA) by the FAD-dependent 2-keto-D-gluconate dehydrogenase (2KGDH) (Gupta et al., 2001; Matsushita et al., 1994), which is a flavohemoprotein with three different subunits similar to GADH that only uses 2KGA as substrate (Adachi et al., 2007). D-gluconate can also be oxidized to 5KGA by a PQQ-dependent membrane-bound glycerol or polyol dehydrogenase. This enzyme has been elusive and now is known that has a broad substrate preference towards several sugar alcohols (D-glycerol, D-sorbitol, D-arabitol, or D-mannitol) and is identical to D-arabitol dehydrogenase (ARDH) or D-sorbitol dehydrogenase (SLDH) (Adachi et al., 2007; Matsushita et al., 2003).

It is important to highlight that the oxidation of D-glucose depends on the pH of the medium, the initial D-glucose concentration and the particular conditions used for cultivation (Asai 1968; Olijve and Kok, 1979, Qazi et al., 1991). Each enzyme involved in the D-glucose oxidation has a specific pH that must be considered during the oxidation process (Silberbach et al., 2003). De Ley et al. (1984) found that the optimal pH for AAB growth is 5.5. On the other hand, some authors reported that D-glucose was quantitatively converted into D-gluconate and no production of keto-D-gluconate was detected when there was no pH control (Beschkov et al., 1995; Weenk et al., 1984). Instead, when pH of the medium was controlled (pH 5.5), although D-glucose was also practically quantitatively converted to D-gluconate, the accumulation of 2KGA and 5KGA was observed (Weenk et al., 1984). Some years later, Qazi et al. (1991), described the

pH 5.5 as the optimal for D-glucose dehydrogenase activity and the range 3.5-4.0 for the activity of gluconate and keto-D-gluconate dehydrogenases. However, other studies have reported different pH for the optimal activity of these enzymes, suggesting that it is heavily dependent on the strain and medium conditions used and also on the aim targeted (Elfari et al., 2005; Silberbach et al., 2003). In general, an acidic pH, between 3.0-6.0, is considered good for membrane-bound dehydrogenase enzymes activity and an alkaline pH, between 8.0-11.0, for cytosolic enzymes activity (Adachi et al., 2001; Matsushita et al., 1994).

Keto-D-gluconates production is highly dependent on the initial concentration of D-glucose and the strain used (Weenk et al., 1984). These authors demonstrated that with initial D-glucose concentrations of 50 and 250 mM, the content of this substrate in the medium should drop below 10 mM and 80 mM, respectively, to boost the synthesis of keto-D-gluconates. In another study, three different D-glucose concentrations (56 mM, 500 mM and 1160 mM) were tested, and while the low concentrations resulted in a quantitatively conversion of D-glucose into D-gluconate, the highest initial concentration of D-glucose (1160 mM), reduced the yield of D-gluconate production (65%) (Beschkov and Velizarov, 1995). D-glucose oxidation is mostly located in the membrane when the concentration of D-glucose in the medium is above 15 mM and the pH is below 3.5, since, in these conditions, the pentose phosphate pathway is inhibited (Olijve and Kok, 1979; Ramachandran et al., 2006).

The oxygen concentration in the medium can also interfere with the D-glucose oxidation. An increase of the dissolved oxygen in the medium positively influences both the AAB growth as the oxidation process by stimulating the enzymes involved (Buse et al., 1992; Macauley et al., 2001). Similar benefits due to a high aeration rate have been observed by other authors (Silberbarch et al., 2003; Sonoyama et al., 1982).

1.3.1.1. Uses of D-gluconic acid and keto-D-gluconic acids

INTRODUCTION

D-gluconic acid is a nontoxic and noncorrosive weak acid soluble in water. It is considered a safe food additive, used in different food industries (Ramachandran et al., 2006; Rogers et al., 2013). It could be used in its free form, calcium or sodium salt and also like a lactone (Gupta et al., 2001). Different industries like pharmaceutical, food, fodder and concrete benefit from its use. D-gluconic acid present chelating properties, especially with calcium and iron (Gupta et al., 2001; Mattey, 1992). It can be used for precious metal cleaning (Gupta et al., 2001), and in dairy industry, as an additive in cleaning products to clean milk storages vessels and machinery and to prevent accumulation of calcium salts (Rogers et al., 2013). Furthermore, it is widely spread in natural products like in fruit juices, honey, yoghurt, bread, cottage cheese and meat. It gives a refreshing sour taste to wine and fruit juices (Ramachandran et al., 2006), can be added in food to give smooth acidity (Rogers et al., 2013) and it has the property to prevent bitterness in foodstuffs (Ramachandran et al., 2006). It is extensively used as flavoring agent and to reduce the fat absorption (Ramachandran et al., 2006). Its low toxicity, high stability and solubility help to achieve high concentrations of calcium or iron salts, when used in medical treatment for anemia (Mattey, 1992). Moreover, D-gluconic acid has been reported to have some beneficial effects upon intestinal microbiota (Asano et al., 1994, 1997; Tsukahara et al., 2002).

From a biotechnological point of view, 5KGA is important for the chemical industry as a precursor of L-tartaric acid (Muynck et al., 2007; Yamada et al., 1971), which can be produced in few steps (Klasen et al., 1991; Matzerath et al., 1995). It is important to highlight that AAB are only involved in the first step of the process, the oxidation of D-glucose to 5KGA. The subsequent oxidation of 5KGA to L-tartaric acid needs the presence of ammonium vanadate (NH_4VO_3) to catalyze this reaction, which is independent on the AAB activity (Klasen et al., 1991; Matzerath et al., 1995). L-tartaric acid has diverse functions as acidulant in wine, fruit and beverages, as taste enhancer and antioxidant in food industry and as acidic reducing agent in textile industry (Herrmann et al., 2004; Matzerath et al., 1995). Moreover, L-tartaric acid could be an

alternative to citric acid as an acidulant in food additives due to its superior organoleptic properties (Matzerath et al., 1995).

Furthermore, different products from D-glucose oxidation are intermediates of the synthesis pathway of L-ascorbic acid (vitamin C). At present, a considerable part of commercially manufactured of L-ascorbic acid is still synthesized via the seven-step Reichstein process that includes six chemical stages and only one microbial step (Bremus et al., 2006; Liu and Zhang, 2010). However, the high energy consumption together with the high temperatures and pressures required at some stages, makes the process very expensive for the industry. Therefore, in the last twenty years, different strategies to overcome these difficulties based on the microbial biotransformation have been investigated. Most of these approaches have been focused on the production of 2-keto-L-gulonate (2KLG), as the key intermediate that can be later converted to L-ascorbic acid by conventional chemical catalysis (Bremus et al., 2006). Figure 3 shows the different routes for the production of L-ascorbic acid indicating which steps can be done by AAB, specially *Gluconobacter* strains. AAB are involved in two possible pathways for the production of 2KLG from D-glucose, oxidation of D-glucose to 5KGA or to 2,5-DKGA. The latter is especially interesting since it could be directly converted to 2KLG by *Corynebacterium* (Sonoyama et al., 1982).

For the chemical industry and especially for the production of organic acids, the microbial fermentation presented more advantages than petroleum-based chemical production (Alonso et al., 2015). Thus, fermentative production is trustworthy, eco-friendly, achievable, sustainable and above anything, it is economically competitive. Therefore, the development and improvement of processes based on microbial fermentation are now one of the challenges in biotech industry.

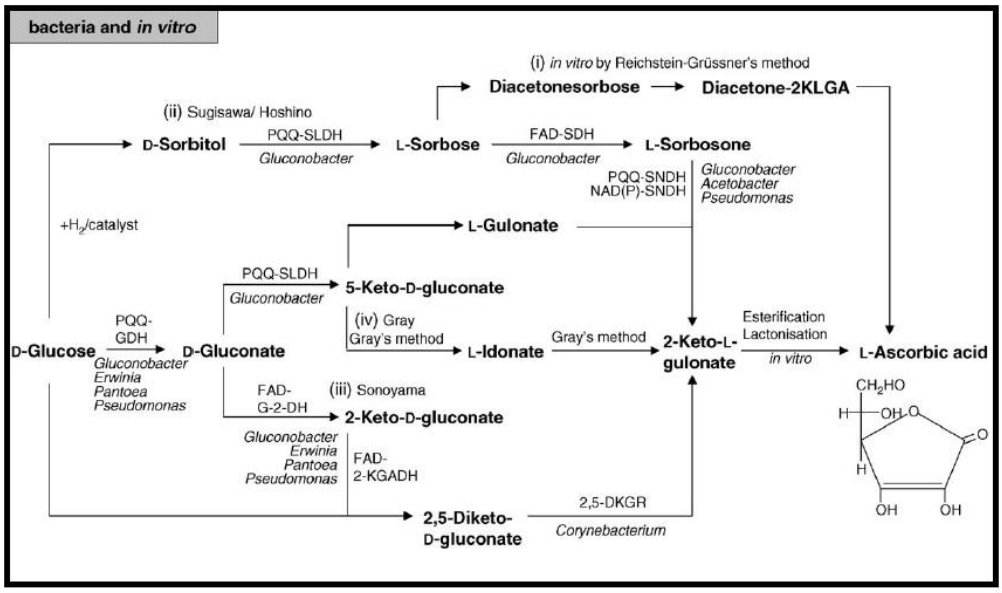

Figure 3. Different methods used for the production of L-Ascorbic acid. (Bremus et al., 2006)

1.3.2. Nitrogen metabolism

Little information is known about both the metabolic pathways involved in nitrogen assimilation as the nitrogen requirements of AAB. Most of AAB are supposed to be able to use inorganic ammonium as the only nitrogen source and to synthetize amino acids and nitrogen compounds (Belly and Claus, 1972; De Ley et al., 1984). However, some studies have reported that some amino acids are essential for AAB growth, while others described a stimulatory or inhibitory function (Belly and Claus, 1972). Anyway, all these studies are quite old (mainly in the 1950s).

In recent years, studies about nitrogen have been focused on the characterization of the consumption pattern of amino acids and ammonium during wine vinegar production and differences between acetification processes (Álvarez-Cáliz et al., 2012, 2014; Maestre et al., 2008; Valero et al., 2005). In addition, possible nitrogen limitations in the raw substrate that could compromise the process have been lately analyzed (Callejón et al., 2010). Wine due to the previous alcoholic fermentation (AF) carried out by yeast could be a poor medium for AAB development during vinegar production. However, the autolysis of yeast at the end of AF release amino acids and vitamins to the medium, which may be beneficial for the growth of AAB (Fleet, 2001). In the case of must grape,

high concentrations of arginine and proline are present in the AF beginning. During alcoholic fermentation, arginine is one of the principal nitrogen sources for yeast, while proline cannot be used in anaerobic conditions. For this reason, wine present high concentrations of proline, which seems to be a good nitrogen source for AAB, followed by leucine and ammonium ion. Anyway, this nitrogen pattern could change due to the raw substrates used for vinegar production (Valero et al., 2005). As expected after acetification, nitrogen content of vinegars is lower than in wines. In fact, in a study done by Maestre et al. (2008), a decrease in total nitrogen content (9.99 mM in wine *vs.* 6.58 mM in vinegar), as well as in nitrogen source diversity (14 amino acids detected in wine *vs.* 9 in vinegar) was observed. Furthermore, wines that has been subjected to biological aging contain lower available nitrogen than young wines (Álvarez-Cáliz et al., 2014), due to nitrogen consumption by flor yeast (Berlanga et al., 2004, 2006). However, the content present in these wines seems not to be a limiting factor for acetification. Moreover, the urea synthesized by the flor yeast during the aging process that could pose a problem is suppressed by the AAB during the acetification (Álvarez-Cáliz et al., 2014).

On the other hand, the pattern of consumption and synthesis of nitrogen sources by AAB was heavily dependent on the environmental conditions (Álvarez-Cáliz et al., 2012). These authors reported that AAB preferred to consume free amino acids rather than ammonium ion when unexpected changes occurred in the environment, while in favorable conditions, AAB used more ammonium ion because they probably synthetize amino acids that are partly stored as a reserve for future unfavorable conditions. Important differences in the consumption pattern of nitrogen sources were also observed when different vinegar production systems such as superficial and submerged methods were compared (Callejón et al., 2008). The consumption of amino acid was much lower in submerged that in surface acetifications, and for instance, proline, the most consumed amino acid in surface acetifications, was not used in submerged cultures. These differences could be explained for two main reasons: acetification time and AAB species involved in the processes.

1.4. AAB Isolation and growth

One of the biggest problems found in AAB is their isolation, lower percentage of recovery and maintenance in culture media, in particular in samples that were isolated from extreme media with high ethanol and/or acetic acid concentration, such as wine or vinegar (Entani et al., 1985; Gullo et al., 2006; Sievers et al., 1992). This low AAB recovery was initially associated with the absence of specific synthetic media for these microorganisms. Therefore, different media with diverse carbon sources were developed to try to solve this problem. In Table 2, different media used for the AAB isolation are shown. Among them, GY, GYC and YPM have been the most suitable media for the isolation of AAB from samples of musts, wines (Bartowsky et al., 2003; Du Toit and Lambrechts, 2002a; González et al., 2004) and diverse vinegars (Gullo et al., 2006; Prieto et al., 2007; Valera et al., 2011; Vegas et al., 2010). In the case of samples from vinegars with high concentration of acetic acid, the most suitable media are AE and RAE with a double layer agar (Entani et al., 1985; Sokollek and Hammes, 1997). However, the total AAB recovery in this media is still low when compared with the microscopy counting (Sokollek et al., 1998; Trček, 2005). These differences can be accounted for the formation of aggregates and because some AAB cells could enter into a viable but non-culturable state (VBNC) (Millet and Lonvaud-Funel, 2000), underestimating, therefore, the real population. To solve these problems, some culture-independent techniques have been developed for AAB detection and quantification, like epifluorescence (Baena-Ruano et al., 2006; Fernández-Pérez et al., 2010; Mesa et al., 2003), quantitative PCR (Jara et al., 2013; Torija et al., 2010; Vegas et al., 2013), fluorescence in situ hybridization FISH (Franke et al., 2000) and metagenomics or massive sequencing (Portillo and Mas., 2016; Valera et al., 2016).

Table 2. Most common media to isolate AAB

Media	Quantity	Media	Quantity
[a]GYC agar		**[b]GY Medium**	
D-glucose	5.0% (w/v)	D-glucose	2.0% (w/v)
Yeast extract	1.0% (w/v)	Yeast extract	1.0% (w/v)
Calcium Carbonate	0.5% (w/v)	Agar	2.0% (w/v)
Agar	2.0% (w/v)		
GYC Medium		**[d]AE Medium**	
D-glucose	10.0% (w/v)	D-glucose	0.5% (w/v)
Yeast extract	1.0% (w/v)	Yeast extract	0.3% (w/v)
Calcium Carbonate	2.0% (w/v)	Peptone	0.4% (w/v)
Agar	1.5% (w/v)	Agar	0.9% (w/v)
		Absolute ethanol	3 ml (v/v)
		Acetic acid	3 ml (v/v)
[c]YPM Medium		**[e]RAE Medium**	
Yeast extract	0.5% (w/v)	D-glucose	0.4% (w/v)
Peptone	0.3% (w/v)	Yeasy Extract	0.1% (w/v)
Mannitol	2.5% (w/v)	Peptone	0.1% (w/v)
Agar	1.2% (w/v)	Absolute ethanol	0-4% (w/v)
		Citric acid	0.015% (w/v)
		Na2HPO4	0.038% (w/v)
		Agar	0.5-1% (w/v)
V50			
Yeast extract	0.4% (w/v)		
Glycerol	0.2% (w/v)		
L-Tartaric acid	0.2% (w/v)		
K2HPO4	0.05% (w/v)		
MgSO4.7H2O	0.05% (w/v)		
Na acetate	0.1% (w/v)		
MnSO4	0.02% (w/v)		
CaCl	0.01% (w/v)		
Ethanol	6.0% (w/v)		
pH5			

[a]D-glucose yeast extract Calcium carbonate medium
[b]D-glucose yeast extract medium
[c]Yeast extract peptone mannitol medium
[d]Acetic acid ethanol medium
[e]Reinforced-AE medium

1.5. AAB Molecular Techniques

Both the identification at species level as well as the typing of AAB have been benefited by the development and progress of molecular techniques. During the last decades, diverse techniques have been tested for AAB analysis, showing interesting results for both taxonomy studies and biotechnology industry. The phenotypic properties were in

the past a mainstay of bacterial identification, however, nowadays they are complemented or replaced by molecular techniques like, sequence analysis or PCR-based methods. The latter are the most used in AAB identification, showing quickness, specificity, reliability and sensitivity. Obviously, these PCR-based methods have different levels of discrimination (genus, species or strain) (Trček and Barja 2015), being very important to choose the most suitable for each application.

1.5.1. Genotyping

Different culture-dependent techniques, such as RAPD-PCR, ERIC-PCR or REP-PCR, have been developed for AAB typing, allowing to differentiate among strains. Random amplified polymorphic DNA-PCR (RAPD-PCR) amplifies randomly the genomic DNA by using low hybridization temperatures and a short primer with arbitrary sequence, and strain-specific band pattern is obtained. This technique was applied to study AAB populations in different kinds of vinegars (Nanda et al., 2001; Trček et al., 1997). However, the most used techniques for AAB typing are ERIC-PCR, REP-PCR or $(GTG)_5$-rep-PCR. These methods are based on the amplification of repetitive sequences distributed along the genome, that are highly conserved and generate different size fragments, producing a unique pattern for each strain. This technique has been successfully used in diverse substrates such as wines (González et al., 2004), vinegars (Hidalgo et al., 2010b, 2012, 2013a, 2013b; Nanda et al., 2001; Vegas et al., 2010, 2013; Wu et al., 2010) and grapes (Mateo et al., 2014; Navarro et al., 2013; Valera et al., 2011).

1.5.2. Genera and species identification

Different culture-dependent and independent techniques have been developed for the identification at genera and species level of AAB. Among the dependent-culture methods, one of the most used is the analysis of 16S rDNA. Although the restriction analysis of the amplification of 16S rDNA is an effective tool for routine AAB species grouping, the subsequent sequencing of the gene is mandatory to have an accurate identification. However, this technique cannot differentiate between closely related

species, as for example between *A. cerevisiae* and *A. malorum* (Valera et al., 2011), due to the high homology between these species, up to 99.7% in this gene (Cleenwerck and De Vos, 2008). In these cases, the use of the 16S-23S rDNA internal transcribed spacer region (ITS) has been demonstrated to be efficient (González and Mas, 2011; Valera et al., 2011). Because of the importance of this technique for our work, it will be discussed with more detail in a separate section.

Two culture-independent techniques have been the most used for AAB species identification: DGEE and RT-PCR (Real Time PCR). The first one is commonly used to determine biodiversity in the samples, amplifying a fragment of the 16S rDNA that is separated according to its sequence not to its size. This technique has been applied to characterize microorganisms present in wines (Andorrà et al., 2008; Lopez et al., 2003), vinegars (De Vero et al., 2006; Gullo et al., 2009; Haruta et al., 2006) and vinegar biofilms (Valera et al., 2015a).

On the other hand, RT-PCR is a fast, accurate and sensitive tool for the detection and enumeration of microorganisms. In this case, it is possible to quantify only those microorganisms on which specific primers o probes have been designed. This technique has been applied in AAB in different studies. Some of them have quantified the total AAB in wines and vinegars using general primers for AAB and SYBR-Green as fluorescent reporter (Andorrá et al., 2008; González et al., 2006b; Torija et al., 2010). Other authors have designed specific TaqMan or TaqMan-MGB probes for the detection and quantification of different genera or species of AAB (Gammon et al., 2006; Torija et al., 2010; Valera et al., 2013) and some have applied these probes for species detection in vinegar samples (Jara et al., 2013; Vegas et al., 2013) and in vinegar biofilms (Valera et al., 2015a).

In the recent years, the development of next-generation sequencing system, such as pyrosequencing, has provided the possibility to have a more complete information about the whole complexity of the communities present in fermented products (Ercolini, 2013; Illeghems et al., 2012). However, few studies (Nie et al., 2013; Valera et al., 2015b) have

been yet done to study the microbiota present in vinegar (liquid or biofilm) using this technology.

1.5.2.1. Analisis of 16S–23S rRNA ITS region

The rRNA genetic loci of eubacterial consist of three genes: 16S, 23S and 5S that are separated by an internal transcribed spacer region (ITS). According to Barry et al. (1991), during the evolution, ITS regions suffered minimal selective pressure, which generated a greater sequence variation in these regions than in genes with functional roles. More than one copy of these regions are usually found in the genome and present some conserved regions coding for functional roles like tRNA genes and antitermination sequences. The ITS region are well known to be characterized by sequence and length variations (Barry et al., 1991; Dolzani et al., 1995), being an appropriate tool for differentiation at species (Barry et al., 1991; Jensen et al., 1993) or strain level (Dolzani et al., 1995; Kostman et al., 1992).

In AAB, different studies have used this region to identify AAB species (González et al., 2006a; González and Mas, 2011; Prieto et al., 2007; Ruiz et al., 2000; Sievers et al., 1996; Trček, 2005; Trček and Teuber, 2002). In most of them, the ITS was amplified and subsequently digested with several restriction enzymes (PCR-RFLP). Sievers et al. (1996) sequenced the 16S-23S rRNA ITS region of three strains belonging to *K. europaeus* and *K. xylinus* species (formely named *Acetobacter europaeus* and *A. xylinum*) and detected the sequences coding for $tRNA^{Ile}$ and $tRNA^{Ala}$, that were identical in all the strains. Moreover, they were able to determine that the genome of these strains contained four copies of the ITS and that all the copies were also identical. Ruiz et al. (2000) compared the results obtained by PCR-RFLP 16S-23S rRNA with the PCR-RFLP 16S rRNA to identify different AAB strains (reference strains and natural wine isolates). The use of the ITS region using diverse restriction enzymes showed the same degree of species differentiation as that of PCR-RFLP 16S rRNA when reference strains where analysed. However, the natural isolates showed different restriction patterns regarding

to the ones obtained with the reference strains, suggesting that this technique may be more adequate for detecting intraspecific differentiation than for identification at species level. However, subsequent studies have demonstrated its usefulness for species identification. Thus, Trček and Teuber. (2002) were able to make a database with 12 groups of 16S-23S ITS restriction profiles of AAB reference strains, that was successfully tested with isolates from wine vinegar (Trček and Teuber, 2002) and alcohol vinegar (Trček, 2005). Moreover, the use of ITS region has been often used to differentiate species closely related that were not able to be differentiate using the gene 16S rRNA. Thus, González et al. (2006a) proposed to combine both techniques (PCR-RFLP of 16S rRNA and 16S-23S rRNA ITS) using diverse restriction enzymes for an accurate identification of AAB species in a shorter time and in Prieto et al. (2007), the ITS region was very useful to correctly identify some isolates from Chilean healthy grapes badly classified using the PCR-RFLP 16S rRNA. Later, González and Mas. (2011) proposed to include the ITS region as a part of the polyphasic approach for AAB identification, since it was a good tool for the discrimination of AAB involved in food processes. Moreover, they also proposed this region as suitable for the design of primers and probes for AAB identification. In fact, probes designed in this region has allowed to discriminate between wild isolates of *A. cerevisiae* and *A. malorum*, that cannot be differentiated by 16S rRNA gene (Valera et al., 2013).

1.5.3. Genome sequencing

In 1977, Frederick Sanger and Alan R. Coulson published the methodology for the determination of DNA sequence (Sanger et al., 1977). The procedure was known as Sanger sequencing and was based on chain-termination method. On the same year, Maxam and Gilbert. (1977) developed another sequencing method that was based on chemical modification of DNA and subsequent cleavage at specific bases (Liu et al., 2012). The sequencing technology proposed by Sanger et al. (1977) showed significant improvement to the Maxam and Gilbert method, due to its higher efficiency and low use

radioactivity, making that Sanger sequencing was adopted as the only technology used for DNA sequencing during the following years. In 1995, the first two bacterial genome (*Haemophilus influenza* and *Mycoplasma genetalium*) were completely sequenced (Fleischmann et al., 1995; Fraser et al., 1995) and in 2001, using the Sanger sequencing technology together with the automatic sequencing instruments the completion of Human Genome Project was possible. This project encouraged technical improvements in sequencing methods to be faster, with more accuracy and cheaper. Moreover, it also stimulated the development of next-generation sequencing methods (NGS). These technologies are different from Sanger sequencing in some aspects such as the high throughput, massively sequencing reactions in parallel and reduced cost (Liu et al., 2012; Van Dijk et al., 2014). Important advances in bioinformatics tools for the data processing have been also necessary since the relatively short reads given by NGS made genome assembly more difficult (Morozovo et al., 2008; Van Dijk et al., 2014).

The vast reduction in the sequencing price has allowed to a great number of labs and research groups to consider sequencing as a basic routine tool for any biological study (Binnewies et al., 2006; Buermans and Den Dunnen, 2014; Van Djik et al., 2014). According to Land et al. (2015), the number of sequenced genomes continue increasing dramatically, and nowadays, more than 90.000 sequenced bacterial genomes are available in the National Center for Biotechnology Information (NCBI, 2016). This high amount of genomic data helps to understand different aspects such as bacterial diversity, population characteristics, mobile genetic elements, horizontal gene transfer (Binnewies et al., 2006), detection of pathogenic genes, and evolution studies (Liu et al., 2012). Furthermore, Ramasamy et al. (2014) suggested incorporating the genomic information in bacterial taxonomic studies for complementing phenotypic and chemotaxonomic parameters, in a new approach named “taxono-genomics”.

Until June 2016, 120 genomes of AAB are publicly available (NCBI, 2016). Of the 19 AAB genera, only 11 have genomes assembly, being *Acetobacter*, *Gluconobacter* and *Komagataeibacter*, those with the highest number of whole genome sequence

published. The genus *Acetobacter* has 51 published genomes of 18 different species, having some species more than one sequenced strain. *A. pasteurianus* is the species with the highest number of genome assemblies (15), followed by *A. malorum*, *A. tropicalis*, *A. aceti* and *A.cerevisiae* with 6, 5, 4 and 3, respectively. In the case of *Gluconobacter* and *Komagataeibacter*, there are published 31 and 6 genomes assemblies, respectively. A strain of *G. oxydans* species (strain 621H) was the first AAB to be completely sequenced by Prust et al. (2005), and nowadays, this species is the second one with more sequenced strains (12). During the years from 2005 to 2008, just one AAB was sequenced per year, while only in 2009, 8 strains of *A.pasteurianus* were sequenced. It is important to note, that till the date (NCIB, June 2016), this year, 40 new AAB genomes have been published, twice the published genomes in the last year. *Acetobacter* and *Gluconobacter* are the genera with the highest number of registered genomes in this year, with 18 sequences each.

2. FERMENTED PRODUCTS

2.1. Fermentation

Fermentation is a process where primary food products suffer biochemical modifications carried out by microorganisms and their enzymes. The fermentation process is the oldest form of food preservation in the world and is an inexpensive technology. There are records of fermentation of milk, meat and vegetables that are dated before 6000 BC. Fermentation is a handcraft process, in which, over the centuries, the methodologies and knowledge associated with the process have passed generation to generation in local communities, monasteries and feudal states, without knowing that those responsible for the process were microorganisms (Caplice and Fitzgerald, 1999). It was in the mid-19th century with the industrial revolution, urbanization and the development of modern science that the role of microorganisms in the process was uncovered, representing a significant impact on food fermentation. The production of food increased in size, and it

was necessary to industrialize the manufacturing process and expand foods to distant markets. Moreover, microorganisms were recognized as responsible for the fermentation process and then it was possible to characterize and isolate starter cultures to produce food on a large scale in industries. The use of selected starter cultures was a revolution in the food industry with an immediate repercussion in quality and safety. Then, fermentation was not only a process for preservation, but also the fermented products started to be important for their unique flavor, aroma and texture attributes that were more appreciated by consumers. Fermentation intensifies aroma, taste, texture, nutritional value (Nount and Mortarjemi, 1997) and other attractive properties as appearance, elimination of undesirable components and protection against food poisoning and development of pathogens (Farnworth, 2004). Thus, consumers perceive that fermented foods promote health and prevent nutritional diseases, mostly due to the relation of fermented foods with safety, digestibility and probiotic effects.

Fermented foods are consumed worldwide and wide range of substrates is used. Diverse substrates, including plant and animal origin, can be used. Furthermore, the composition of substrates, water and the microorganisms used have great impact on the final product. In the 21st century, one of the main problems of world population is the food waste, being meat, fruit, vegetables and bakery products, the main wasted foods (Melikoglu and Webb, 2013). Fermentation could be a solution to reduce food waste, using its potential to transform raw material in new products with added value and attractive for the consumer.

2.2. Vinegar

Historically, the appearance of vinegar goes back with the beginning of the agriculture and the discovery of the alcoholic fermentation from fruit, vegetables and cereals. The word vinegar comes from the latin *vinum acre* or *vinum acetum* (sour wine), referring to a kind of wine dominated by high volatile acidity. Thus, the word vinegar, since ancient times, has been always related with strong tasting, intense or acid. Vinegar has not been

very well considered as fermented food. However, it has been extensively used as a food condiment, preservative agent and in some places, vinegar is considered a healthy drink (Solieri and Giudici, 2009). During the Middle Ages and the Renaissance, the medical use of vinegar expanded and it was used as a digestive, prophylactic for liver problems, sore throats, anthelmintic and hair loose (Plessi, 2003). Moreover, vinegar is known for their anti-bacterial activity. Thus, some recent studies have linked the consumption of vinegar as drink with lower blood pressure, anti-oxidant activity, reduced diabetes effects, prevention of cardiovascular diseases and refreshment after exercise (Fushimi et al., 2002; Johnston et al., 2004; Kondo et al., 2001; Nishidai et al., 2000; Ogawa et al., 2000; Shau-mei Ou and Chang, 2009; Shimoji et al., 2002; Sugiyama et al., 2003;).

The definition for vinegar in Codex Alimentarius (1987) says that "*vinegar is a liquid, fit for human consumption, and produced from a suitable raw material of agricultural origin, containing starch, sugars or starch and sugar, by the process of double fermentation, first alcoholic and the acetous"*. Therefore, vinegar could be produced from any fermentable sugar by two fermentation steps; in the first one, carbohydrates are metabolized into ethanol by yeast and afterwards, AAB oxidize the ethanol into acetic acid in an aerobic process. Different kind of raw materials could be used for vinegar production, including rice, malt, grapes, apple, potatoes, and any other sugary food (Bamforth, 2005; Solieri and Giudici, 2009).

In general, vinegar is an inexpensive product, where low cost material is used, mostly surpluses raw substrates from seasonal agriculture, substandard and waste fruit or from processed food (Table 3). However, there are some expensive vinegars, such as Traditional Balsamic Vinegar or Sherry vinegar, that are produced according to established methods, in specific geographical regions and generally protected by "Apellation of origin" systems.

INTRODUCTION

Table 3. Overview of vinegars from around the world: raw materials, intermediate product, vinegar name and geographical distribution. (Solieri and Giudici, 2009).

Category	Raw material	Intermediate	Vinegar name	Geographical distribution
Vegetable [a]	Rice	Moromi	Komesu, kurosu (Japanese) Heicu (Chinese)	East and Southeast Asia
	Bamboo sap	Fermented bamboo sap	Bamboo vinegar [b]	Japan, Korea
	Malt	Beer	Malt vinegar	Northern Europe, USA
	Palm sap	Palm wine (Toddy, tari, tuack, tuba)	Palm vinegar, Toddy vinegar	Southeast Asia, Africa
	Barley	Beer	Beer vinegar	Germany, Austria, Netherlands
	Millet	Koji	Black vinegar	China, East Asia
	Wheat	Koji	Black vinegar	China, East Asia
	Sorghum	Koji	Black vinegar	China, East Asia
	Tea and sugar	Kombucha	Kombucha vinegar	Russia, Asia (China, Japan, Indonesia)
	Onion	Onion alcohol	Onion vinegar	East and Southeast Asia
	Tomato	-	Tomato vinegar	Japan, East Asia
	Sugarcane	Fermented sugar cane juice Basi	Cane vinegar Sukang iloko Kibizu	France, USA Philippines Japan
Fruit	Apple	Cider	Cider vinegar	USA, Canada
	Grape	Raisin Red or white wine Sherry wine Cooked must	Raisin (grape) vinegar Wine vinegar Sherry (Jerez) vinegar Balsamic vinegar	Turkey and Middle East Widespread Spain Italy
	Coconut	Fermented Coconut water	Coconut water vinegar	Philippines, Sri Lanka
	Date	Fermented date juice	Date vinegar	Middle East
	Mango	Fermented mango juice	Mango vinegar	East and Southeast Asia
	Red date	Fermented jujube juice	Jujube vinegar	China, East Asia
	Raspberry	Fermented raspberry juice	Raspberry vinegar	East and Southeast Asia
	Blackcurrant	Fermented blackcurrant juice	Blackcurrant vinegar	East and Southeast Asia
	Blackberry	Fermented blackberry juice	Blackberry vinegar	East and Southeast Asia
	Mulberry	Fermented mulberry juice	Mulberry vinegar	East and Southeast Asia
	Plum	Umeboshi [c] fermented juice	Ume-su	Japan
	Cranberry	Fermented cranberry juice	Cranberry vinegar	East and Southeast Asia
	Kaki	Fermented persimmon juice	Persimmon vinegar Kakisu	South Korea Japan
Animal	Whey	Fermented whey	Whey vinegar	Europe
	Honey	Diluted honey wine, tej	Honey vinegar	Europe, America, Africa

[a] Vegetable is not a botanical term and it used to refer to an edible plant part; some botanical fruits, such as tomatoes, are also generally considered to be vegetables.
[b] Obtained by bamboo sap fermentation.
[c] Umeboshi are pickled *ume* fruits. *Ume* is a species of fruit-bearing tree of the genus Prunus, which is often called a plum but is actually more closely related to the apricot

2.2.1. Fruits and fruit vinegars

Fruits and fruit products are vital for human diet and have played an important role in nutrition. Foods in general, and especially fruits, have different nutrients that are essential for growth, maintenance, body tissue repair and giving nourishment. These nutrients are divided in macronutrients (energy substrates required in large quantities), micronutrients (essential for humans in small quantities) and bioactive compounds (Sánchez-Moreno et al., 2012).

Fruits contain between 70% and 90% of water and carbohydrates are the main nutrients after water. The principal monosaccharides are D-glucose and D-fructose and two different types of fiber could be found, water-soluble fiber and insoluble fiber. Fruits are the best sources of vitamins (vitamin C, vitamin E, vitamin B1, B2, B3, B6); folate and diverse types of minerals (iron, calcium, phosphorus, magnesium, potassium, zinc, copper, selenium). The concentrations of all these macro and micronutrients vary according to the kind of fruit. Bioactive compounds or phytochemicals like carotenoids, flavonoids and phytosterols are present in low quantities. However, their consumption is important due to their ability to decrease the risk of diverse degenerative diseases such heart disease, cancer and aging.

Nowadays, people care more about the food health benefits and “natural” aspects, and consequently, the demand for high quality products is growing. Fermentation is the process that could give the consumer exceptional flavor, taste, bioactive compounds and other benefits in a 100% natural way (Hugenholtz, 2013). In some countries of Africa and especially in Asia, the demand of fruit vinegar products is growing due to their relation to health. Large variety of fruits could be used for the production of vinegar. It is possible to use second quality and waste fruit that can produce high quality juices (Monspart-Sényi, 2006). The advantage of this type of vinegars is that keep the subtle flavors and aroma of the raw material (Plessi, 2003). However, fruits with low concentration of sugar are more difficult to be used for these products. Thus, in the elaboration of some fruit

vinegars, fruit or fruit juice are added to alcohol or rice vinegars; so that vinegar acts as a solvent for the extraction of aromas and nutrients of the fruit, resulting in a final sweet-sour taste (Shao-meu Ou and Chang, 2009; Solieri and Giudici, 2009). In a study done by Chang et al. (2005), these authors analyzed 44 Taiwan local fruit vinegars and only nine samples were directly obtained from the fermentation of the fruit juice.

Several studies have analyzed fruit vinegars; however, they are mainly focused on their organoleptic characteristics and their quality parameters. Some examples involve rabbiteye blueberry (Min-Sheng and Po-Jung, 2010), blueberry (Hidalgo et al., 2013a), apple (Liu et al., 2008; Sakanaka and Ishihara, 2008), lemon, peach (Liu et al., 2008), persimmon (Hidalgo et al., 2010a; Sakanaka and Ishihara, 2008; Ubeda et al., 2011b), plum (Liu and He, 2009), and strawberry (Hidalgo et al., 2010a; Ubeda et al., 2011a; Ubeda et al., 2012;) vinegars.

2.3. Strawberry drink

As previously mentioned, one of the problems of fresh fruit and vegetables production is the large amount of waste, due to both the surplus of production and the second or third quality fruit that for its appearance is impossible to be sold in the market. A possible solution is the use of this low-quality fruit for the production of different products like beverages, fruit juices, jams purees, etc.. However, the market of these products is quite saturated, and therefore, it is not able to absorb all this surplus production, which implies that part of the harvest is left in the fields, creating serious ecological and economic problems. Thus, the waste takes not only the fruits, but also the agrochemicals and the labor used. Moreover, some fruits are quickly perishable. Some alternatives to these problems include the transformation by fermentations. For instance, the alcoholic fermentation of fruit sugars, obtaining as a final product, a fruit wine with variable alcohol concentration. Consumption of fruit wine is not extended around the world, however some apricot (Joshi et al 1990), apple (Joshi et al., 1991), banana (Akubor et al., 2003),

acerola (Santos et al., 2005), mango (Reddy and Reddy, 2005), gabiroba (Duarte et al., 2009) wines have been reported to be popular in some countries.

Other strategy could be to transform these fruit surplus by microbiological processes in new products with added value that are attractive and healthy for consumers. This was the main objective of two projects (AGL2007-66417-C02/ALI and AGL2010-22152-C03-02) developed by the Oenological Biotechnology group from URV in collaboration with other research groups. In the first one, the aim was to elaborate fruit vinegars while in the second one, the purpose was the elaboration of a strawberry drink by selective fermentation using yeasts and AAB. This drink would be the result of a mixture of two bioprocesses; being the first one a strawberry vinegar, produced by alcoholic fermentation and then the acetification of the strawberry puree. The second one would be the oxidation of the natural D-glucose of the strawberry into D-gluconic acid by AAB, without consuming D-fructose. This “gluconated strawberry juice” could have stability problems due to the presence of a fermentable sugar (D-fructose) and the low acidity. However, the addition of strawberry vinegar will solve this limitation. Furthermore, the low pH of the drink will help to prevent the growth of spoilage microorganisms. The new beverage will have a refreshing sour taste, but sweetened by the natural D-fructose of the fruit. Therefore, in this new non-alcoholic beverage the goal is the equilibrium between the acidity (which provides microbial stability), D-fructose sweetness (acid balance in taste) and the D-gluconic acid fixed acidty (which will intensify the taste).

3. HEALTH IMPACT OF THE STRAWBERRY DRINK

Several studies report the relation between rich diet in vegetables and fruits and lower occurrence of obesity, neurologic and cardiovascular diseases, cancer and infections (Etminan et al., 2004; Vauzour et al., 2010). The fruit consumption will increase in the population if the sensory characteristics of the products are attractive and include some health benefits. According to Prosinska and Bartles. (2007), when a new fruit product

appears in the market, the success will be due to the attitude of the consumer towards the product depending on the expected and real taste, quality and healthiness.

Many studies have reported the healthy and nutritional effects of strawberries. According to Giampieri et al. (2012a), the presence of high levels of vitamin C, folate and phenolic compounds, increases the nutritional quality of strawberries. Most of the bioactive compounds of strawberries are phenolic compounds that are known for their antioxidant and anti-inflammatory actions, anti-hypertensive, anti-allergic properties (Alvarez-Suarez, 2011), anti-cancer and antineurodegenerative effects (Tulipani et al., 2009). The principal class of phenolic compounds found in strawberries are flavonoids, especially anthocyanins, flavonols such as quercetin and kaempferol, and flavanols (Giampieri et al., 2012a, 2012b; Tulipani et al., 2009). Furthermore, strawberries contain a huge concentration of phenolic acids and ellagic acid/ ellagitannins, a recently characterized compound in food and only found in berries from the *Rosaceae* family (Giampieri et al., 2012a, 2012b).

There are no studies on the health benefits of strawberry vinegar or D-gluconic acid fermentation of strawberry, but it has been demonstrated the presence of different phenolic compounds and antioxidant activities in these products (Álvarez-Fernández et al., 2014; Hornedo-Ortega et al., 2016; Ubeda et al., 2012). In the previous project (AGL2007-66417-C02/ALI), strawberry vinegar was produced using different conditions and treatments and a decrease in the total phenols, total monomeric anthocyanins and anti-oxidant activity during the process was observed. However, when acetification was done in wood barrels, an improvement in all the parameters was detected. It seems that the use of wood barrels and especially cherry barrels, is beneficial for a rich concentration of phenols and the increase of the antioxidant activity in strawberry vinegars. Álvarez-Fernández et al (2014) identified 44 non-anthocyanin phenolic compounds in D-gluconic fermentation of strawberry and most of these compounds increased during the process.

An important feature of the strawberry drink is to maintain the natural sweetness of the D-fructose of the fruit. The main sources of D-fructose are fruits, some vegetables, honey, and table sugar (sucrose). However, in the early 1970, a major source of D-fructose was developed: a high fructose corn syrup (HFCS), which was produced enzymatically by converting starch in a mixture of D-fructose and D-glucose. Two variants of HFCS exist: HFCS-55 (50% D-fructose + 50% D-glucose) and HFCS-42 with 42% of D-fructose (Johnson and Muray, 2010). Nowadays, the major consumption of D-fructose is through the intake of products that contain HFCS, like sweetens drinks, soft drinks, juice fruits and pre-packaged food (Basciano et al., 2005; Bray and Popkin, 2013). Forty years ago, D-fructose was considered better sweetener for diabetes patients, because it had no influence on D-glucose plasma level, being used in patients with limited insulin production (Basciano et al., 2005; Tappy et al., 2010). However, several studies related the consumption of D-fructose with weight gain, type 2 diabetes mellitus, non-alcoholic fatty liver disease, increase of triglyceride levels, hypertension, metabolic syndrome, insulin resistant and D-glucose intolerance (Basciano et al., 2005; Cruz et al., 2007; Gaby, 2005; Johnson and Muray, 2010; Montonen et al., 2007; Tappy et al., 2010). However, it has to be emphasized that these aspects were related with the consumption of high quantities of HFCS. Thus, most investigations focused on the high consumption of D-fructose by humans (Faeh et al., 2005; Ka et al., 2009; Montonem et al., 2007; Pérez-Pozo et al., 2010), due the greater intake of sweetened beverage in the last years, which has contributed to increase obesity or metabolic disorders. Moderate consumption of D-fructose does not cause harmful health effects (Cruz et al., 2007; Gaby, 2005). Those deleterious effects are related to the amounts consumed as well as the individual tolerance, age, body fat mass and genetic background (Bray and Popkin, 2014; Tappy and Lê, 2015). Bray and Popkin. (2014), highlight the large amount of studies related with D-fructose but that none of them compares the consumption of D-fructose with that of the other sugars.

The product that we propose has only the natural D-fructose of the strawberry, without any further addition. The presence of sugar in the proposed strawberry drink is much lower than the content in any soft drink and that of natural or industrial fruit juices. However, the moderate consumption of natural fruit juices is considered a healthy option (Sartorelli, et al., 2009), and, thus, this beverage contains the same healthy components as well as low sugar content. Vasdev et al. (2002), demonstrated that rats fed with D-fructose and vitamin C did not develop metabolic syndrome. Moreover, strawberries contain high concentrations of vitamin C, minimizing the possible adverse effects caused by D-fructose (Johnson and Murray, 2010).

REFERENCES

Adachi, O., Tayama, K., Shinagawa, E., Matsushita, K., Ameyama, M., 1980. Purification and characterization of membrane-bound aldehyde dehydrogense from *Gluconobacter suboxydans*. Agric. Biol. Chem. 44, 503-515.

Adachi, O., Fuji, Y., Ano, Y., Moonmangmee, D., Toyama, H., Shinagawa, E., Theeragool, G., Lotong, N., Matsushita, K., 2001. Membrane-bound sugar alcohol dehydrogenase in acetic acid bacteria catalyzes L-ribulose formation and NAD-dependent ribitol dehydrogenase is independent of the oxidative fermentation. Biosci. Biotechnol. Biochem. 65, 115-125.

Adachi, O., Ano, Y., Toyama, H., Matsushita., 2007. Biooxidation with PQQ- and FAD-Dependent Dehydrogenases. In: Rolf D. Schmid and Vlada B. Urlacher (Eds.), Modern Biooxidation. Enzymes, Reactions and Applications. Weinheim: Wiley-vch, Verlag GmbH & Co. KGaA.

Akubor, P.I., Obio, S.O., Nwadomere, K.A., Obiomah, E., 2003. Production and quality evaluation of banana wine. Plant Foods Hum. Nutr. 58, 1–6.

Alonso, S., Rendueles, M., Díaz, M., 2015. Microbial production of specialty organic acids from renewable and waste materials. Crit. Rev. Biotechnol. 4, 497-513.

Álvarez-Cáliz, C., Santos-Dueñas, I.M., Cañete-Rodriguez, A.M., García-Martínez, T., Mauricio, J.C., García-García, I., 2012. Free amino acids, urea and ammonium ion contents for submerged wine vinegar production: influence of loading and air-flow rate. Acetic Acid Bacteria. 1, 1-5.

Álvarez-Fernández, M.A., Hornedo-Ortega, R., Cerezo, A.B., Tronoco, A.M., García-Parrilla, M.C., 2014. Non-anthocyanin phenolic compounds and antioxidant activity of beverages obtained by gluconic fermentation of strawberry. Innov. Food Sci. Emerg.Technol. 26, 469-481.

Alvarez-Suarez, J.M., Dekansi, D., Ristic, S., Nevena, V., Radonjic, V., Petronijevic, N.D., …Battino, M., 2011. Strawberry polyphenols attenuate etanol-induced gastric lesions in rats by activation of antioxidant enzymes and attenuation of MDA increase. PLoS One. 6, e25878.

Ameyama, M., Shinagawa, E., Matsushita, K., Adachi, O., 1981. D-Glucose Dehydrogenase of *Gluconobacter suboxydans*: Solubilization, Purification and Characterization. Agr. Biol. Chem. 45, 851-861.

Andorrà, I., Landi, S., Mas, A., Guillamón, J.M., Esteve-Zarzoso, B., 2008. Effect of oenological practices on microbial populations using culture-independent techniques. Food Microbiol. 25, 849–856.

Andrés-Barrao, C., Benaglib, C., Chappuisa, M., Pereza, R.O., Tonollab, M., Barjaa, F., 2013. Rapid identification of acetic acid bacteria using MALDI-TOF mass spectrometry fingerprinting. Syst. Appl. Microbiol. 36, 75-81.

Asai, T., 1935. Taxonomic studies on acetic acid bacteria and allied oxidative bacteria isolated from fruits: a new classification of the oxidative bacteria. J. Agric. Chem. Soc. Japan. 11, 674–708.

Asai, T. 1968. Acetic acid bacteria: classification and biochemical activities. Tokyo: University of Tokyo Press.

Asano, T., Yuasa, K., Kunugita, K., Teraji, T., Mitsuoka, T., 1994. Effects of Gluconic Acid on Human Faecal Bacteria. Microb. Ecol. Health D. 7, 247-256.

Asano, T., K. Yuasa., Y. Yoshimura., S. Takenawa., H. Fukuba., 1997. Digestion, absorption and intestinal residue of various gluconic acids in rats. Journal of Japan Society of Nutrition and Food Science. 50, 287–294.

Baena-Ruano, S., Jimenez-Ot, C., Santos-Dueñas, I.M., Cantero-Moreno, D., Barja, F., Garcia-Garcia, I., 2006. Rapid method for total, viable and non-viable acetic acid bacteria determinationduring acetification process. Process Biochem. 41, 1160-1164.

Bamforth, C.W., 2005. Vinegar In: Bamforth, C.W. (Ed.), Food, fermentation and micro-organisms. Blachwell Science, Kundli, pp. 154-159.

Barja, F., Mesa, M. M., Macías, M., Bermudez, I., Cantero, D., Lopez, J. 2003. Aspectos bioquímicos, moleculares y morfológicos de las bacterias acéticas. In: Mas, A., Guillamón, J. M. (Eds.), Primeras jornadas en I+D+i en la elaboración de vinagre de vino. Tarragona, Spain: Servei de publicacions, pp. 17-20.

Barry, T., Colleran, G., Glennon, M., Dunican, L.K., Gannon, F., 1991. The 16s/23s Ribosomal Spacer Region as a Target for DNA Probes to Identify Eubacteria. PCR Methods Appl. 1, 149.

Bartowsky, E. J., Xia, D., Gibson, R. L., Fleet, R. L., Henschke, P. A., 2003. Spoilage of bottled red wine by acetic acid bacteria. Lett. Appl. Microbiol. 36, 307-314.

Basciano, H., Federico, L., Adeli K. 2005. Fructose, insulin resistance, and metabolic dyslipidemia. Nutr.Metab. 2, 1-14.

Beijerinck, M.W., 1898. Über die Arten der Essigbakterien. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene. Zweite Abteilung 4, 209–216.

Belly, R.T., Clauss, G.W., 1972. Effect of amino acids on the growth of *Acetobacter suboxydans*. Arch. Microbiol. 83, 237-245.

Berlanga, T.M., Peinado, R., Millán, C., Mauricio, J.C., Ortega, J.M., 2004. Influence of blending on the content of different compounds in the biological aginf of sherry dry wines. J. Agr. Food Chem. 52, 2577-2581.

Berlanga, T.M., Millán, C., Mauricio, J.C., Ortega, J.M., 2006. Influence of nitrogen on the biological aging of sherry wine. J. Sci. Food Agr. 86, 2113-2118.

Beschkov, V., Velizarov, S., Peevva, L. 1995., Some kinetic aspects and modelling of biotransformation of D-glucose to Keto-D-Gluconate. Bioprocess Biosyst. Eng. 6, 301-305.

Binnewies, T.T., Motro, Y., Hallin, P.F., Lund, O., Dunn, D., La, T., Hampson, D.J., Bellgard, M., Wassenaar, T.M., Ussery, D.W., 2006. Ten years of bacterial genome sequencing: comparative-genomic-based discoveries. Funct. Integr. Genomics, 6, 165-185.

Bray, G.A., Popkin, B.M., 2013. Calorie-sweetened beverages and fructose: what have we learned 10 years later. Pediatr. Obes. 8, 242-248.

Bray, G.A., Popkin, B.M., 2014. Dietary sugar and body weight: have we reached a crisis in the epidemic of obesity and diabetes? Health be damned! Pour on the sugar. Diabetes Care. 37, 950-955.

Bremus, C., Herrmann, U., Bringer-Meyer, S., Sahm, H., 2006. The use of microorganisms in L-ascorbic acid production. J. Biotechnol. 124, 196-205.

Buermans, H.P.J., Den Dunnen, J.T., 2014. Next generation sequencing technology: Advances and applications. Biochim. Biophy. Acta. 1842, 1932-1941.

Buse, R., Onken, U., Qazi, G.N., Sharma, N., Parshad, R., Verma, V., 1992. Influence of dilution rate and dissolved oxygen concentration on continuous keto acid production *by Gluconobacter oxydans* subsp. melanogenum. Enzyme Microb. Tech. 12, 1001-1006.

Callejón, R.M., Tesfaye, W., Torija, M.J., Mas, A., Troncoso, A.M., Morales, M.L., 2008. HPLC determination of amino acids with AQC derivatization in vinegars along submerged and surface acetifications and its relation to the microbiota. Eur. Food Res. Technol. 227, 93-102.

Callejón R.M., Troncoso, A.M., Morales, M.L., 2010. Determination of amino acids in grape-derived products: A review. Talanta. 81, 1143-1152.

Caplice, E., Fitzgerald, G.F., 1999. Food fermentations: role of microorganisms in food production and preservation. Int. J. Food Microbiol. 50, 131-149.

Chang, R.C., Lee, H.C., Ou, A.S.M., 2005. Investigation of the physicochemical properties of concentrated vinegar. J. Food Drug. Anal. 13, 348-356.

Cleenwerck, I., De Vos, P., 2008. Polyphasic taxonomy of acetic acid bacteria: an overview of the currently applied methodology. Int. J. Food Microbiol. 125, 2–14.

Codex Alimentarius., 1987. Codex Regional Standard for vinegar. Codex Standard 162. Geneva: FAO/OMS.

Crotti, E., Rizzi, A., Chouaia, B., Ricci, I., Favia, G., Alma, A., Sacchi, L., Bourtzis, K., Mandrioli, M., Cherif, A., Bandi, C., Daffonchio, D., 2010. Acetic acid bacteria, newly emerging symbionts of insects. Appl. Environ. Microb. 76, 6963–6970.

Cruz, E.P., Zúñiga, A.E.S., Mier, G.M., 2007. Efectos benefices y deltéreos del consumo de fructosa. Revista de Endocrinologia y Nutricion. 15, 67-74.

De Ley, J., Gillis, M., Swings, J., 1984. Family VI. *Acetobacteraceae*. Bergey's Manual of Systematic Bacteriology 1, 267–278.

De Vero, L., Gala, E., Gullo, M., Solieri, L., Landi, S., Giudici, P., 2006. Application of denaturing gradient gel electrophoresis (DGGE) analysis to evaluate acetic acid bacteria in traditional balsamic vinegar. Food Microbiol. 23, 809–813.

Deppenmeier, U., Hoffmeister, M., Prust, C., 2002. Biochemistry and biotechnological applications of Gluconobacter strains. Appl. Microbiol. Biotechnol. 60, 233-242.

Dolzani, L., Tonin, E., Lagatolla, C., Prandin, L, Monti-Bragadin, C., 1995. Identification of Acinetobacter Isolates in the *A.calcoaceticus – A.baumannii* Complex by Restriction Analysis of the 16S-23S rRNA Intergenic Spacer Sequences. J. Clin. Microbiol. 33, 1108-1113.

Du Toit, W. J., Lamberchts, M. G., 2002a. The enumeration and identification of acetic acid bacteria from South African red wine fermentations. Int. J. Food Microbiol. 74, 57-64.

Du Toit, W.J., Pretorius, I.S., 2002b. The occurrence, control and esoteric effect of acetic acid bacteria in winemaking. Ann. Microbiol. 52,155–179.

Duarte, W.F., Dias, D.R., De Melo Pereira, G.V., Gervásio, I.M., Schwan, R.F., 2009. Indigenous and inoculated yeast fermentation of gabiroba (*Campomanesia pubescens*) pulp for fruit wine production. J. Ind. Microbiol. Biotechnol. 36, 557–569.

Elfari, M., Ha, S.W., Bremus, C., Merfort, M., Khodaverdi, V., Herrmann, U., Sahm, H., Görisch, H., 2005. A *Gluconobacter oxydans* mutant converting glucose almost quantitatively to 5-keto-D-gluconic acid. Appl. Microbiol. Biotechnol. 66, 668-674.

Entani, E., Ohmori, S., Masai, H., Suzuki, K. I., 1985. *Acetobacter polyoxogenes* sp. nov., a new species of an acetic acid bacterium useful for producing vinegar with high acidity. J. Gen. Appl. Microbiol. 31, 475-490.

Ercolini, D., 2013. High-Throughput Sequencing and Metagenomics: Moving Forward in the Culture Independent Analysis of Food Microbial Ecology. Appl. Environ. Microbiol. 79, 3138-3155.

Etminan, M., Takkouche, B., Caamano-Isorna, F., 2004. The role of tomato products ans lycopene in the prevention of prostate cáncer: a meta-analysis of observational studies. Cancer Epidemiol. Biomarkers Prev. 13, 340-345.

Faeh, D., Minehira, K., Schwarz, J., Periasami, R., Seongus, P., Tappy, L., 2005. Effect of fructose overfeeding and fish oil administration on hepatic de novo lipo génesis and insulin sensitivity in healthy meals. Diabetes. 54, 1907-1913.

FAOSTAT. Food and Agriculture Organization of the United Nations Statistics Division. 2012 http://faostat.fao.org

Farnworth, E.R., 2004. The Beneficial Health Effects of Fermented Foods–Potential Probiotics Around the World. J. Nutraceut. Function. Med. Foods. 4, 93-117.

Fernández-Pérez, R., Torres, C., Sanz, S., Ruiz-Larrea, F., 2010. Strain typing of acetic acid bacteria responsible for vinegar production by the submerged elaboration method. Food Microbiol. 27, 973–978.

Fleet, G.H., 2001. Wine. In: Doyle, M.P., Beuchat, L.R., Montville, T.J (Eds.), Food Microbiology Fundamentals and Frontiers, ASM Press, Washington, DC, pp. 267-288.

Fleischmann, R.D., Adams, M.D., White, O., Clayton, R.A., Kirkness, E.F., Kerlavage, A.R., Bult, C.J., Tomb, J.F., Dougherty, B.A., Merrick, J.M., McKenney, K., Sutton, G., FitzHugh W., Fields, C., Gocyne, J.D., Scott, J., Shirley, R., Liu, L.I., Glodek, A., Kelley, J.M., Weidman, J.F., Phillips, C.A., Spriggs, T., Hedblom, E., Cotton, M.D., Utterback, T.R., Hanna, M.C., Nguyen, D.T., Saudek, D.M., Brandon, R.C., Fine, L.D., Fritchman, J.L., Fuhrmann, J.L., Geoghagen, N.S.M., Gnehm, C.L., McDonald, L.A., Small, K.V., Fraser, C.M., Smith, H.O., Venter, J. C., 1995. Whole genome random sequencing and assembly of Haemophilus influenzae Rd. Science. 269, 496–498.

Franke, I.H., Fegan, M., Hayward, C., Leonard, G., Sly, L.I., 2000. Molecular detection of Gluconacetobacter sacchari associated with the pink sugarcane mealybug *Saccharicoccus sacchari* (Cockerell) and the sugarcane leaf sheath microenvironment by FISH and PCR. FEMS Microbiol. Ecol. 31, 61–71.

Fraser, C.M., Gocayne, J.D., White, O., Adams, M.D., Clayton, R.A., Fleischmann, R.D., Bult, C.J., Kerlavage, A.R., Sutton, G., Kelley, J.M., Fritchman, R.D., Weidman, J.F., Small, K.V., Sandusky, M., Fuhrmann, J., Nguyen, D., Utterback, T.R., Saudek, D.M., Phillips, C.A., Merrick, J.M., Tomb, J.F., Dougherty, B.A., Bott, K.F., Hu, P.C., Lucier, T.S., Peterson, S.N., Smith, H.O., Hutchison, C.A., Venter, J.C., 1995. The minimal gene complement of *Mycoplasma genitalium*. Science, 270, 397–403.

Frateur, J., 1950. Essai sur la systématique des Acetobacters. Cellule. 53, 287–392.

Fuentes-Ramírez, L.E., Bustillos-Cristales, R., Tapia-Hernández, A., Jiménez-Salgado, T., Wang, E.T., Martínez-Romero, E., Caballero-Mellado, J., 2001. Novel nitrogen-fixing acetic acid bacteria, *Gluconacetobacter johannae* sp. nov. and *Gluconacetobacter azotocaptans* sp. nov., associated with coffee plants. Int. J. Syst. Evol. Microbiol. 51, 1305–1314.

Fushimi, T., Tayama, K., Fukaya, M., Kitakoshi, K., Nakai, N., Tsukamoto, Y., Sato, Y., 2002. The efficacy of acetic acid for glycogen repletion in liver and skeletal muscle of rats. J. Nutr. 131, 1973-1977.

Gaby, A.R., 2005. Advance effects of dietary fructose. Altern. Med. Rev. 10, 294-306.

Gammon, K.S., Livens, S., Pawlowsky, K., Rawling, S.J., Chandra, S., Middleton, A.M., 2006. Development of real-time PCR methods for the rapid detection of low concentrations of *Gluconobacter* and *Gluconacetobacter* species in a electrolyte replacement drink. Lett. Appl. Microbiol. 44, 262–267.

Giampieri, F., Alvarez-Suarez, J.M., Mazzoni, L., Romandini, S., Bompadre, S., Diamanti, J., Capocasa, F., Mezzeti, B., Quiles, J.L., Ferreiro, M.S., Tulipani, S., Battino, M., 2012a. The potential impact of strawberry on human health. Nat. Prod. Res. 1-8.

Giampieri, F., Tulipani, S., Alvarez-Suarez, J.M., Quiles, J.L., Mezzetti, B., Battino, M., 2012b. The strawberry: compostion, nutritional quality, and impacto n human health. Nutrition. 28, 9-19.

González, A., Hierro, N., Poblet, M., Rozes, N., Mas, A., Guillamon, J. M., 2004. Application of molecular methods for the differentiation of acetic acid bacteria in a red wine fermentation. J. Appl. Microbiol. 96, 853-860.

González, A., Guillamón, J. M., Mas, A., Poblet, M., 2006a. Application of molecular methods for routine identification of acetic acid bacteria. Int. J. Food Microbiol., 108: 141-146.

González, A., Hierro, N., Poblet, M., Mas, A., Guillamón, J.M., 2006b. Enumeration and detection of acetic acid bacteria by real-time PCR and nested PCR. FEMS Microbiol. Lett. 254, 123–128.

González, A., Mas, A., 2011. Differentiation of acetic acid bacteria based on sequence analysis of 16S-23S rRNA gene internal transcribed spacer sequences. Int. J. Food Microbiol. 147, 217–222.

Greenberg, D.E., Porcella, S.F., Stock, F., Wong, A., Conville, P.S., Murray, P.R., Holland, S.M., Zelazny, A.M., 2006.*Granulibacter bethesdensis* gen. nov., sp. nov., a distinctive pathogenic acetic acid bacterium in the family *Acetobacteraceae*. Int. J. Syst. Evol.Microbiol. 56, 2609–2616.

Gullo, M., Caggia, C., De Vero, L., Giudici, P., 2006. Characterization of acetic acid bacteria in "traditional balsamic vinegar". Int. J. Food Microbiol., 106, 209-212.

Gullo, M., De Vero, L., Giudici, P., 2009. Succession of selected strains of *Acetobacter pasteurianus* and other acetic acid bacteria in traditional balsamic vinegar. Appl. Environ. Microbiol. 75, 2585–2589.

Gullo, M., Mamlouk, D., De Vero, L., Giudici, P., 2012. Acetobacter pasteurianus strain AB0220: cultivability and phenotypic stability over 9 years of preservation. Curr. Microbiol. 64, 576–580.

Gupta, A., Singh, V.K., Qazi, G.N., Kumar, A., 2001. *Gluconobacter oxydans*: It´s biotechnological applications. J. Mol. Microbiol. Biotechnol. 3, 445-456.

Haruta, S., Ueno, S., Egawa, I., Hashiguchi, K., Fujii, A., Nagano, M., Ishii, M., Igarashi, Y., 2006. Succession of bacterial and fungal communities during a traditional pot fermentation of rice vinegar assessed by PCR-mediated denaturing gradient gel electrophoresis. Int. J. Food Microbiol. 109, 79–87.

Herrmann, U., Merfort, M., Jeude, M., Bringer-Meyer, S., Sahm, H., 2004. Biotransformation of glucose to 5-keto-D-gluconic acid by recombinant *Gluconobacter oxydans* DSM 2343. Appl. Microbiol. Biotechnol. 64, 86–90.

Hidalgo, C., Mateo, E., Cerezo, A.B., Torija, M.J., Mas, A., 2010a. Technological process for production of persimmon and strawberry vinegars. International Journal of Wine Research. 2, 55-61.

Hidalgo, C., Vegas, C., Mateo, E., Tesfaye, W., Cerezo, A.B., Callejón, R.M., Poblet, M., Guillamón, J.M., Mas, A., Torija, M.J., 2010b. Effect of barrel design and the inoculation of *Acetobacter pasteurianus* in wine vinegar production. Int. J. Food Microbiol. 141, 56–62.

Hidalgo, C., Mateo, E., Mas, a, Torija, M.J., 2012. Identification of yeast and acetic acid bacteria isolated from the fermentation and acetification of persimmon (*Diospyros kaki*). Food Microbiol. 30, 98–104.

Hidalgo, C., García, D., Romero, J., Mas, A, Torija, M.J., Mateo, E., 2013a. *Acetobacter* strains isolated during the acetification of blueberry (*Vaccinium corymbosum* L.) wine. Lett. Appl. Microbiol. 57, 227–232.

Hidalgo, C., Torija, M.J., Mas, A, Mateo, E., 2013b. Effect of inoculation on strawberry fermentation and acetification processes using native strains of yeast and acetic acid bacteria. Food Microbiol. 34, 88–94.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T., 1994. Bergey's Manual of Determinative Bacteriology, Bergey's Manual of Determinative Bacteriology. Williams & Wilkins, Baltimore, Maryland.

Hornedo-Ortega, R., Álvarez-Fernández, A., Cerezo, A.B., Troncoso, A.M., García-Parrilla, M.C., 2016. Influence of storage conditions on the anthocyanin profile and color of an innovative beverage elaborated by gluconic fermentation of strawberry. J. Funct. Food. 23, 198-209.

Hugenholtz, J., 2013. Traditional biotechnology for new foods and beverages. Curr. Opin. Biotech. 24, 155-159.

Illeghems, K., De Vuyst, L., Papalexandratou, Z., Weckx, S., 2012. Phylogenetic Analysis of a Spontaneous Cocoa Bean Fermentation Metagenome Reveals New Insights into Its Bacterial and Fungal Community Diversity. Plos Onne, 7, e38040.

Jara, C., Mateo, E., Guillamón, J.M., Mas, A., Torija, M.J., 2013. Analysis of acetic acid bacteria by different culture-independent techniques in a controlled superficial acetification. Ann. Microbiol. 63, 393–398.

Jensen, M.A., Webster, J.A., Straus, N., 1993. Rapid Identification of Bacteria on the Basis of Polymerase Chain Reaction- Amplified Ribosomal DNA Spacer Polymorphisms. Appl. Environ. Microbiol., 59, 945-952.

Johnson, R., Murray, R., 2010. Fructose, Excercise, and health. Curr. Sports Med. Rep. 9, 253-258.

Johnston, C.S., Kim, C.M., Buller, A.J., 2004. Vinegar improves insulin sensitivity to a high-carbohydrate meal in subjects with insulin resistance or type 2 diabetes. Diabetes Care. 27, 281-282.

Jojima, Y., Mihara, Y., Suzuki, S., Yokozeki, K., Yamanaka, S., Fudou, R., 2004. *Saccharibacter floricola* gen. nov., sp. nov., a novel osmophilic acetic acid bacterium isolated from pollen. Int. J. Syst. Evol. Microbiol. 54, 2263-2267.

Joshi, V.K., Bhutani, V.P., Sharma, R.C., 1990. Effect of dilution and addition of nitrogen sourcenin chemical, mineral and sensory qualities of wild apricot wine. Am. J. Enol. Vitic. 41, 229-231.

Joshi, V.K., Sandhu, D.K., Attri, B.L., Walla, R.K., 1991. Cider preparation from apple juice concentrate and its consumer acceptability. Indian J. Hort. 148-321.

Joyeux, A., Lafon-Lafourcade, S., Ribéreau-Gayon, P., 1984. Evolution of acetic acid bacteria during fermentation and storage of wine. Appl. Environ. Microbiol. 48, 153-156.

Ka, L., Ith, M., Kreis, R., Faeh, D., Bortolotti, M., Tran, C., Boesch, C., Tappy, L., 2009. Fructose overconsumption causes dyslipidemia and ectopic lipid deposition in healthy subjects with and without a family history type 2 diabetes. Am. J. Clin. Nutr. 89, 1760-1765.

Klasen, R., Bringer Meyer, S., Sahm, H., 1991. Incapability of *Gluconobacter oxydans* to produce tartaric acid. Biotechnol. Bioeng. 40, 183-186.

Klasen, R., Bringer-Meyer, S., Sahm, H., 1995. Biochemical characterization and sequence analysis of the gluconate-NADP-5-oxidoreductase gene from *Gluconobacter oxydans*. J. Bacteriol. 177, 2637-2643.

Kondo, S., Tayama, K., Tsukamoto, Y, Ikeda, K., Yamori, Y., 2001. Antihypertensive effects of acetic acid and vinegar spontaneous hypertensive rats. Biosci. Biotechnol. Biochem. 65, 2690-2694.

Kostman, J. R., T. D. Edlind, J. J. LiPuma, and T. L. Stull., 1992. Molecular epidemiology of *Pseudomonas cepacia* determined by polymerase chain reaction ribotyping. J. Clin. Microbiol. 30, 2084–2087.

Krajewski, V., Simic, P., Mouncey, N.J., Bringer, S., Sahm, H., Bott, M., 2010. Metabolic Engineering of Gluconobacter oxydans for Improved Growth Rate and Growth Yield on Glucose by Elimination of Gluconate Formation. Appl. Environ. Microbiol. 76, 4369-4376.

Land, M., Hauser, L., Jun, S.R., Nookaew, I., Leuze, M.R., Ahn, T.H., Karpinets, T., Lund, O., Kora, G., Wassenaar, T., Pouder, S., Ussery, D.W., 2015. Insights from 20 years of bacterial genome sequencing. Funct. Integr. Genomics. 15, 141-161.

Li, L., Prat, J., Borremans, W., Nunes, O.C., Manaia, C.M., Cleenwerck, I., Meeus, I., Smagghe, G., De Vuyst, L., Vandamme, P., 2015. *Bombella intestini* gen. Nov., sp. nov., acetic acid bacterium isolated from bumble bee crop. Int. J. Syst. Evol. Microbiol. 65, 267-273.

Lisdiyanti, P., Kawasaki, H., Widyastuti, Y., Saono, S., Seki, T., Yamada, Y., Uchimura, T., Komagata, K., 2002. *Kozakia baliensis* gen. nov., sp. nov., a novel acetic acid bacterium in the α-Proteobacteria. Int. J. Syst. Evol. Microbiol. 52, 813-818.

Liu, F., He, Y., Wang, L., 2008. Determination of effective wavelengths for discrimination of fruit vinegars using near infrared spectroscopy and multivariate analysis. Anal. Chim. Acta. 615, 10-17.

Liu, F., He, Y., 2009. Application of successive projections algorithm for variable selection todetermine organic acids of plum vinegar. Food Chem. 115,1430-1436.

Liu, D., Zhang, W., 2010. Some progress in Organic Synthesis of Pharmaceutical in China. In: Shioiri, T., Izawa, K., Konoike, T. (Eds.), Pharmaceutical Process Chemistry, Whiley-VCH. Wheinheim, Germany.

Liu, L., Li, Y., Li, S., Hu, N., He, Y., Pong, R., Lin, D., Lu, L., Law, M., 2012. Comparion of next-generation sequencinf systems. J. Biomed. Biotechnol. 2012, 251-364.

Loganathan, P., Nair, S., 2004. *Swaminathania salitolerans* gen. nov., sp. nov., a salt-tolerant, nitrogen-fixing and phosphate solubilizing bacterium from wild rice (Porteresia coarctata Tateoka). Int. J. Syst. Evol. Microbiol. 54, 1185–1190.

Lopez, I., Ruiz-Larrea, F., Cocolin, L., Orr, E., Philster, T., Marshall, M., Vander- Gheynst, J., Mills, D., 2003. Design and evaluation of PCR primers for analysis of bacterial populations in wine by denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. 69, 6801–6807.

Macauley, S., McNeil, B., Harvey, L.M., 2001. The genus *Gluconobacter* and its applications in Biotechnology. Crit. Rev. Biotechnol. 1, 1-25.

Maestre, O., Santos-Dueñas, I.M., Peinado, R., Jiménez-Ot, C., García-García, I., Mauricio, J.C., 2008. Changes in amino acid composition during wine vinegar production in a fully automatic pilot acetator. Process Biochem. 8, 803-807.

Malimas, T., Chaipitakchonlatarn, W., Thi Lan Vu, H., Yukphan, P., Muramatsu, Y., Tanasupawat, S., Potacharoen, W., Nakagawa, T., Tanticharoen, M., Yamada, Y., 2013. *Swingsia samuiensis* gen. nov., sp. nov., an osmotolerant acetic acid bacteriumin the α-Proteobacteria. J. Gen. Appl. Microbiol. 59, 375-384.

Mamlouk, D., Gullo, M., 2013. Acetic Acid Bacteria: Physiology and Carbon Sources Oxidation. Indian J. Microbiol. 53, 377-384.

Mateo, E., Torija, M.J., Mas, A., Bartowsky, E.J., 2014. Acetic acid bacteria isolated from grapes of South Australian vineyards. Int. J. Food Microbiol. 178, 98-106.

Matsushita, K., Toyama, H., Adachi, O., 1994. Respiratory chains and bioenergetics of acetic acid bacteria. In: Rose, A.H., Tempest, D.W. (Eds.), Advances in Microbial Physiology, Academic Press Ltd, London, pp. 247-301.

Matsushita, K., Fujii, Y., Ano, Y., Toyama, H., Shinjoh M., Tomiyama, N., Miyazaki, T., Sugisawa, T., Hoshino, T., Adachi, O., 2003. 5-Keto-D-Gluconate production is catalyzed by a quinoprotein glycerol dehydrogenase, major polyol dehydrogenase, in *Gluconobacter* species. Appl. Environ. Microbiol., 69, 1959-1966.

Mattey, M., 1992. The production of organic acids. Crit. Rev. Biotechnol. 12, 87-132.

Matzerath, I., Kläui, W., Sahm, H., 1995. Vanadate catalysed oxidation of 5-keto-D-gluconic acid to tartaric acid: the unexpected effect of phosphate and carbonate on rate and selectivity. Inorg. Chim. Acta. 1, 237-205.

Maxam, A.M., Gilbert, W., 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. 74, 560-564.

Melikoglu, M., Webb, C., 2013. Use of Waste Bread to Produce Fermentation Products, In: Food Industry Wastes, Kosseva, M.R., Webb, C. (Eds.), Elsevier, United States of America, pp. 63-76.

Mesa, M.M., Macías, M., Cantero, D., Barja, F., 2003. Use of the direct epifluorescence filter technique for the enumeration of viable and total acetic acid bacteria from vinegar fermentation. J. Fluoresc. 13, 261-265.

Millet, V., Lonvaud-Funel, A., 2000. The viable but non-culturable state of wine microorganisms during storage. Lett. Appl. Microbiol. 30, 126-141.

Min-Sheng, S., Po-Jung, C., 2010. Aroma impact components of rabbiteye blueberry (*Vaccinium ashei*) vinegars. Food Chem. 119, 923-928.

Monspart-Sényi, J., 2006. Fruit Processing Waste Management. In: Hui, Y. H. (Eds.), Handbook of Fruits and Fruit Processing, Blackwell Publishing, Iowa, pp 205-215.

Montonen, J., Jarvinem, R., Knekt, P., Heliovaara, M., Reunanen, A., 2007. Consumption of sweetened beverages and intakes of fructose and glucose predict type 2 diabetes occurence. J. Nutr. 137, 1447-1453.

Morozovo, O., Marra, M., 2008. Applications of next-generation sequencing technologies in functional genomics. Genomics. 92, 255-265.

Muynck, C., Pereira, C.S.S., Naessens, M., Parmentier, S., Soetaert, W., Vandamme, E.J., 2007. The genus *Gluconobacter oxydans*: comprehensive overview of biochemistry and biotechnological applications. Crit. Rev. Biotechnol. 27, 147-171.

Nanda, K., Taniguchi, M., Ujike, S., Ishihara, N., Mori, H., Ono, H., Murooka, Y., 2001. Characterization of acetic acid bacteria in traditional acetic acid fermentation of rice vinegar (komesu) and unpolished rice vinegar (kurosu) produced in Japan. Appl. Environ. Microbiol. 67, 986-990.

Navarro, D., Mateo, E., Torija, M.J., Mas, A., 2013. Acetic acid bacteria in grape must. Acetic Acid Bact. 2, 19-23.

NCBI, National Center for Bioechnology Information., 2016, http://www.ncbi.nlm.nih.gov/genome/browse/

Ndoye, B., Lebecque, S., Dubois-Dauphin, R., Tounkara, L., Guiro, A.T., Kere, C., Diawara, B., Thonart, P., 2006. Thermoresistant properties of acetic acids bacteria isolated from tropical products of Sub-Saharan Africa and destined to industrial vinegar. Enzyme Microb. Technol. 39, 916-923.

Nie, Z., Zheng, Y., Wang, M., Han, Y., Wang, Y., Luo, J., Niu, D., 2013. Exploring mi- crobial succession and diversity during solid-state fermentation of Tianjin duliu mature vinegar. Bioresour. Technol. 148, 325-333.

Nielsen, D.S., Teniola, O.D., Ban-Koffi, L., Owusu, M., Andersson, T.S., Holzapfel, W.H., 2007. The microbiology of Ghanaian cocoa fermentations analysed using culture-dependent and culture-independent methods. Int. J. Food Microbiol. 114, 168–186.

Nishidai, S., Nakamura, Y., Torikai, K., Yamamoto, M., Ishihara, N., Mori, H., Ohigashi, H., 2000. Kurosu, a traditional vinegar produced from unpolished rice, supresses lipid peroxidation in vitro and in mouse skin. Biosci. Biotechnol. Biochem. 64, 1909-1914.

Nount, M. J. R., Mortarjemi, Y., 1997. Assessment of fermentation as a household technology for improving food safety. A joint FAO/WHO workshop. Food Control. 8, 221-226.

Ogawa, N., Satsu, H., Watanabe, H., Fukaya, M., Tsukamato, Y., Miyamoto, Y., Shimizu, Y., 2000. Acetic acid suppresses the increase in desaccharidase activity that occurs during culture of Caco-2 cells. J. Nutr. 130, 507-513.

Olijve, W., Kok, J.J., 1979. Analysis of growth of *Gluconobacter oxydans* in glucose containing media. Arch. Microbiol. 121, 283-290.

Ordóñez. J., Sainz, F., Callejón, R.M., Troncoso, A.M., Torija, M.J., García-Parrilla, M.C., 2015. Impact of gluconic fermentation of strawberry using acetic acid bacteria on amino acids and biogenic amines profile. Food Chem. 178, 221-228.

Park, J.K., Jung, J.Y., Park, Y.H., 2003. Cellulose production by *Gluconacetobacter hansenii* in a medium containing ethanol. Biotechnol. Lett. 25, 2055–2059.

Pérez-Pozo, S.E., Schold, J., Nakagawa, T., Sánchez-Lozada L.G., Johnson, R.J., Lillo, J.L., 2010. Excessive fructose intake induces the features of metabolic síndrome in healthy adult men: role of uric acid in the hypertensive response. Int. J. Obes. 34, 454-461.

Plessi, M., 2003. Vinegar. In: Caballero, B., Trugo, L.C., and Finglas, P.M. (Eds.), Encyclopedia of food, science and nutririon, Academic Press, Oxford.

Portillo, M.C., Mas, A., 2016. Analysis of microbial diversity and dynamics during wine fermentation of Grenache grape variety by high-throughput barcoding sequencing. LWT Food. Sci. Technol. 72, 317-321.

Prieto, C., Jara, C., Mas, A., Romero, J., 2007. Application of molecular methods for analyzing the distribution and diversity of acetic acid bacteria in Chilean vineyards. Int. J. Food Microbiol. 115, 348-355.

Pronk, J.T., Levering, P.R., Olijve, W., Vandiken, J.P., 1989. Role of NADP-dependent and quinoprotein glucose dehydrogenases in gluconic acid production by *Gluconobacter oxydans*. Enzyme Microb. Technol. 3, 160-164.

Prosinska, M., Bartles, J., 2007. Theorical framework on consumer innovativeness for fruit. Warsaw Agricultural University Research Report, Wageningen, Netherlands.

Prust, C., Hoffmeister, M., Liesegang, H., Wiezer, A., Fricke, W.F., Ehrenreich, A., Gottschalk, G., Deppenmeier, U., 2005. Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydans*. Nat. Biotechnol. 23,195-200.

Qazi, G.N., Parshad, R., Verma, V., Chopra, C.L., 1991. Diketo-gluconate fermentation by *Gluconobacter oxydans*. Enzyme Microb. Technol. 6, 504-507.

Ramachandran, S., Fontanille, P., Pandei, A., Larroche, C., 2006. Gluconic acid: properties, applications and microbial production. Food Technol. Biotech. 2, 185-195.

Ramasamy, D., Mishra, A.K, Lagier, J.C., Padhmanabhan, R., Rossi, M., Sentausa, E., Raoult, D., Fournier, P.E., 2014. A polyphasic strategy incorporating genomic data for the taconomic description of novel bacterial species. Int. J. Syst. Evol. Micr. 64, 384-391.

Ramírez-Bahena, M.H., Tejedor, C., Martín, I., Velázquez, E., Peix, A., 2013. *Endobacter medicaginis* gen. nov., sp. nov., isolated from alfalfa nodules in an acidic soil. Int. J. Syst. Evol. Microbiol. 63, 1760-1765.

Raspor, P., Goranovic, D., 2008. Biotechnological applications of acetic acid bacteria. Crit. Rev. Biotechnol. 28, 101-124.

Reddy, L.V.A., Reddy, O.V.S., 2005. Production and characterization of wine from mango fruit (*Mangifera indica* L.). World J. Microbiol. Biotechnol. 21, 1345-1350.

Rogers, P., Chen, J.S., Zidwick, M.J., 2013. Organic acid and solvent production: acetic, lactic, gluconic, succinic and polyhydroxyalkanoic acids. In: Rosenber, E., F. De Long, E., Lory, S., Stackebrandt, E., Thompson, F (Eds.), The prokaryotes. Applied bacteriology and biotechnology. Springer Berlin Heidelberg, pp. 3-75.

Roh, S.W., Nam, Y.D., Chang, H.W., Kim, K.H., Kim, M.S., Ryu, J.H., Kim, S.H., Lee, W.J., Bae, J.W., 2008.Phylogenetic characterization of two novel commensal bacteria involved with innate immune homeostasis in Drosophila melanogaster. Appl. Environ. Microbiol. 74, 6171-6177.

Ruiz, A, Poblet, M., Mas, A., Guillamón, J.M., 2000. Identification of acetic acid bacteria by RFLP of PCR-amplified 16S rDNA and 16S-23S rDNA intergenic spacer. Int. J. Syst. Evol. Micr. 50, 1981-1987.

Saeki, A., Theeragool, G., Matsushita, K., Toyama, H., Lotong, N., Adachi, O., 1997. Development of thermotolerant acetic acid bacteria useful for vinegar fermentation at higher temperatures. Biosci. Biotechnol. Biochem. 61, 138-145.

Sakanaka, S., Ishihara, Y., 2008. Comparison of antioxidant properties of persimmon vinegar and some other commercial vinegars in radical-scavenging assays and on lipid oxidation in tuna homogenates. Food Chem. 107, 739-744.

Sánchez-Moreno, C., De Pascual-Teresa, S., De Ancos, B., Cano, M.P., 2012. Nutritional quality of fruits. In: Nirmal, K.H., Jiwan, S.S., József, B., James, S.B. W and Cano. M.P (Eds.), Handbook of fruits and fruit processing. John Wiley & Sons, Ldt.

Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. 74, 5463-5467.

Santos, S.C., Almeida, S.S., Toledo, A.L., Santana, J.C.C., De Souza, R.R., 2005. Making and sensorial analysis of Malpighia Punicifolia L. wine. Braz. J. Food Technol. 10, 47-50.

Sartorelli, D.S., Franco, L.J., Gimeo, S.G.A., Ferreira, S.R.G., Cardoso, M.A., 2009. Dietary fructose, fruits, fruit juices and glucose tolerance status in Japanese-Brazilians. Nutr. Metab. Cardiovasc. Dis. 19, 77-83.

Shau-mei Ou, A., Chang, R.C., 2009. Taiwan fruit vinegar. In: Solieri, L., Giudici, P (Eds.), Vinegars of the world. Springer, Milan, Italy, pp. 223-242.

Shimoji, Y., Tamua, Y., Nakamura, Y., Nanda, K., Nishidai, S., Nishikawa, Y., Ishihara, N., Uenakai, K., Ohigashi, H., 2002. Isolation and identification of DPPH radical scavenging compunds in Kurosu (Japanese unpolished rice vinegar). J. Agri. Food Chem. 50, 6501-6503.

Shinagawa, E., Matsushita, K., Toyama, H., Adachi, O., 1999. Production of 5-keto-D-gluconate by acetic acid bacteria is catalyzed by pyrroloquinoline quinone (PQQ)-dependent membrane-bound D-gluconate dehydrogenase. J. Mol. Catal. B: Enzym. 6, 341-350.

Sievers, M., Sellmer, S., Teuber, M., 1992. Acetobacter europaeus sp-nov a main component of industrial vinegar fermenters in Central-Europe. Syst. Appl. Microbiol. 15, 386-392.

Sievers, M., Ludwig, W., Teuber, M., 1994a. Phylogenetic positioning of *Acetobacter*, *Gluconobacter*, *Rhodopila* and *Acidiphilium* species as a branch of acidophilic bacteria in the α-subclass of Proteobacteria based on 16S ribosomal DNA sequences. Syst. Appl. Microbiol. 17, 189-196.

Sievers, M., Ludwig, W., Teuber, M., 1994b. Revival of the species *Acetobacter methanolicus* (ex Uhlig et al., 1986) nom. rev. Syst. Appl. Microbiol. 17, 352-354.

Sievers, M., Lorenzo, A., Gianotti, S., Boesch, C., Teuber, M., 1996. 16-23S ribosomal RNA spacer región of *Acetobacter europaeus* and *A.xylinum*, tRNA genes and antitermination sequences. FEMS Microbiol. Lett. 142, 43-48.

Silberbach, M., Maier, B., Zimmermann, M., Buchs, J., 2003. Glucose oxidation by *Gluconobacter oxydans*: characterization in shaking-flasks, scale-up and optimization of the pH profile. Appl. Microbiol. Biotechnol. 1, 92-98.

Sokollek, S.J., Hammes, W.P., 1997. Description of a starter culture preparation for vinegar Fermentation. Syst. Appl. Microbiol. 20, 481-491.

Sokollek, S.J., Hertel, C., Hammes, W.P., 1998. Description of *Acetobacter oboediens* sp. nov. and *Acetobacter pomorum* sp. nov., two new species isolated from industrial vinegar fermentation. Int. J. Syst. Bacteriol.48, 935-940.

Solieri, L., Giudici, P., 2009. Vinegars of the World. In: Solieri, L., Giudici, P. (Eds.), Vinegars of the world. Springer, Milan, Italy, pp. 1-14.

Sonoyama, T., Tani, H., Matsuda, K., Kageyama, B., Tanimoto, M., Kobayashi, K., Yagi, S., Kyotani, H., Matsushita, K., 1982. Production of 2-kerto-L-gluconic acid from D-glucose by two stages fermentation. Appl. Environ. Microbiol. 5, 1064-1069.

Sugiyama, A. Saitoh, M., Takahara, A., Satoh, Y., Hashimoto, K., 2003. Acute cardivascular effects of a new beverage made of wine vinegar and grape juice, assessed using in vivo rat. Nutr. Res. 23, 1291-1296.

Tappy, L., Lê, K.A., Tran, C., Paquot, N., 2010. Fructose and metabolic disease: new findings, new questions. Nutrition. 26, 1044-1049.

Tappy, Luc., Lê, K.A., 2015. Health effects of fructose and fructose- containing caloric sweeteners: where do we stand 10 years after the initial whistle blowings?. Curr. Diab. Rep. 15, 1-12.

Thi Lan Vu, H., Yukphan, P., Chaipitakchonlatarn, W., Malimas, T., Muramatsu, Y., Thi Tu Bui, U., Tanasupawat, S., Cong Duong, K., Nakagawa, Y., Thanh Pham, H., Yamada, Y., 2013. *Nguyenibacter vanlangensis* gen. nov., sp. nov., an unusual acetic acid bacterium in the α-Proteobacteria. J. Gen. Appl. Microbiol. 59, 153-166.

Torija, M.J., Mateo, E., Guillamón, J.M., Mas, A., 2010. Identification and quantification of acetic acid bacteria in wine and vinegar by TaqMan-MGB probes. Food Microbiol. 27, 257-265.

Toyama, H., Furuya, N., Saichana, I., Ano, Y., Adachi, O., Matsushita, K., 2007. Membrane-Bound, 2-Keto-D-Gluconate-Yielding D-Gluconate Dehydrogenase from "*Gluconobacter dioxyacetonicus*" IFO 3271: Molecular Properties and Gene Disruption. Appl. Environ. Microbiol. 73, 6551-6556.

Trček, J., Ramus, J., Raspor, P., 1997. Phenotypic characterization and RAPD-PCR profiling of Acetobacteri sp. isolated from spirit vinegar production. Food Technol. Biotech. 35, 63-67.

Trček, J., Teuber, M., 2002. Generic resctriction analysis of the 16S-23S rDNA internal transcribed spacer región of the acetic acid bacrteria. FEMS Microbiol. Lett. 208, 69-75.

Trček, J., 2005. Quick identification of acetic acid bacteria based on nucleotide sequence of the 16S-23S rDNA internal transcribed spacer región and of the PQQ-dependent alcohol dehydrogenase gene. Syst. Appl. Microbiol. 28, 735-745.

Trček, J., Jernejc, K., Matsushita, K., 2007. The highly tolerant acetic acid bacterium *Gluconacetobacter europaeus* adapts to the presence of acetic acid by changes in lipid composition, morphological properties and PQQ-dependent ADH expression. Extremophiles. 11, 627-635.

Trček, J., Barja, F., 2015. Uptades on quick identification of acetic acid bacteria with focus on the 16S-23S rRNA gene internal transcribed spacer and the analysis of cell protein by MALDI-TOF mass spectrometry. Int. J. Food Microbiol. 196, 137-144.

Tsukahara, T., Koyama, H., Okada, M., Ushida, K., 2002. Stimulation of butyrate production by gluconic acid in batch culture of pig cecal digesta and identification of butyrate-producing bacteria. J. Nutr. 132, 2229-2234.

Tulipani, S., Mezzetti., B., Battino, M., 2009. Impact of strawberry on human health: insight into matginally discussed bioactive compounds for the mediterranean diet. Public Health Nutri. 12, 1656-1662.

Ubeda, C., Callejón, R. M., Hidalgo, C., Torija, M. J., Mas, A., Troncoso, A. M., Morales, M. L., 2011a. Determination of major volatile compounds during the production of fruit vinegars by static gas chromatography–mass spectrometry method. Food Res. Int. 44, 259-268.

Ubeda, C., Hidalgo, C., Torija, M. J., Mas, A., Troncoso, A. M., Morales, M. L., 2011b. Evaluation of antioxidant activity and total phenols index in persimmon vinegars produced by different processes. Food Sci. Technol. 44, 1591-1596.

Ubeda, C., Callejón, R. M., Hidalgo, C., Torija, M. J., Troncoso, A. M., Morales, M. L., 2012. Employment of different processes for the production of strawberry vinegars: Effects on antioxidant activity, total phenols and monomeric anthocyanins. LWT- Food. Sci. Technol. 52, 139-145.

Urakami, T., Tamaoka, J., Suzuki, K.I., Komagata, K., 1989. *Acidomonas* gen. nov., incorporating *Acetobacter methanolicus* as *Acidomonas methanolica* comb. nov. Int. J. Syst. Bacteriol. 39, 50–55.

Valera, M. J., Laich, F., González, S. S., Torija, M. J., Mateo, E., Mas, A., 2011. Diversity of acetic acid bacteria present in healthy grapes from the Canary Islands. Int. J. Food Microbiol. 151, 105-112.

Valera, M.J., Torija, M.J., Mas, A., Mateo, E., 2013. *Acetobacter malorum* and *Acetobacter cerevisiae* identification and quantification by Real-Time PCR with TaqMan-MGB probes. Food Microbiol. 36, 30-39.

Valera, M.J., Torija, M.J., Mas, A., Mateo, E., 2015a. Acetic acid bacteria from biofilm of strawberry vinegar visualized by microscopy and detected by complementing culture-dependent and culture-independent techniques. Food Microbiol. 46, 452-462.

Valera, M.J., Poehlein, A., Torija, M.J., Haack, F.S., Rolf Daniel, R., Streit, W.R., Mateo, E., Mas, A. 2015b. Draft Genome Sequence of *Komagataeibacter europaeus* CECT 8546, Cellulose-Producing Strain of Vinegar Elaborated by the Traditional Method. Genome Announcements, 3, e01231-15.

Valera, M.J., Mas, A., Streit, W.R., Mateo, E., 2016. GqqA, a novel protein in *Komagataeibacter europaeus* involved in bacterial quorum quenching and cellulose formation. Micro. Cell Fact. 15:88. doi: 10.1186/s12934-016-0482-y.

Valero, E., Berlanga, M.T., Roldán, P.M., Jiménez, C., García, I., Mauricio, J.C., 2005. Free amino acids and volatile compounds in vinegars obtain from different types of substrate. J. Sci. Food Agr. 85, 603-608.

Van Dijk, E.L., Auger, H., Jaszczyszyn, Y., Thermes, C., 2014. Ten years of next-generation sequencinf technology. Trends in Genetis. 30, 418-426.

Vasdev, S., Gill, V., Parai, S., Longerich, L., Gadag, V., 2002. Dietary vitamin E and C supplementation prevents fructose induced hypertension in rats. Mol. Cell Biochem. 241, 107-114.

Vauzour, D., Vafeiadou, K., Pendeiro, C., Corona, G., Spenser, J.P.E., 2010. The inhibitory effects of Berry-derivated flavonoids against neurodegenerative processes. J. Berry. Res. 1, 45-52.

Vegas, C., Mateo, E., González, A., Jara, C., Guillamón, J.M., Poblet, M., Torija, M.J., Mas, A., 2010. Population dynamics of acetic acid bacteria during traditional wine vinegar production. Int. J. Food Microbiol. 138, 130-136.

Vegas, C., González, Á., Mateo, E., Mas, A., Poblet, M., Torija, M.J., 2013. Evaluation of representativity of the acetic acid bacteria species identified by culture-dependent method during a traditional wine vinegar production. Food Res. Int. 51, 404-411.

Velizarov, S., Beschkov, V., 1998. Biotransformation of glucose to free gluconic acid by *Gluconobacter oxydans*: substrate and product inhibition situations. Process Biochem. 5, 527-534.

Weenk, G., Olijve, W., Harder, W., 1984. Ketogluconate formation by *Gluconobacter* species. Appl. Microbiol. Biotechnol. 20,400-405.

Wieme, A.D., Spitaels, F., Aerts, M., De Bruyne, K., Van Landschoot, A., Vandamme, P., 2014. Effects of Growth Medium on Matrix-Assisted Laser Desorption–Ionization Time of Flight Mass Spectra: a Case Study of Acetic Acid Bacteria. Appl. Environ. Microbiol. 80, 1528-1538.

Wu, J.J, Gullo, M., Chen, F.S., Giudici., P., 2010. Diversity of *Acetobacter pasteurianus* strains isolated from solid-state fermentation of cereal vinegars. Curr. Microbiol. 60, 280-286.

Yamada, k., Kotera, U., Obata, T., Takahasi, N., 1971. Microbial formation of tartaric acid from glucose. J. Ferment. Technol. 49, 85-92.

Yamada Y, Kondo K., 1985. *Gluconoacetobacter*, a new subgenus comprising the acetate-oxidizing acetic acid bacteria with ubiquinone-10 in the genus *Acetobacter*. J. Gen. Appl. Microbiol. 30, 297-303.

Yamada, Y., Hoshino, K., Ishikawa, T., 1997a. Taxonomic studies of acetic acid bacteria and Allied Organisms. Part XI. The Phylogeny of Acetic Acid Bacteria Based on the Partial Sequences of 16S Ribosomal RNA: The Elevation of the Subgenus *Gluconoacetobacter* to the Generic Level. Biosci. Biotechnol. Biochem. 61, 1244-1251.

Yamada, Y., Hoshino, K., Ishikawa, T., 1997b. The phylogeny of acetic acid bacteria based onthe partial sequences of 16S ribosomal RNA: the elevation of the subgenus *Gluconoacetobacter* to the generic level. Biosci. Biotechnol. Biochem. 61, 1244-1251.

Yamada, Y., Katsura, K., Kawasaki, H., Widyastuti, Y., Saono, S., Seki, T., Uchimura, T., Komagata, K., 2000. Asaia bogorensis gen. nov., sp. nov., an unusual acetic acid bacte-rium in the α-Proteobacteria. Int. J. Syst. Evol. Microbiol. 50, 823-829.

Yamada, Y., Yukphan, P., 2008. Genera and species in acetic acid bacteria. Int. J. Food Microbiol. 125, 15-24.

Yamada, Y., Yukphan, P., Vu, H.T.L., Muramatsu, Y., Ochaikul, D., Nakagawa, Y., 2012a. Subdivision of the genus *Gluconacetobacter* Yamada, Hoshino and Ishikawa 1998: the proposal of *Komagatabacter* gen. nov., for strains accomodated to the *Gluconacetobacter xylinus* group in the α-Proteobacteria. Ann. Microbiol. 62, 849– 859.

Yamada, Y., Yukphan, P., Vu, H.T.L., Muramatsu, Y., Ochaikul, D., Tanasupawat, S., Nakagawa, Y., 2012b. Description of *Komagataeibacter* gen. nov., with proposals of new combinations (*Acetobacteraceae*). J. Gen. Appl. Microbiol. 58, 397-404.

Yukphan, P., Malimas, T., Potacharoen, W., Tanasupawat, S., Tanticharoen, M., Yamada, Y., 2005. *Neoasaia chiangmaiensis* gen. nov., sp. nov., a novel osmotolerant acetic acid bacterium in the α-Proteobacteria. J. Gen. Appl. Microbiol. 51, 301-311.

Yukphan, P., Malimas, T., Muramatsu, Y., Takahashi, M., Kaneyasu, M., Tanasupawat, S., Nakagawa, Y., Suzuki, K., Potacharoen, W., Yamada, Y., 2008. *Tanticharoenia sakaeratensisgen*. nov., sp. nov., a new osmotolerant acetic acid bacterium in theα-Proteobacteria. Biosci. Biotechnol. Biochem. 72, 672-676.

Yukphan, P., Malimas, T., Muramatsu, Y., Takahashi, M., Kaneyasu, M., Potacharoen, W., Tanasupawat, S., Nakagawa, Y., Hamana, K., Tahara, Y., Suzuki, K., Tanticharoen, M., Yamada, Y., 2009. *Ameyamaea chiangmaiensis* gen. nov., sp. nov., an acetic acid bacterium in the α-Proteobacteria. Biosci. Biotechnol. Biochem. 73, 2156-2162.

Yukphan, P., Malimas, T., Muramatsu, Y., Potacharoen, W., Tanasupawat, S., Nakagawa, Y., Tanticharoen, M., Yamada, Y., 2011. *Neokomagataea* gen. nov., with descriptions of *Neokomagataea thailandica* sp.nov.and *Neokomagataea tanensis* sp. nov., osmotolerant acetic acid bacteria of the α-Proteobacteria. Biosci. Biotechnol. Biochem. 75, 419-426.

UNIVERSITAT ROVIRA I VIRGILI
SELECTION AND OPTIMIZATION OF ACETIC ACID BACTERIA FOR D-GLUCONIC ACID PRODUCTION
Florencia Sainz Perez

CHAPTER 1

Comparison of D-gluconic acid production in selected strains of acetic acid bacteria

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International Journal of Food Microbiology 222, 40-47 (2016)

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ABSTRACT

The oxidative metabolism of acetic acid bacteria (AAB) can be exploited for the production of several compounds, including D-gluconic acid. The production of D-gluconic acid in fermented beverages could be useful for the development of new products without D-glucose. In the present study, we analyzed nineteen strains belonging to eight different species of AAB to select those that could produce D-gluconic acid from D-glucose without consuming D-fructose. We tested their performance in three different media and analyzed the changes in the levels of D-glucose, D-fructose, D-gluconic acid and the derived gluconates. D-glucose and D-fructose consumption and D-gluconic acid production were heavily dependent on the strain and the media. The most suitable strains for our purpose were *Gluconobacter japonicus* CECT 8443 and *Gluconobacter oxydans* Po5. The strawberry isolate *Acetobacter malorum* (CECT 7749) also produced D-gluconic acid; however, it further oxidized D-gluconic acid to keto-D-gluconates.

Keywords: D-glucose, Strawberry, Keto-D-gluconic acids, *Acetobacter, Gluconobacter*

1. INTRODUCTION

Acetic acid bacteria (AAB) belong to the *Acetobacteraceae* family and are now classified into nineteen genera: *Acetobacter, Gluconobacter, Gluconacetobacter, Komagataeibacter, Asaia, Neokomagataea, Granulibacter, Kozakia, Neoasaia, Swaminathania, Saccharibacter, Acidomonas, Tanticharoenia*, *Ameyamaea, Nguyenibacter, Swingsia, Commensalibacter*, *Endobacter* and *Bombella* (Lan Vu et al., 2013; Malimas et al., 2013; Trček and Barja, 2015). AAB are strictly aerobic, gram-negative, rod-shaped bacteria (Deppenmeier et al., 2002; Sievers and Swings, 2005) that are widespread in nature, occurring in sugary and/or alcoholic niches, such as fruits, flowers or alcoholic beverages (wine, cider, beer, sake and soft drinks) (Trček and Teuber, 2002). From a biotechnological point of view, AAB are very interesting due to their ability to perform oxidative fermentation through incomplete oxidation of alcohols or sugars (such as D-glucose, glycerol, D-sorbitol, ethanol), resulting in a near-quantitative excretion of the corresponding oxidation products in the culture medium (Adachi et al., 2003; Deppenmeier et al., 2002; Matsushita et al., 2003). Some examples of this metabolism are the production of acetic acid from ethanol or D-gluconic acid from D-glucose (Deppenmeier et al., 2002; Lino et al., 2012; Prust et al., 2005).

D-gluconic acid is a non-corrosive, non-volatile, non-toxic, mild organic acid and a natural constituent of fruits, plants, wine and honey that provides a refreshing sour taste. It is listed as a generally permitted food additive (E574) by the EFSA, and it is listed as a GRAS (Generally Recognized As Safe) additive by the US FDA (Ramachandran et al., 2006). Although, D-gluconic acid can be produced chemically or by enzymatic processes using purified enzymes, mainly from *Aspergillus niger*, the most common method for its synthesis is oxidative fermentation (Papagianni, 2011). In the case of AAB, D-gluconate can be synthesized by two different pathways. In the first pathway, D-glucose is directly oxidized in the periplasmic space by membrane-bound pyrroquinoline quinone (PQQ)-dependent glucose dehydrogenase (GDH) (Matsushita et al., 1994; Merfort et al., 2006; Olijve and Kok, 1979; Silberbach et al., 2003). In this pathway, D-glucose is oxidized to

glucono-δ-lactone and then to D-gluconate. This acid could be further oxidized to 2-keto-D-gluconate (2-KGA) and 2,5-di-keto-D-gluconate by a flavin-dependent gluconate-2-dehydrogenase (GA2DH) and 2-keto-D-gluconate dehydrogenase (2KGDH), respectively (Matsushita et al., 1994). Additionally, D-gluconate could also be oxidized to 5-keto-D-gluconic acid (5-KGA) by a membrane-bound PQQ-dependent gluconate-5-dehydrogenase (GA5DH) (Shinagawa et al., 1999). The second pathway is located in the cytoplasm; therefore, D-glucose is first taken into the cells and then converted to D-gluconate by a soluble $NADP^+$-dependent glucose dehydrogenase, which is further dissimilated in the pentose phosphate cycle (Herrmann et al., 2004; Muynck et al., 2007). In the cytoplasm, this D-gluconate can also be oxidized to 2-KGA or 5-KGA by 2-keto-D-gluconate reductase (2KGR) and 5-keto-D-gluconate reductase (5KGR), respectively. 2-KGA can also be reduced back to D-gluconate by the cytosolic 2KGR (Deppenmeier et al., 2002; Matsushita et al., 1994; Muynck et al., 2007). In either case, the production of D-gluconic acid is mainly the result of the direct oxidation in the periplasmic space, as the membrane-bound GDH activity is 30-fold higher than that of the cytosolic GDH (Pronk et al., 1989).

Strawberries are very popular berries and have a high nutritional value due to the presence of micronutrients and phenolic substances, of which the potential health benefits have been widely studied (Giampieri et al., 2012; Hannum, 2004). However, they are also an easily perishable fruit, which are generally consumed fresh, and, therefore, must be processed very quickly after harvest. Some alternatives to direct consumption have been developed, such as juice, jelly, nectar, puree, concentrate or jams (Barrett et al., 2005; Sinha, 2006). Recently, the production of strawberry vinegars by double fermentation has been proposed as a good alternative to the current processing methods because fermentation extends the shelf life, can add some value to the product and is one of the more environmentally friendly processes (Hidalgo et al., 2010, 2013). Nevertheless, the consumption of vinegar is low, and other strategies that preserve the fruit's natural sweetness and compensate for acidity using AAB are being

studied. One such process is the production of a beverage that is mainly based on D-glucose oxidation to D-gluconic acid, where D-fructose is not fermented. Thus, the aim of this work is the selection of AAB strains to carry out this process. Although strains of the *Gluconobacter* genus initially seemed to be the most suitable for this purpose (because they prefer sugary environments and have been well studied with regard to D-glucose metabolism in AAB), the only AAB strain we isolated from strawberries is a strain of *Acetobacter malorum* (Hidalgo et al., 2013). Therefore, strains from species of both the *Gluconobacter* and *Acetobacter* genera were tested in this study. Different media and conditions were used to select the best strain for the production of a non-alcoholic fermented beverage made from strawberries.

2. MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions

Nineteen strains of eight species of AAB were used in this study (Table 1). Fifteen of these strains were isolated by our research group in previous studies (Hidalgo et al., 2013; Navarro et al., 2013; Valera et al., 2011; Vegas et al., 2010), and the remaining four were obtained from different culture collections. The AAB were grown in GY medium (1% (w/v) yeast extract; 1% (w/v) D-glucose; Panreac, Barcelona, Spain) at 28°C with shaking (125 rpm) for 48 h.

Table 1. Strains used in this study.

Species	Strain	Origin or isolation source	References
Gluconobacter japonicus	CECT 8443	Grape must	Navarro et al. (2013)
	Gj1	Grape	Valera et al. (2011)
	Gj2	Grape	Valera et al. (2011)
	Gj3	Grape	Valera et al. (2011)
	6	Grape must	Navarro et al. (2013)
	13	Grape must	Navarro et al. (2013)
Gluconobacter oxydans	DSM 7145^T	Beer	(Henneberg 1897) De Ley (1961)
	17	Grape must	Navarro et al. (2013)
	Po5	Vinegar	Vegas et al. (2010)
Gluconobacter albidus	14	Grape must	Navarro et al. (2013)
Gluconobacter cerinus	16	Grape must	Navarro et al. (2013)
Gluconobacter thailandicus	15	Grape must	Navarro et al. (2013)
Acetobacter pasteurianus	DSM 3509 ^{T}	Beer	(Hansen 1879) Beijerinck and Folpmers (1916)
	11	Grape must	Navarro et al. (2013)
Acetobacter cerevisiae	LMG 1625 ^{T}	Beer (ale) in storage	Cleenwerck et al. (2002)
	19	Grape must	Navarro et al. (2013)
Acetobacter malorum	LMG 1746 ^{T}	Rotting apple	Cleenwerck et al. (2002)
	9	Grape must	Navarro et al. (2013)
	CECT 7749	Strawberry vinegar	Hidalgo et al. (2013)

^{T} Type Strains

2.2. D-gluconic acid production

Two experiments were performed to study the production of D-gluconic acid by the AAB. In the first experiment, all AAB strains were screened for the production of D-gluconic acid in duplicate in three different media: Minimal medium (MM; 1% (w/v) yeast nitrogen base w/o amino acids (Becton, Dickinson & Co, Franklin Lakes, NJ, USA) and sugars), Synthetic must (SM) prepared according to Riou et al. (1997) and 2x strawberry concentrate (2x-SC, HUDISA, S.A, Huelva, Spain). The sugar concentrations (D-glucose and D-fructose) and pH in the MM and SM were adjusted to the natural sugar concentration (192 mM of D-glucose and 246 mM of D-fructose) and the pH (3.3) of the

strawberry concentrate (2x-SC). The pH was adjusted with 10 M hydrochloric acid. Only one strain of each species of *Acetobacter* genera was tested in 2x-SC. The criteria used for the selection of the strains were as follows: a) maximal concentration of D-gluconic acid, b) D-glucose depletion, and c) minimal consumption of D-fructose. According to these parameters, the three best strains were selected, and the experiments were repeated in triplicate and monitored over time.

In the second experiment, the strains selected from the previous results were tested in triplicate in 3x strawberry concentrate (3x-SC; 333 mM D-glucose and 356 mM D-fructose; HUDISA, Huelva, Spain) at two different pH values: 3.3 and 4.8. The pH of the 3x-SC was adjusted to 4.8 by adding calcium carbonate (Sigma-Aldrich, St. Louis, MO, USA).

In both experiments, the fermentations were performed in sterile 250 mL Erlenmeyer flasks in 100 mL of medium inoculated with 2×10^6 cells/mL of the corresponding strain grown in GY medium. All of the experiments were stopped after 30 days. Although D-glucose depletion and the maximal D-gluconic consumption were expected to occur rapidly, the experiment was extended (until 30 days) to determine whether the different AAB strains were able to use other carbon sources, such as D-fructose and/or D-gluconic acid. The media had been previously sterilized with dimethyldicarbonate (DMDC; Velcorin, Sigma-Aldrich) when the strawberry concentrates were used (2x-SC and 3x-SC). Additionally, 100 mg/L of natamycin E-235/Delvocid (DSM; Delft, The Netherlands) were added to prevent the growth of yeast and fungi. The effectiveness of these treatments was confirmed by microscopy and plating on GYA (GY plus 2% (w/v) agar (Panreac) and YPDA (2% (w/v) D-glucose, 2% (w/v) bacteriological peptone (Panreac), 1% (w/v) yeast extract, and 2% (w/v) agar) media. Moreover, in the case of 3x-SC, 20 µL of pectolytic enzymes (ROHAPECT®, AB Enzymes, Darmstadt, Germany) were used to reduce the viscosity and allow better homogenization of the medium. All of the Erlenmeyer flasks were incubated at 28°C with shaking (125 rpm).

Samples were taken at different times of the process to monitor the sugar consumption (D-glucose and D-fructose) and production of D-gluconic acid, 2-keto-D-gluconic acid (2-KGA) and 5-keto-D-gluconic acid (5-KGA). Microbiological controls of the processes were performed at different levels, depending on the complexity of the media. In MM and SM, the optical density (OD) was measured throughout the processes. In the case of the strawberry concentrates, microscopic evaluation was performed because it was not possible to measure the OD. Moreover, the presence of solid particles in these concentrates made it very difficult to properly evaluate the AAB population with microscopy because these insoluble particles resulted in an overestimation of the population, with high standard deviations.

2.3. Chemical analyses

The sugar concentrations (D-glucose and D-fructose) were measured with an enzymatic kit (Boehringer Mannhein, Germany). The pH was measured using a Crison micro pH meter (Crison Instruments, S.A., Barcelona, Spain).

D-gluconate, 2-KGA and 5-KGA were determined and quantified by the Centre for Omic Sciences. To prepare the sample, dilutions of the aqueous extracts (1:10) were centrifuged at 15,000 rpm and 4°C for 10 min and then serially diluted in ultrapure LC-MS water (Milli-Q system – Millipore) to a final dilution of 1:10000. The following analytical standards were used: D-gluconic acid, 2-KGA and 5-KGA (Sigma-Aldrich).

A 1290 UHPLC Series Liquid Chromatograph coupled to a 6490 QqQ/MS (Agilent Technologies, Palo Alto, U.S.A.) was used for the D-gluconic acid, 2-KGA and 5-KGA determinations. The ion exchange chromatographic column was a Hi-Plex H, 6.5 x 300 mm, 8 µm (Agilent Technologies). The mobile phases were water (solvent A) and ACN (solvent B). The flow rate was 0.25 mL/min, and the column compartment was set at an isothermal temperature of 65°C. Elution gradient was 0-10 min 15% B isocratic, 10-12 min 0% B, 12-18 min 0% B isocratic, and 19 min 15% B. A post run of 3 min was applied. The injected sample volume was 2 µL. The retention times of D-gluconate, 2-KGA and

5-KGA were 11.10 min, 9.63 min and 10.28 min, respectively. The calibration curves, linearity, precision, accuracy and the method detection and quantification limits were studied by analyzing serial standard dilutions prepared in ultrapure LC-MS water and pooled samples spiked with standard solutions. The obtained validation parameters of this method allowed us to quantify D-gluconic acid, 2KGA and 5KGA in the extract samples. Additionally, in some samples, the D-gluconic acid quantification using this methodology was compared to that with the enzymatic kit (Boehringer Mannhein, Germany) and obtained very similar concentrations, with standard deviation values that were always lower than 10%.

2.4. Statistical analysis

All statistical analyses were performed using the statistical software package SPSS version 17.0 for Windows. One-way analysis of variance and Sheffe's post tests were performed to evaluate the significant differences between media (significance levels $p < 0.05$).

3. RESULTS AND DISCUSSION

3.1. Initial selection of the acetic acid bacteria strains for D-gluconic acid production

The ability of nineteen AAB strains to produce D-gluconic acid from D-glucose without using D-fructose was tested in three different media (MM, SM and 2x-SC) (Table 2). The different compositions of these media were considered appropriate to test the background effect on D-gluconic acid production. However, all of the media had the same sugar concentration and pH as strawberry puree for comparative purposes. The strawberry concentrates were industrially pasteurized; however, this treatment efficiently reduces the population size of the microorganisms present in the puree, but it does not completely eliminate them. For this reason, the strawberry concentrates were sterilized with DMDC. The effectiveness of the DMDC treatment was tested by microscopic

evaluation and growth on GYA and YPDA. No colonies grew on any media, and no cells were observed by optical microscopy. Moreover, sugar consumption and D-gluconic acid production were not observed without AAB inoculation. D-glucose and D-fructose consumption and D-gluconic acid production were dependent on the strain and the media. This is consistent with previous studies, (Asai, 1968; Olijve and Kok, 1979; Weenk et al., 1984), which reported that the rate of D-glucose oxidation was dependent on the particular characteristics of the strain and the conditions of the culture. However, as expected, all strains presented a preference for D-glucose rather than D-fructose. D-fructose dissimilation seemed to be adaptive and is repressed by the presence of D-glucose, while the D-glucose utilization systems were constitutive and did not depend on the substrate on which the microorganisms grew (Benziman and Rivetz, 1972). The utilization of D-fructose by the AAB was higher in the strawberry concentrate, where only four strains consumed less than 15% (w/v) of the initial D-fructose, compared to thirteen strains in the other two media. Moreover, in 2x-SC, eight strains oxidized more than 50% (w/v) of the D-fructose, while in the other media, no strain was able to oxidize more than 42.5% (w/v) (Table 2).

Among the strains tested, most belonged to the *Gluconobacter* genus. This genus was reported to prefer sugar-rich environments (Raspor and Goranovic, 2008), showing high oxidation activity with sugar and sugar alcohols (D-glucose, D-gluconic acid, D-sorbitol, and glycerol). All strains tested produced D-gluconic acid, although D-glucose depletion was medium-dependent because some strains exhausted D-glucose in one media but not in the others. In MM, the D-gluconic acid production in all *Gluconobacter* strains was similar, while in SM, higher divergence was observed, resulting in two and even four times greater accumulation in some strains compared to the others.

Table 2. Production of D-gluconic acid by acetic acid bacteria in minimal medium, synthetic must and 2x strawberry concentrate

	MINIMAL MEDIUM				SYNTHETIC MUST				2X-STRAWBERRY CONCENTRATE			
Strain	**Glucose[a] (mM)**	**% Fru consumed[b]**	**Max. Gln[c] (mM)**	**% Gln consumed[d]**	**Glucose (mM)**	**% Fru consumed**	**Max. Gln (mM)**	**% Gln consumed**	**Glucose (mM)**	**% Fru consumed**	**Max. Gln (mM)**	**% Gln consumed**
CECT 8443	41.1	0.0	132.1	0.0	-	5.0	159.2	45.2	-	10.5	105.6	0.0
Gj1	37.8	0.0	132.7	0.0	-	10.0	146.9	65.3	9.4	18.6	85.2	29.6
Gj2	28.9	0.0	136.2	0.0	-	1.2	164.3	56.9	7.2	17.2	81.6	17.8
Gj3	23.3	1.0	141.8	0.0	-	9.7	153.1	53.3	12.8	0.0	77.6	7.3
6	27.8	42.5	112.2	0.0	105.6	15.0	35.7	0.0	11.1	53.5	71.4	42.9
13	-	12.5	120.9	14.3	-	2.5	96.9	23.7	-	54.6	86.7	29.9
DSM 7145	33.3	25.0	102.0	0.0	116.7	15.0	30.6	0.0	-	100.0	71.4	71.4
17	-	23.0	134.7	14.4	-	27.5	95.9	0.0	12.8	75.6	41.8	0.0
Po5	66.7	0.0	102.0	0.0	-	0.0	165.8	0.0	-	11.2	153.1	0.0
14	-	3.8	119.4	19.7	-	20.2	79.1	26.5	5.6	72.1	66.3	53.8
16	-	23.2	138.8	0.0	-	40.7	79.1	35.5	27.8	79.1	67.3	0.0
15	-	0.0	120.4	0.0	-	11.2	115.3	9.7	19.4	21.9	23.0	0.0
DSM 3509	-	2.2	107.7	4.1	-	12.7	103.6	14.7	n.d.[e]	n.d.	n.d.	n.d.
11	134.4	13.2	38.3	0.0	-	14.7	109.7	48.9	11.1	52.1	55.6	74.1
LMG 1625	151.7	10.0	32.7	0.0	122.2	5.0	15.3	0.0	n.d.	n.d.	n.d.	n.d.
19	137.2	15.5	49.0	0.0	-	19.0	106.1	0.0	12.2	85.5	41.0	63.6
LMG 1746	150.6	10.0	8.7	0.0	158.9	8.5	1.5	0.0	n.d.	n.d.	n.d.	n.d.
9	134.4	18.0	49.2	0.0	-	19.0	61.2	0.0	n.d.	n.d.	n.d.	n.d.
CECT 7749	65.0	0.0	102.0	0.0	-	33.5	127.6	100	-	10.0	121.9	8.6

[a] Concentration of D-glucose not used by AAB after 30 days of process
[b] % Fru consumed: percentage of D-fructose consumed along the process
[c] Max. Gln: Maximum concentration of D-gluconic acid that was accumulated
[d] % Gln consumed: percentage of D-gluconic acid that was consumed after its maximum accumulation
[e] n.d.: not determined

In 2x-SC, the *Gluconobacter* strains produced the least amount of D-gluconic acid, and *Gluconobacter oxydans* Po5 was the only strain that accumulated as much or more D-gluconate in this medium as in the other two. Interestingly, some strains consumed D-gluconic acid, but this behavior was also dependent on the medium. For example, strains of *Gluconobacter japonicus* CECT 8443, Gj1, Gj2 and Gj3 oxidized approximately 50% of the D-gluconic acid produced (45-65%) in SM but not in MM. Moreover, these same strains produced less D-gluconic acid in 2x-SC and exhibited little or no oxidation of this acid.

Out of the strains that exhausted all of the D-glucose, the D-gluconic acid yield that accumulated from the initial D-glucose was highly variable between strains and media, ranging from 72% to 83% in MM, 47-99% in SM and 43-92% in 2x-SC. AAB are recognized for their rapid oxidation of sugars and alcohols ("overflow metabolism") and the near-quantitative excretion of the resulting acids to the medium (Deppenmeier et al., 2002; Deppenmeier and Ehrenreich, 2009). Although D-glucose can also be metabolized via the pentose phosphate pathway, the pH of the medium (< 3.5) and the initial D-glucose content (> 15 mM) strongly repressed this pathway (Olijve and Kok, 1979). Therefore, the low rate of D-gluconic acid accumulation in some cases may be related to its further oxidation to ketogluconates because D-gluconic acid metabolization via the pentose phosphate pathway may be almost completely prevented due to the low pH (Olijve and Kok, 1979).

Some of the strains used in this study belonged to the *Acetobacter* genus. This genus was reported to prefer alcohol-rich environments (Raspor and Goranovic, 2008), exhibiting highly active ethanol oxidation reactions and reduced oxidation activity with sugar or sugar alcohols (Matsushita et al., 2003). This statement is generally consistent with the results, but as in the case of *Gluconobacter,* these results were highly dependent on the strain and medium. In general, low levels of D-glucose degradation were observed in MM with only one strain, *Acetobacter pasteurianus* DSM 3509, which consumed all of the D-glucose. In contrast, in SM, 70% of the strains exhausted all of the D-glucose, with

the *A. malorum* and *Acetobacter cerevisiae*-type strains as the only ones that exhibited low consumption (Table 2). Consequently, the D-gluconic acid production was higher in SM than in MM. Interestingly, *A. malorum* CECT 7749 showed the highest accumulation of D-gluconic acid in SM, although this acid was then rapidly and completely oxidized. However, this strain behaved differently in the other two media and produced large amounts of D-gluconic acid without relevant oxidation to other products.

From the results of this first experiment, the best strains were selected using the total depletion of D-glucose, a high rate of D-gluconic acid production from D-glucose and minimal consumption of D-fructose as the main criteria. Although all of the data obtained in the previous experiments were considered, the results from 2x-SC medium were considered more relevant for the selection of the strains due to the great divergence observed in these parameters between the three media in most of the strains. Therefore, according to these criteria, three strains were selected, *G. oxydans* Po5, *G. japonicus* CECT 8443 and CECT 7749, a strain of *A. malorum*, which was the only one in this study that was isolated from strawberry.

3.2. Production of D-gluconic acid and its derived gluconates in the selected strains

In the selected strains, the production of D-gluconic acid and consumption of D-glucose and D-fructose in the three media were monitored throughout the entire process (Fig. 1). In all of the strains, D-glucose consumption was significantly faster in SM and was completely exhausted after an average of 5 days. As expected, this time point coincided with the maximal accumulation of D-gluconic acid during fermentation. Furthermore, the D-glucose depletion seemed to be correlated with the start of the D-gluconic acid oxidation in *G. japonicus* CECT 8443 (Fig. 1a) and *A. malorum* CECT 7749 (Fig. 1c) but not *G. oxydans* Po5 (Fig. 1b). In any case, the D-gluconic acid oxidation was quite different between the two strains. In *G. japonicus* CECT 8443, only half of the D-gluconic acid was oxidized, and the rest remained stable for more than 20 days, while all of the

D-gluconic acid was depleted in the *A. malorum* CECT 7749 strain after 20 days. Furthermore, in this strain, the slow consumption of D-fructose overlapped with that of D-gluconic acid, although when the acid was depleted, D-fructose oxidation stopped. In the 2x-SC medium, the D-gluconic acid production was slower for all strains but obtained maximum concentrations similar to those obtained in SM, with the exception of *G. japonicus* CECT 8443. Moreover, no clear D-gluconic acid oxidation was observed, likely due to the presence of small quantities of D-glucose until the end of the process (<11 mM). Very slow and low level D-gluconic acid consumption only occurred in *A. malorum* CECT 7749. In this medium, low levels of D-fructose were also consumed in the early days, but afterwards, the D-fructose levels remained stable until the end of the process. Finally, in MM, a very high rate of conversion of D-glucose into D-gluconic acid was achieved (higher than 90% in all the strains) in the first days of the process, although almost no growth was observed (data not shown). Low growth yields and high oxidation rates have been described for these bacteria. (Olijve and Kok, 1979; Sievers and Swings, 2005). The main problem of the MM was an abrupt arrest that occurred when there was still between 50 and 75 mM D-glucose in the medium, although initially, the process was very similar to that in SM. Under these conditions, a large amount of D-gluconic acid accumulated outside the cells and created an acidic environment, dropping the pH to below 2.2 (data not shown). Due to the lack of buffering substrates in the medium, we presume that this low pH led to a slower growth, a gradual decrease in cell viability (Velizarov and Beschkov, 1998), and a strong repression of D-gluconic acid production. Concomitant with the decrease in the pH, this arrest in the D-gluconic acid production could also be due to the inactivation of the D-glucose membrane-bound dehydrogenase, similar to what has been described for *Gluconobacter suboxydans* alcohol dehydrogenase in acidic growth conditions (Matsushita et al., 1994).

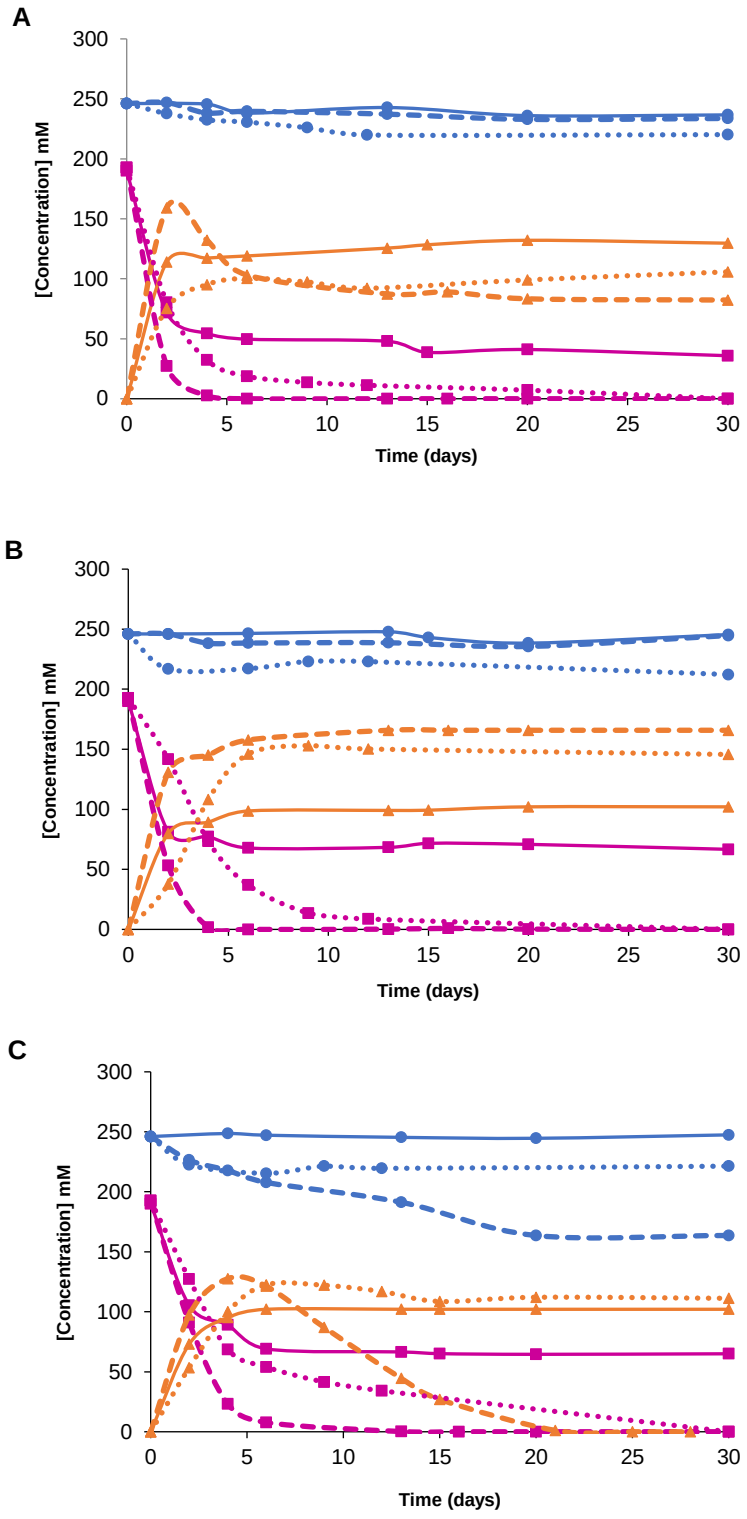

Figure 1. D-glucose (■), D-fructose (●) and D-gluconic acid (▲) concentrations in different strains of acetic acid bacteria in different media: minimal medium (MM; ─), synthetic must (SM;╌) and 2x strawberry concentrate (2x-SC;┅). A. *G. japonicus* CECT 8443; B. *G. oxydans* Po5; and C. *A. malorum* CECT 7749. The concentrations plotted are the means of triplicates. The standard deviations were always below 10%.

The production of 5-KGA and 2-KGA in the selected strains was also evaluated in the SM medium (Fig. 2). In strains *G. japonicus* CECT 8443 (Fig. 2a) and *A. malorum* CECT 7749 (Fig. 2c), the accumulation of 2-KGA started before D-gluconic acid achieved the maximum concentration observed in this medium, while 5-KGA was only detected when D-gluconic acid oxidation began. Most of the 2-KGA had accumulated when degradation of the acid was observed, with the final concentration of 2-KGA being higher than that of 5-KGA. The arrest of the production of both ketoacids coincided with the cessation of D-gluconic acid consumption, and further utilization of both ketoacids was even observed in *A. malorum* CECT 7749. On one hand, these results agree with those of Weenk et al. (1984), who linked the start of the keto-D-gluconate formation to the decrease in the D-glucose concentration in the media below a threshold of approximately 30 mM. In our case, 2-KGA was first detected when the amount of D-glucose in the media was approximately this concentration. On the other hand, these results also agree with those of Silberbach et al. (2003), who reported that small amounts of 2-KGA were formed during the oxidation of D-glucose to D-gluconate, but 5-KGA production could not be observed until D-glucose was completely oxidized to D-gluconic acid. The production of 5-KGA by *A. malorum* CECT 7749 was surprising because when this species was first described, one of its features was its inability to produce 5-KGA from D-glucose (Cleenwerck et al., 2002). Conversely, *G. oxydans* Po5 produced the highest concentration of D-gluconic acid, but very small amounts of keto-D-gluconic acids were produced (concentrations < 2.5 mM) (Fig. 2b). Similar results were obtained with other strains, such as *G. oxydans* DSM 3503, as reported by Silberbach et al. (2003). The production of 2-KGA and 5-KGA under acidic conditions and in the absence of $NADP^+$ was demonstrated to require two different types of membrane-bound D-gluconate dehydrogenases (GADH); these enzyme activities seemed to be largely dependent on the culture conditions (pH, temperature, shaking, etc.) (Shinagawa et al., 1999). These two enzymes compete with each other to oxidize D-gluconate; therefore, the selective

expression of either dehydrogenase can increase the production of either of the keto-D-gluconates (Elfari et al., 2005; Matsushita et al., 2003).

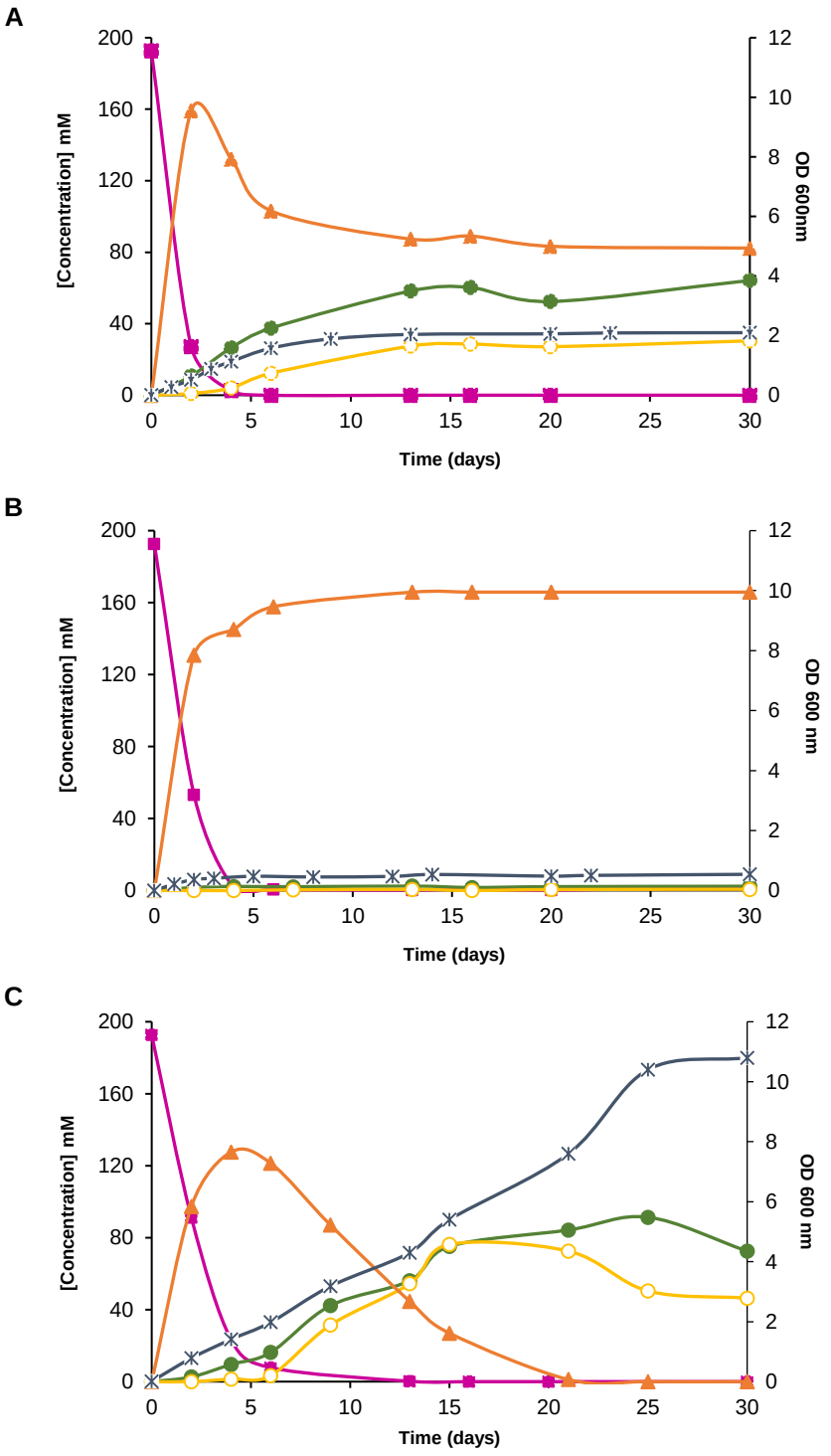

Figure 2. Evolution of the metabolites derived from D-glucose oxidation and bacterial growth by three different strains of acetic acid bacteria throughout the fermentation of synthetic must (SM). A. *G. japonicus* CECT 8443; B. *G. oxydans* Po5; C. *A. malorum* CECT 7749; D-glucose (■); D-gluconic acid (▲); 2-keto-D-gluconic acid (●); 5-keto-D-gluconic acid (○); OD (✳). The concentrations plotted are the means of triplicates. The standard deviations were always below 10%.

Different studies have reported that *Gluconobacter* strains (most of the studies referred to *G. oxydans* strains) presented differences in the ratio of both keto-D-gluconates obtained from D-glucose (Elfari et al., 2005; Herrmann et al., 2004; Silberbarch et al., 2003; Weenk et al., 1984). This is consistent with our findings because the three strains tested yielded quite different product spectra.

Bacterial growth was also monitored during this process (Fig. 2). Clear differences between strains were observed with the maximal OD that was achieved. Strains *G. oxydans* Po5 and *A. malorum* CECT 7749 presented the lowest and highest growth, respectively; the latter was twenty-fold higher. In strains *A. malorum* CECT 7749 and *G. japonicus* CECT 8443, the bacterial growth increased during the utilization of D-glucose or D-gluconic acid, and even 5-KGA in *A. malorum* CECT 7749. On the other hand, in *G. oxydans* Po5, as no D-gluconic acid utilization was observed, the growth was arrested when D-glucose was exhausted. It has been reported that *Gluconobacter* strains are unable to grow rapidly, even in a complete medium (Macauley et al., 2001), and, as mentioned above, there is a negative correlation between product accumulation and biomass formation. Therefore, the higher the biomass, the lower the amount of D-glucose that was used for product formation (Elfari et al., 2005). This agrees with our results, where *G. oxydans* Po5, the strain with the lowest biomass production, accumulated the highest content of D-gluconic acid (40% more than the strain with the highest biomass production, *A. malorum* CECT 7749). However, D-gluconate and keto-D-gluconate (mainly 5-KGA) utilization seemed to be associated with a large increase in biomass.

3.3. Effect of pH on the production of D-gluconic acid and the derived gluconates by the selected strains

The selected strains were tested in 3x-SC (Fig. 3) using two different initial pH values (pH_i): 3.3, the natural pH of strawberry concentrate, and 4.8, the pH with a maximal empirical activity for the oxidation from D-glucose to D-gluconate, according to

Silberbach et al. (2003). It is important to note that the pH was monitored but not controlled, and, therefore, pH decreased due to microbial acid production. *G. japonicus* CECT 8443 (Fig. 3a) accumulated more D-gluconic acid (ca. 50 mM) at pH_i 3.3 and did so faster than at pH_i 4.8 (Fig. 3b). At this pH_i, the lower amount of D-gluconic acid was compensated for by a higher concentration of both keto-D-gluconic acids (the sum of both ketoacids was 134 mM at pH_i 4.8, whereas it was 77 mM at pH_i 3.3). Other differences with this strain were that at pH_i 3.3, D-glucose was not exhausted, and the concentrations of both ketoacids were similar, while at pH_i 4.8, more 2-KGA was produced (82 mM 2-KGA *vs.* 51 mM 5-KGA). At both pH_i values, the synthesis of the ketoacids overlapped with the D-gluconic acid production, which was more evident at pH_i 4.8. However, the strain *G. oxydans* Po5 was the only one that presented a similar pattern at both pH_i values (Fig. 3c and 3d), producing high levels of D-gluconic acid (ca. 255 mM) and no further oxidation. Another characteristic of this strain is that it produced a higher concentration of 5-KGA than 2-KGA, which was the inverse of the other two strains. Finally, fermentations with strain *A. malorum* CECT 7749 exhibited changes that were similar to strain *G. japonicus* CECT 8443 in the beginning of the process, with more accumulation of D-gluconic acid at pH_i 3.3 (Fig. 3e), although slower than the *G. japonicus* CECT 8443 strain, and with more production of keto-D-gluconic acids at pH_i 4.8 (Fig. 3f). However, at this latter pH_i, the D-gluconic acid began to be slowly oxidized on day 7, along with the remaining D-glucose, while the concentration of 2-KGA increased rapidly to reach 166 mM on day 16 (seven-fold higher than 5-KGA). At this time point, no D-glucose was available, and all of the metabolites derived from its oxidation (D-gluconate, 2-KGA and 5-KGA) were rapidly and completely metabolized. The lack of these acids resulted in a sharp increase in the pH of the medium, reaching pH values near 8. No significant modifications of the pH were observed in any of the other fermentations. On the other hand, at pH_i 3.3, these compounds were not metabolized, retaining similar concentrations of both ketoacids and a high concentration of D-glucose (*ca.* 67 mM) in the medium.

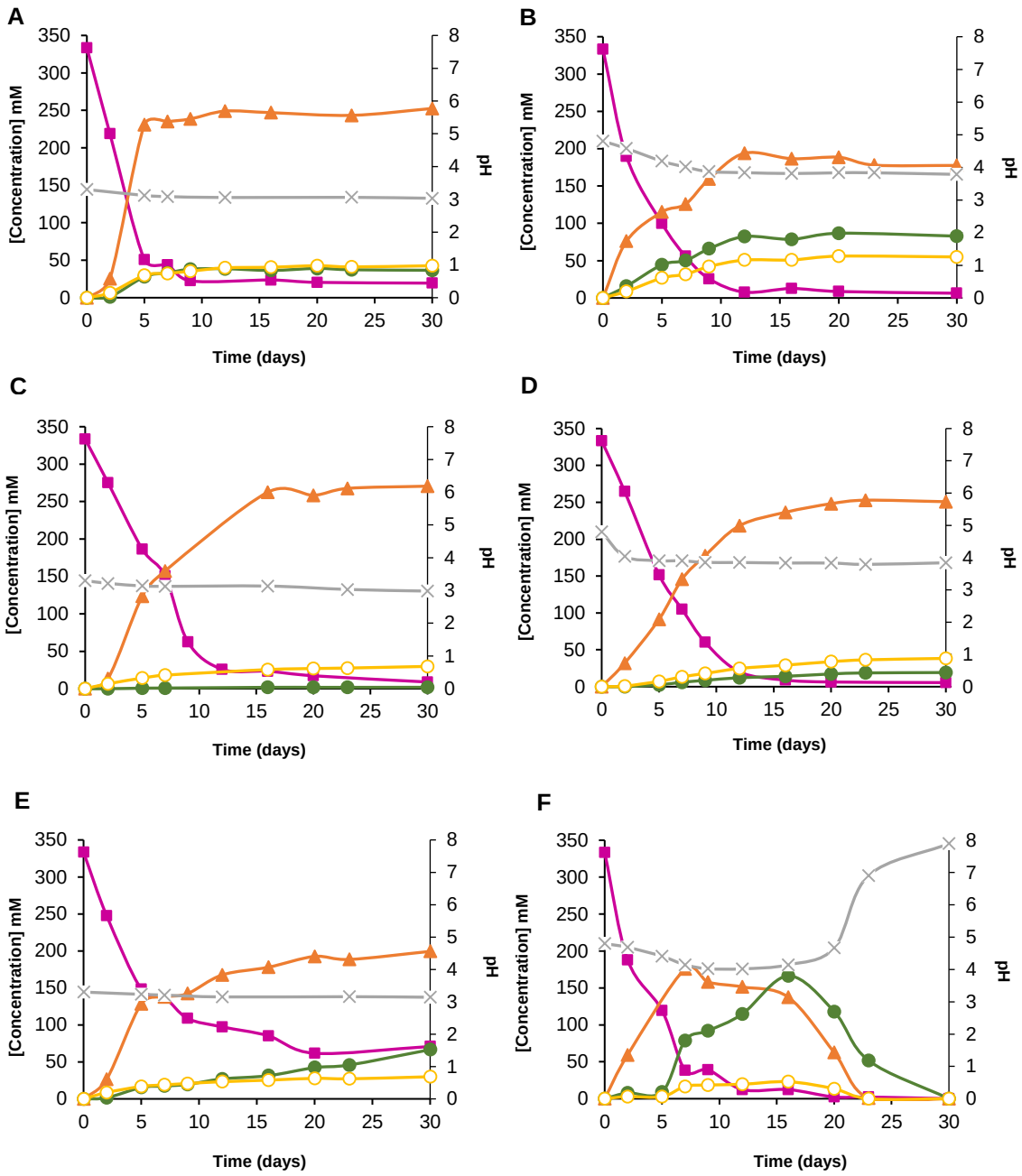

Figure 3. Evolution of the metabolites derived from D-glucose oxidation by three different strains of acetic acid bacteria (*G. japonicus* CECT 8443 (A, B); *G. oxydans* Po5 (C, D); *A. malorum* CECT 7749 (E, F)) throughout the fermentation of 3x strawberry concentrate (3x-SC) at two initial pHs: 3.3 (A, C, E) and 4.8 (B, D, F). D-glucose (■); D-gluconic acid (▲); 2-keto-D-gluconic acid (●); 5-keto-D-gluconic acid (○); pH (×). The concentrations plotted are the means of triplicates. The standard deviations were always below 10%.

These results differ markedly from other previously reported results; the accumulation of D-gluconic acid was higher at pH 3.3. In fact, there is no agreement about the optimal pH for the formation of D-glucose oxidation products, due to its dependence on medium conditions. For example, Ano et al. (2011) found that the pH of the medium was the most

important factor for selective production, with pH values of nearly 3, between 3 and 5, and nearly 5 as the optimal pH values for D-gluconate, 5-KGA and 2-KGA production, respectively. This suggests that the optimal pH for the production of D-gluconic acid is quite far from the pH proposed by Silberbach et al. (2003) and is more in agreement with our results. In most cases, the synthesis of both keto-D-gluconates started when high concentrations of D-glucose were still present in the medium (> 28 mM), which contradicted the results observed in this study with other media and also those reported by other research groups (Weenk et al., 1984; Silberbach et al., 2003). Therefore, the beginning of the synthesis of keto-D-gluconates seemed to be linked to more than simply the amount of D-glucose present in the medium, as observed by Beschkov et al. (1995). Moreover, in the *A. malorum* CECT 7749 strain, there was a simultaneous decrease in the extracellular concentrations of D-gluconic acids, 2-KGA and 5-KGA at pH_i 4.8, resulting in an increase of the pH. Ano et al. (2011) described an increase in the pH and cell growth in *G. suboxydans* IFO 12528 when 5-KGA was metabolized. They suggested that 5-KGA had been taken into the cell and utilized by the assimilative pathway. In our case, two explanations are possible: (i) 2-KGA could be further oxidized to 2,5-di-keto-D-gluconate by the membrane 2-keto-D-gluconate dehydrogenase enzyme or (ii) 2-KGA was taken up and dissimilated by oxidation via the pentose phosphate pathway (Matsushita et al., 1994). Nevertheless, the large increase in the pH to values up to 8 suggested that the latter occurred.

Modifications to the medium pH_i did not have the same effect in all strains; *G. oxydans* Po5 was not affected, while the other two strains exhibited higher production of D-gluconic acid and lower accumulation of keto-D-gluconates at pH_i 3.3. It is important to note that the pH_i was adjusted to 4.8 with $CaCO_3$, and the addition of this salt at the beginning of the conversion is considered essential for the increased production of keto-D-gluconates (Beschkov et al., 1995). Therefore, the presence of this salt could be responsible for the increased synthesis at this pH_i. Furthermore, while *G. japonicus* CECT 8443 and *A. malorum* CECT 7749 accumulated larger amounts of 2-KGA than of

5-KGA, *G. oxydans* Po5 primarily synthesized 5-KGA, with 2-KGA being a minor product. The preference for the synthesis of one or the other keto-D-gluconate seemed to be closely linked to the pH and its control (Ano et al., 2011; Silberbach et al., 2003). However, in our case, the pH was not controlled, and the different experiments were developed at pH values of approximately 3 (for experiments with an initial pH of 3.3) or 4 (for experiments with an initial pH of 4.8); the main product should have been 5-KGA and not 2-KGA, according to the optimal pH values reported for the synthesis of both keto-D-gluconates (Ano et al., 2011; Shinagawa et al., 1999). Therefore, in our case, keto-D-gluconate synthesis seemed to be more dependent on the strain.

3.4. Conclusions

It was possible to achieve selective conversion of D-glucose into D-gluconic acid without fermenting D-fructose in strawberry concentrates using AAB. *G. japonicus* CECT 8443 and *G. oxydans* Po5, belonging to the *Gluconobacter* genus, were the best strains for this process. The choice of the strain will depend on the final concentration of D-gluconic acid desired because *G. oxydans* Po5 generally produced higher amounts of D-gluconic acid in all media tested. These strains may be used in industrial conditions (continuous fermentations with pH and oxygen controls) in the future. The *A. malorum* CECT 7749 strain, isolated from strawberry, also showed good performance in fruit concentrate at its natural pH. However, its ability to further oxidize D-gluconic acid in certain conditions discouraged its selection for this process.

ACKNOWLEDGMENTS

This work was supported by the grant AGL2010-22152-C03-02 from the Spanish Ministry of Science and Innovation. F. Sainz holds an FI Fellowship from the AGAUR, Government of Catalonia. The authors thank the Centre for Omic Sciences (COS) for their help in the determination of D-gluconic acid and its derivatives.

REFERENCES

Adachi, O., Moonmangmee, D., Toyama, H., Yamada, M., Shinagawa, E., Matsushita, K., 2003. New developments in oxidative fermentation. Applied Microbiology and Biotechnology 60, 643-653.

Ano, Y., Shinawaga, E., Adachi, O., Toyama, H., Yakushi, T., Matsushita, K., 2011. Selective, high conversion of D-Glucose to 5-Keto-D-gluconate by *Gluconobacter oxydans*. *Bioscience,* Biotechnology and Biochemistry 73, 586-589.

Asai, T., 1968. Acetic acid bacteria: classification and biochemical activities. University of Tokyo Press, Tokyo.

Barrett, D.M., Somogyi, L., Ramaswamy, H., 2005. Processing fruits: Science and Technology, second ed. CRC Press, United States of America.

Beijerinck, M.W., Folpmers, T., 1916. Verslagen van de gewone vergadering der wis- en natuurkundige afdeeling. Koninklijke Akademie van Wetenschappen 18, 1198-1200.

Benziman, M., Rivetz, B., 1972. Factors affecting hexose phosphorylation in *Acetobacter xylinum.* Journal of Bacteriology 111, 325-353.

Beschkov, V., Velizarov, S., Peeva, L., 1995. Some kinetic aspects and modelling of biotransformation of D-glucose to keto-D-gluconates. Bioprocess Engineering 13, 301-305.

Cleenwerck, I., Vandemeulebroecke, K., Janssens, D., Swings, J., 2002. Re-examination of the genus *Acetobacter*, with descriptions of *Acetobacter cerevisiae* sp. nov. and *Acetobacter malorum* sp. nov. International Journal of Systematic and Evolutionary Microbiology 52, 1551-1558.

De Ley, J., 1961. Comparative carbohydrate metabolism and a proposal for the phylogenetic relationship of the acetic acid bacteria. Journal of General Microbiology 24, 31-50.

Deppenmeier, U., Hoffmeister, M., Prust, C., 2002. Biochemistry and biotechnological applications of *Gluconobacter* strains. Applied Microbiology and Biotechnology 60, 233-242.

Deppenmeier, U., Ehrenreich, A., 2009. Physiology of acetic acid bacteria in light of the genome sequence of *Gluconobacter oxydans.* Journal of Molecular Microbiology and Biotechnology 16, 69-80.

Elfari, M., Ha, S.W., Bremus, C., Merfort, M., Khodaverdi, V., Herrmann, U., Sahm, H., Görisch, H., 2005. A *Gluconobacter oxydans* mutant converting glucose almost quantitatively to 5-keto-D-gluconic acid. Applied Microbiology and Biotechnology 66, 668-674.

Giampieri, F., Tulipane, S., Alvarez-Suarez, J.M., Quiles, J.L., Mezzetti, B., Battino, M., 2012. The strawberry: composition, nutritional quality, and impact on human health. Nutrition 28, 9-19.

Hannum, S.M., 2004. Potentional impact of strawberries on human health: a review of the science. Critical Reviews in Food Science and Nutrition 44, 1-17.

Herrmann, U., Merfort, M., Jeude, M., Bringer-Meyer, S., Sahm, H., 2004. Biotransformation of glucose to 5-keto-D-gluconic acid by recombinant *Gluconobacter oxydans* DSM 2343. Applied Microbiology and Biotechnology 64, 86-90.

Hidalgo, C., Mateo, E., Cerezo, A.B., Torija, M.J., Mas, A., 2010. Technological process for production of persimmon and strawberry vinegars. International Journal of Wine Research 2, 55-61.

Hidalgo, C., Torija, M.J., Mas, A., Mateo, E., 2013. Effect of inoculation on strawberry fermentation and acetification processes using native strains of yeast and acetic acid bacteria. Food Microbiology 34, 88-94

Lan Vu, H.T., Yukphan, P., Chaipitakchonlatarn, W., Malimas, T., Muramatsu, Y., Thi Tu Bui, U., Tanasupawat, S., Cong Duong, K., Nakagawa, Y., Thanh Pham, H., Yamada, Y., 2013. *Nguyenibacter vanlangensis* gen. nov., sp. nov., an unusual acetic acid bacterium in the α-Proteobacteria. The Journal of General and Applied Microbiology 59, 153-166.

Lino, T., Suzuki, R., Kosako, Y., Ohkuma, M., Komagata, K., Uchimura, T., 2012. *Acetobacter okinawensis* sp. nov., *Acetobacter papayae* sp. nov., and *Acetobacter persicus* sp. nov.; novel acetic acid bacteria isolated from stems of sugarcane, fruits, and a flower in Japan. The Journal of General and Applied Microbiology 58, 235-243.

Macauley, S., McNeil, B., Harvey, L.M., 2001. The genus *Gluconobacter* and its applications in biotechnology. Critical Reviews in Biotechnology 21, 1-25.

Malimas, T., Chaipitakchonlatarn, W., Thi Lan Vu, H., Yukphan, P., Muramatsu, Y., Tanasupawat, S., Potacharoen, W., Nakagawa, Y., Tanticharoen, M., Yamada, Y., 2013. *Swingsia samuiensis* gen. nov., sp. nov., an osmotolerant acetic acid bacterium in the α-Proteobacteria. The Journal of General and Applied Microbiology 59, 375-384.

Matsushita, K., Toyama, H., Adachi, O., 1994. Respiratory chains and bioenergetics of acetic acid bacteria. In: Rose, A.H., Tempest, D.W. (Eds.), Advances in Microbial Physiology, vol. 36. Academic Press Ltd, London, pp. 247-301.

Matsushita, K., Fujii, Y., Ano, Y., Toyama, H., Shinjoh M., Tomiyama, N., Miyazaki, T., Sugisawa, T., Hoshino, T., Adachi, O., 2003. 5-Keto-D-Gluconate production is catalyzed by a quinoprotein glycerol dehydrogenase, major polyol dehydrogenase, in *Gluconobacter* species. Applied and Environmental Microbiology 69, 1959-1966.

Merfort, M., Herrmann, U., Bringer-Meyer, S., Sahm, H., 2006. High-yield 5-keto-D-gluconic acid formation is mediate by soluble and membrane-bound gluconate-5-dehydrogenases of *Gluconobacter oxydans*. Applied Microbiology and Biotechnology 73, 443-451.

Muynck, C., Pereira, C.S.S., Naessens, M., Parmentier, S., Soetaert, W., Vandamme, E.J., 2007. The genus *Gluconobacter oxydans*: comprehensive overview of biochemistry and biotechnological applications. Critical Reviews in Biotechnology 27, 147-171.

Navarro, D., Mateo, E., Torija, M.J., Mas, A., 2013. Acetic acid bacteria in grape must. Acetic acid bacteria 2, 19- 23.

Olijve, W., Kok, J.J., 1979. Analysis of growth of *Gluconobacter oxydans* in glucose containing media. Archives of Microbiology 121, 283-290.

Papagianni, M., 2011. Organic acids. In: Moo-Young, M. (Ed.), Comprehensive Biotechnology. Canada, pp. 109-120.

Pronk, J.T., Levering, P.R., Olijve, W., Van Dijken, J.P., 1989. Role of NADP-dependent quinoprotein glucose dehydrogenase in gluconic acid production by *Gluconobacter oxydans*. Enzyme and Microbial Technology 11, 160-164.

Prust, C., Hoffmeister, M., Liesegang, H., Wiezer, A., Fricke, W.F., Ehrenreich, A., Gottschalk, G., Deppenmeier, U., 2005. Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydans*. Nature Biotechnology 23, 195-200.

Ramachandran, S., Fontanille, P., Pandey, A., Larroche, C., 2006. Gluconic acid: properties, applications and microbial production. Food Technology and Biotechnology 44, 185-195.

Raspor, P., Goranovic, D., 2008. Biotechnological applications of acetic acid bacteria. Critical Reviews in Biotechnology 28, 101-124.

Riou, C., Nicaud, J.M., Barre, P., Gaillardin, C., 1997. Stationary-phase gene expression in *Saccharomyces cerevisiae* during wine fermentation. Yeast 13, 903-915.

Shinagawa, E., Matsushita, K., Toyama, H., Adachi, O., 1999. Production of 5-keto-D-gluconate by acetic acid bacteria is catalyzed by pyrroloquinoline quinone (PQQ)-dependent membrane-bound D-gluconate dehydrogenase. Journal of Molecular Catalysis B: Enzymatic 6, 341-350.

Sievers, M., Swings, J., 2005. Family *Acetobacteraceae.* In: Garrity, G., Brenner, D.J., Krieg, N.R., Staley, J.T. (Eds.), Bergey's Manual of Systematic Bacteriology. Springer, New York, USA, pp. 41-95.

Silberbach, M., Maier, B., Zimmermann, M., Buchs, J., 2003. Glucose oxidation by *Gluconobacter oxydans*: characterization in shaking-flasks, scale-up and optimization of the pH profile. Applied Microbiology and Biotechnology 62, 92-98.

Sinha, N.K., 2006. Strawberries and raspberries. In: Hui, Y.H. (Ed.), Handbook of Fruits and Fruit Processing, Part III: Commodity Processing. Blackwell Publishing, Iowa, USA, pp. 581- 595.

Trček, J., Teuber, M., 2002. Genetic and restriction analysis of the 16S-23S rDNA internal transcribed spacer regions of the acetic acid bacteria. FEMS Microbiology letters 208, 69-75.

Trček, J., Barja, F., 2015. Updates on quick identification of acetic acid bacteria with a focus on the 16S–23S rRNA gene internal transcribed spacer and the analysis of cell proteins by MALDI-TOF mass spectrometry. International Journal of Food Microbiology 196, 137-144.

Valera, M.J., Laich, F., González, S., Torija, M.J., Mateo, E., Mas, A., 2011. Diversity of acetic acid bacteria present in healthy grapes from the Canary Islands. International Journal of Food Microbiology 151,105-112.

Vegas, C., Mateo, E., Gonzalez, A., Jara, C., Guillamon, J.M., Poblet, M., Torija, M.J., Mas, A., 2010. Population dynamics of acetic acid bacteria during traditional wine vinegar production. International Journal of Food Microbiology 138, 130-136.

Velizarov, S., Beschkov, V., 1998. Biotransformation of glucose to free gluconic acid by *Gluconobacter oxydans:* substrate and product inhibition situations. Process Biochemistry 33, 527-534.

Weenk, G., Olijve, W., Harder, W., 1984. Ketogluconate formation by *Gluconobacter* species. Applied Microbiology and Biotechnology 20, 400-405

CHAPTER 2

Determination of dehydrogenase activities involved in D-glucose oxidation in *Gluconobacter* and *Acetobacter* strains

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Frontiers in Microbiology, doi: 10.3389/fmicb.2016.01358

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SELECTION AND OPTIMIZATION OF ACETIC ACID BACTERIA FOR D-GLUCONIC ACID PRODUCTION
Florencia Sainz Perez

ABSTRACT

Acetic acid bacteria (AAB) are known for rapid and incomplete oxidation of an extensively variety of alcohols and carbohydrates, resulting in the accumulation of organic acids as the final products. These oxidative fermentations in AAB are catalyzed by PQQ- or FAD-dependent membrane-bound dehydrogenases. In the present study, the enzyme activity of the membrane-bound dehydrogenases (membrane-bound PQQ-glucose dehydrogenase (mGDH), D-gluconate dehydrogenase (GADH) and membrane-bound glycerol dehydrogenase (GLDH)) involved in the oxidation of D-glucose and D-gluconic acid (GA) was determined in six strains of three different species of AAB (three natural and three type strains). Moreover, the effect of these activities on the production of related metabolites [GA, 2-keto-D-gluconic acid (2KGA) and 5-keto-D-gluconic acid (5KGA)] was analyzed. The natural strains belonging to *Gluconobacter* showed a high mGDH activity and low activity in GADH and GLDH, whereas the *A. malorum* strain presented low activity in the three enzymes. Nevertheless, no correlation was observed between the activity of these enzymes and the concentration of the corresponding metabolites. In fact, all the tested strains were able to oxidize D-glucose to GA, being maximal at the late exponential phase of the AAB growth (24 h), which coincided with D-glucose exhaustion and the maximum mGDH activity. Instead, only some of the tested strains were capable of producing 2KGA and/or 5KGA. In the case of *G. oxydans* strains, no 2KGA production was detected which is related to the absence of GADH activity after 24 h, while in the remaining strains, detection of GADH activity after 24h resulted in a high accumulation of 2KGA. Therefore, it is possible to choose the best strain depending on the desired product composition. Moreover, the sequences of these genes were used to construct phylogenetic trees. According to the sequence of *gcd*, gene coding for mGDH, *Acetobacter* and *Komagataeibacter* were phylogenetically more closely related each other than with *Gluconobacter*.

Keywords: Acetic acid bacteria, D-gluconic acid, Keto-D-gluconic acids, strawberry beverage.

1. INTRODUCTION

Acetic acid bacteria (AAB) are gram-negative, ellipsoidal to rod-shape acidophilic bacteria and are obligate aerobes (De Ley and Swings, 1984; Deppenmeier et al., 2002). These bacteria could occur in sugary natural environments such as fruits, honey bees, or flowers and in artificial and manmade environments such as soft drinks, cider, beer, wine, or vinegar (De Ley and Swings, 1984). AAB are well known for the rapid and incomplete oxidation of a broad range of sugars, sugar alcohols, and sugar acids (such as D-glucose, glycerol, D-sorbitol, ethanol, or D-gluconic acid) resulting in the accumulation of high amounts of the oxidized products in the culture medium (Asai, 1968; Deppenmeier et al., 2002; Elfari et al., 2005). This capacity allows for the use of AAB for a variety of biotechnological processes in which they carry out oxidative fermentation to obtain several useful compounds that are difficult to be prepared with chemical processes or to be produced with high yields (Gupta et al., 2001; Deppenmeier et al., 2002). Some examples of this metabolism are the production of acetic acid from ethanol or D-gluconic acid (GA) from D-glucose (Deppenmeier et al., 2002; Prust et al., 2005; Lino et al., 2012). Most of these oxidative reactions are catalyzed by membrane-bound dehydrogenases, with reactive centers that are oriented to the periplasmic space (Matsushita et al., 1994). This implies that transport of substrates inside the cell is unnecessary and accumulation of oxidized products in the medium is rapid and near-quantitative (Deppenmeier et al., 2002; Adachi et al., 2003; Matsushita et al., 2003; Elfari et al., 2005; Merfort et al., 2006).

In AAB, many membrane-bound oxidoreductases have been described, and most of these oxidoreductases are pyrroloquinoline quinone (PQQ-) or flavin (FAD-) dependent proteins (Saichana et al., 2015). The oxidative reaction with these dehydrogenases results in bioenergy for AAB because electrons extracted from the substrates are transferred via ubiquinone to the terminal ubiquinol oxidase (Adachi et al., 2007). In D-glucose oxidation, several enzymes located on the periplasmic face of the cytoplasmic membrane catalyze D-glucose oxidation sequentially. Membrane-bound PQQ-glucose

dehydrogenase (mGDH) oxidizes D-glucose to glucono-δ-lactone, and it is then converted to GA by glucono-δ-lactonase or spontaneously (Matsushita et al., 1994; Shinagawa et al., 1999). Therefore, mGDH is the enzyme responsible for the production of most GA from D-glucose during fermentation (Macauley et al., 2001). GA can be further converted to 2-keto-D-gluconic acid (2KGA) or 5-keto-D-gluconic acid (5KGA) by two different membrane-bound dehydrogenases (Matsushita et al., 1994; Saichana et al., 2015). One protein is D-gluconate dehydrogenase (GADH), which is a FAD-dependent enzyme (flavoprotein-cytochrome *c* complex) reacting with GA as its only substrate and is responsible for the oxidation of GA to 2KGA (Matsushita et al., 1994; Adachi et al., 2007; Toyama et al., 2007). The membrane-bound dehydrogenase involved in the 5KGA production has been unidentified for a long time, and no specific 5KGA-yielding gluconate dehydrogenase has been found in AAB. Instead, it has been shown that this reaction is catalyzed by a glycerol or polyol dehydrogenase (GLDH, membrane-bound glycerol dehydrogenase), which shows a broad substrate specificity towards several sugar alcohols (D-glycerol, D-sorbitol, D-arabitol, or D-mannitol). Therefore, it is concluded that other PQQ-dependent dehydrogenases such as D-arabitol dehydrogenase (ARDH) or D-sorbitol dehydrogenase (SLDH) are identical to GLDH. (Matsushita et al., 2003; Adachi et al., 2007). 2KGA could be further oxidized to 2,5-di-keto-D-gluconate by the FAD-dependent 2-keto-D-gluconate dehydrogenase (2KGDH), which is characterized as a flavoprotein-cytochrome *c* complex with three different subunits similar to GADH.

We have developed a strawberry beverage in which D-glucose is completely fermented to GA or some other acids, yet fruit D-fructose is maintained as a natural sweetener (Cañete-Rodríguez et al., 2015, 2016). GA could be found naturally in fruit juices, honey, yoghurt, bread, cottage cheese and meat. This acid gives a refreshing sour taste to wine and fruit juices and has the property of preventing bitterness in foodstuffs. In the food industry, GA is widely used as flavoring agent and for reducing absorption of fat products and is listed as a generally permitted food additive (E574) by the EFSA, and as a GRAS

(Generally Recognized As Safe) additive by the US FDA (Ramachandran et al., 2006). Moreover, GA has been reported to have some beneficial effects on intestinal microbiota (Asano et al., 1994, 1997; Tsukahara et al., 2002) and it has limited toxicity. This low toxicity makes GA useful for food additives as one of the common counter ions for the administration of some metal cations (Zn, Ca, Na, K) or other chemicals (chlorhexidine). However, the equimolar conversion of D-glucose into GA and the high D-glucose concentrations in some fruits might recommend the reduction of GA by further oxidation. Therefore, the knowledge of the possible transformations of D-glucose into different metabolites would help control the levels of the different compounds in these transformed fruit beverages. In a previous study (Sainz et al., 2016), three natural AAB strains were selected for this GA fermentation using different media and conditions, but especially focusing on the strawberry process. Two of these strains belong to the *Gluconobacter* genus: *Gluconobacter japonicus* strain CECT 8443 isolated from grape must (Navarro et al., 2013) and *Gluconobacter oxydans* strain Po5 isolated from wine vinegar (Vegas et al., 2010). The other strain from *Acetobacter malorum* (CECT 7742) was the only strain isolated from strawberry vinegar (Hidalgo et al., 2013).

The aim of the present study was to compare the enzyme activities of the membrane-bound dehydrogenases responsible for D-glucose and GA oxidations in six strains of three different AAB species (selected strains from our collection and other strains from other culture collection strains). We wanted to analyze the effect of these enzyme activities on the production of the involved metabolites (GA, 2KGA and 5KGA) for better control of the production of these fermented beverages.

2. MATERIALS AND METHODS

2.1. Microorganism and culture conditions

Two strains of each AAB species (*G. oxydans, G. japonicus and A. malorum*) were used in this study (Table 1). For the preparation of the inocula, these strains were previously

grown for 24 h in 5 mL potato media (Matsushita and Ameyama, 1982) with shaking at 28ºC. Experiments were performed in Erlenmeyer flasks of 500 mL with 100 mL media (30 g/L of D-glucose (Wako Pure Chem., Osaka, Japan), 40 g/L of D-fructose (Wako Pure Chem.), 5 g/L of polypeptone (Nihon Pharmaceutical Co., Ltd, Tokyo, Japan) and 5 g/L of yeast extract (Oriental Yeast Co., Ltd, Tokyo, Japan)) and inoculated with 1 mL of the corresponding strain grown in potato media. The experiment was carried out in triplicate, with shaking (200 rpm) at 28ºC and sampled at 24, 48 and 96 h. Bacterial growth was measured by a Klett-Summerson photoelectric colorimeter with a red filter.

Table 1. Strains used in this study

Species	Strain	Source	Reference
Gluconobacter japonicus	CECT 8443	Grape must	Navarro et al., (2013)
	NBRC 3271[T]	*Myrica rubra* (Fruit)	Malimas et al., (2009)
Gluconobacter oxydans	Po5	Vinegar	Vegas et al., (2010)
	621 H	-	(Henneberg, 1987) De Ley ., (1961)
Acetobacter malorum	CECT 7742[a]	Strawberry vinegar	Hidalgo et al., (2013)
	NBRC 108912 [T]	Rotting apple	Cleenwerck et al., (2002)

[T] Type strains.
[a] This strain has been incorrectly named CECT 7749 in previous studies (Hidalgo et al., 2013 and Sainz et al., 2016).

2.2. Preparation of membrane fraction

As explained previously, cells were harvested at 24, 48 and 96 h. The total volume (100 mL) was centrifuged for 5 min at 10.600 x *g*, and the cells were washed twice with 50 mM potassium phosphate buffer, pH 6.5 (1 g wet cells per 4-5 mL buffer). After washing, the pellets were stored for 24 hr at 4ºC and then resuspended in the same volume with the same buffer. The cell suspension was passed twice through a French pressure cell press (SIM AMINCO, Spectronic Instruments, Inc., Rochester, NY, USA) at 16.000 psi. Intact cells were removed with 10.000 x *g* for 10 min, and the supernatant was centrifuged at 100.000 x *g* for 60 min at 4ºC. The resulting precipitate was resuspended

in potassium phosphate buffer [1M dipotassium phosphate (Wako Pure Chem.) and 1M monopotassium phosphate (Wako Pure Chem.), pH 6.5] (20 mL buffer per 1 g pellet) and homogenized with the same buffer in a glass homogenizer. In the case of GLDH, 10 mM MES [2-(N-morpholino) ethanesulfonic acid, (Dojindo, Kumamoto, Japan)] - NaOH buffer was used. The resulting homogenate was considered the membrane fraction.

2.3. Protein determination

The protein concentration was determined by a modified Lowry method (Dulley and Grieve, 1975) using bovine serum albumin (Sigma, Tokyo, Japan) as the standard.

2.4. Assays of enzyme activity

All enzymatic reactions were performed in triplicate and at 25ºC. mGDH and GLDH were assayed in the presence of phenazine methosulfate (PMS) (Wako Pure Chem.) and 2,6-dichlorophenol indophenol (DCIP) (Wako Pure Chem.) as electron acceptors, as described by Matsushita et al. (1980). The 1 mL reaction mixture contains 50 mM potassium phosphate buffer (pH 6.5), 8 mM sodium azide (Wako Pure Chem.), 6.67 mM DCIP, 6 mM PMS, 100 mM D-glucose or glycerol (Wako Pure Chem.) as substrate and the membrane fraction. Some modifications were done for the GLDH assay; 10 mM acetate buffer (10mM sodium acetate trihydrate (Wako Pure Chem) and acetic acid (Wako Pure Chem) (pH 6.0) was used instead of potassium phosphate buffer. For the conversion of apo-enzyme to holo-enzyme, 3 mM calcium chloride anhydrate (Wako Pure Chem.) and 0.1 µM PQQ (Wako Pure Chem.) were added and incubated for 10 min in an ice bath. The enzyme activity was measured by the reduction of DCIP at 600 nm. One unit of enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1 µmol of substrate per min, which was calculated using the millimolar extinction coefficient of DCIP of 13.2 at pH 6.5 and of 11.13 at pH 6.0.

The enzyme activity of GADH and 2KGDH was measured according to Wood et al. (1962), using ferricyanide (Wako Pure Chem.) as an electron acceptor. The reaction mixture consists of 8 mM sodium azide, 100 mM ferricyanide, 100 mM GA (Sigma) or

2KGA (Sigma) as the substrate, the membrane fraction and McIlvaine buffer [a mixture of 0.1 M citric acid (Wako Pure Chem.) and 0.2 M disodium hydrogen phosphate (Wako Pure Chem.), pH 4.5] to a total volume of 1.0 mL. The reaction started with the addition of ferricyanide solution, and after 10 min, the reaction was stopped by adding 500 µL of ferric-Dupanol reagent (Wako Pure Chem.). Twenty min later, 3.5 mL of water was added, and after mixing well, the absorbance at 600 nm was measured by a UV-1700 PharmaSpec spectrophotometer (UV-1700 PharmaSpec, Shimadzu, Kyoto, Japan). Under these assay conditions, 4 absorbance units corresponded to 1 µmol of substrate oxidized.

2.5. Determination of substrates and products by HPLC analysis

All metabolites were analyzed using high performance liquid chromatography (HPLC – Shimadzu). D-glucose and D-fructose were quantified on a Pb^{2+}-loaded cation-exchange column (SUGAR SP0810, 8.0 mm I.D. × 300 mm L, Shodex, Showa denko KK, Kawasaki, Japan) at 80°C using distilled and deionized water as the mobile phase at a flow rate of 0.5 $mL \cdot min^{-1}$. Substances were detected with a refractive index detector. The retention times for D-glucose and D-fructose were 19.5 and 24.7 min, respectively. GA, 5KGA, and 2KGA were quantified on an ion-exclusion column (RSpak KC-811, 8.0 mm I.D. × 300 mm L, Shodex, Showa denko KK, Kawasaki, Japan) at 60°C using 0.1% (w/v) phosphoric acid as the mobile phase at a flow rate of 0.4 $mL \cdot min^{-1}$. Substances were detected with an UV detector (SPD-M20A, Shimadzu SPD-M20A) at 210 nm. The retention times of GA, 5KGA, and 2KGA were 18.8, 18.1, and 17.4 min, respectively.

2.6. Primer design and PCR conditions

Genes coding for mGDH (*gcd*) and large subunits of GADH (*gnd*L), GLDH (*sld*A) and 2KGDH (*kgd*L) were partially amplified to confirm their presence. For this reason, the primers for these genes were designed using the program Primer3Plus (Untergasser et al., 2007) in each species using the sequences available in the GenBank database

(Table 2). The amplification reaction was carried out in a total volume of 50 µL consisting of 1 µL of DNA solution, 5 µL of 10 X buffer, 3 µL of $MgCl_2$, 200 µM each of the four dNTPs (Roche Diagnostic GmBh, Manheim, Germany), 0.4 µL of BSA (20 mg/mL), 4 µL of DMSO, 1 µL of each primer (10 pmol), and 0.4 µL of Taq Polymerase (Biotaq, Bioline - USA). The conditions of the PCR were as follows: initial denaturation at 94ºC for 5 min, followed by 30 cycles of denaturing at 94ºC for 1 min, annealing at 55ºC or 60ºC (depending on the primers) for 30 s, extension at 72ºC for 1 min and a final extension at 72ºC for 10 min and maintained at 4ºC. The amplifications were performed in a Gene Amp PCR System 2700 (Applied Biosystems, Foster city, USA), and the PCR products were detected by electrophoresis gel on 1% agarose in 1X TBE buffer. The gels were stained with ethidium bromide and photographed.

2.7. Sequence alignment and phylogenetic tree construction

The nucleotide sequences of genes *gcd* and *gnd*L of the natural strains used in this study have been sequenced and deposited in the GenBank Database with the following accession numbers: *G. oxydans* Po5 (KU896941, KU896943), *A. malorum* CECT 7742 (Amal_02000, Amal_01874) and *G. japonicus* CECT 8443 (A0J51_02827, A0J51_00901). The *sld*A gene sequence was not found in *A. malorum* and the corresponding sequences for *Gluconobacter* species were A0J51_00428 and A0J51_00622 for *G. japonicus* and KU896942 for *G. oxydans*. These sequences were compared with the sequences from other genera and species available in GenBank database for the phylogenetic analyses. The sequence alignment was performed using the nucleotide sequence with the MUSCLE 3.8.31 software (Edgar 2004a, 2004b). The poorly aligned regions were removed using the Gblocks 0.91b program (Castresana, 2000; Talavera and Castresana, 2007).

The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.1/3.0 aLRT). The HKY85 substitution model was selected assuming an estimated proportion of invariant sites (of 0.248) and 4 gamma-

distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data (gamma=0.770). Reliability for internal branch was assessed using the aLRT test (SH-Like). The tree rendering was performed with the TreeDyn 198.3 graphical editor (http://www.phylogeny.fr/; Dereeper et al., 2008, 2010).

3. RESULTS

In this study, three selected AAB strains, belonging to *G. japonicus*, *G*. *oxydans*, and *A. malorum* species, isolated from vinegar or fruit were examined together with their corresponding culture collection strains in terms of growth, enzyme activities involved in the D-glucose oxidation, and metabolites produced from oxidation. For the *G. japonicus* species, both the isolated and the type culture strains showed very similar growth (Figure 1), achieving a high population at the end of the experiment (320 Klett units at 96 h), without reaching the stationary phase. Both strains presented a high mGDH activity and a similar evolution over time (Figure 1A). In both cases, mGDH activity is maximal at 24 h, although strain CECT 8443 exhibited twice the activity of NBRC 3271, and the activity decreased afterwards. In relation to GADH, both strains presented similar behavior, showing the highest activity at 24 h (Figure 1B). However, strain NBRC 3271 had four-fold higher activity than CECT 8443 during the first 48 h followed by a sharp decline, resulting in a GADH activity being practically absent at 96 h. Instead, the GLDH activity in these strains presented low activity (lower than 0.15 U/mg protein in all the cases) and behaved differently from each other (Figure 1C). Strain NBRC 3271 presented the highest activity at 24 h and decreased afterwards, whereas strain CECT 8443 exhibited the highest activity at 48 h. The *G. oxydans* strains (Figure 2), although they had a very similar initial population, presented huge differences in their growth, mainly due to the first 24 h, when strain 621H achieved twice the population of Po5. After this moment, the evolution in both strains was very similar, showing slower growth and entry in the stationary phase.

Table 2. PCR pair primers used for gene amplification

Strain	GenBank accession No.	Locus tag of gene sequences used for primer design	gene	Product	Primer name	Primer sequence (fwd)	Primer sequence (rev)
Gluconobacter japonicus NBRC 3271	LHZK00000000	AD938_10885	*gcd*	membrane-bound glucose dehydrogenase	*mgdh*	TGGTTTTCCCGGGTGATCTG	GTAGTAGTCCATCGTGCCCG
	LHZK00000000	AD938_08480	*gnd*L	gluconate 2-dehydrogenase, large subunit	*gadh1*	TCCTGAGTGCGTTCCAGTTC	CGCTTTGGCAATGGGTTCAA
	LHZK00000000	AD938_03325	-	gluconate 2-dehydrogenase, large subunit	*gadh2*	GGCCTATCCCTCGTCAATCG	TGCATAACCGCTGCAAAACC
	LHZK00000000	AD938_10275	*sld*A	glycerol dehydrogenase large subunit SldA	*gldh1*	CGGGTGAAGAGAAGTGGGTC	GAGCTGGTCATACATCGGGG
	LHZK00000000	AD938_11075	*sld*A	glycerol dehydrogenase large subunit SldA	*gldh2*	GGTAAGGAGATCTGGCGTCG	TGAAACTGCATTTTCCGCCG
Gluconobacter oxydans 621H	CP000009	GOX0265	*gcd*	membrane-bound glucose dehydrogenase	*mgdh*	CTCGTGTACATCCCGATGGG	ACCACCCCACTCGAACATTC
	CP000009	GOX1231	-	gluconate 2-dehydrogenase, large subunit	*gadh*	TATTGCAGCGGCTATGACTG	CATGGTCGAAATTCATGCTG
	CP000009	GOX0854	*sld*A	glycerol dehydrogenase large subunit SldA	*gldh*	GCGACGGGTAAGGAGATCTG	TTTCTTCAGGGCTACGCAGG
Gluconobacter oxydans NBRC 3293	AB985494	-	*kgd*L	large subunit of 2-keto-D-gluconate dehydrogenase	*2kgdh*	GGAAAACTGGCGCAACATGTCG	CCCGAACGGGATCATGTC
Acetobacter malorum strain DmCS_005	JOJU00000000	AmDm5_2097	-	membrane-bound glucose dehydrogenase	*mgdh*	ATGTTTGAATGGGGCGGTCT	CGTCATACGCCCGGATGTAA
	JOJU00000000	AmDm5_1995	-	gluconate 2-dehydrogenase, large subunit	*gadh*	CGGGTGAAGCCTATACGGTC	AGAATGACAAGTCCGGCAGG
	JOJU00000000	AmDm5_0421	*kgd*L	large subunit of 2-keto-D-gluconate dehydrogenase	*2kgdh*	ACCTGCCGTCAGACTTTGAG	ATACAATGCGCGGCAATCAC

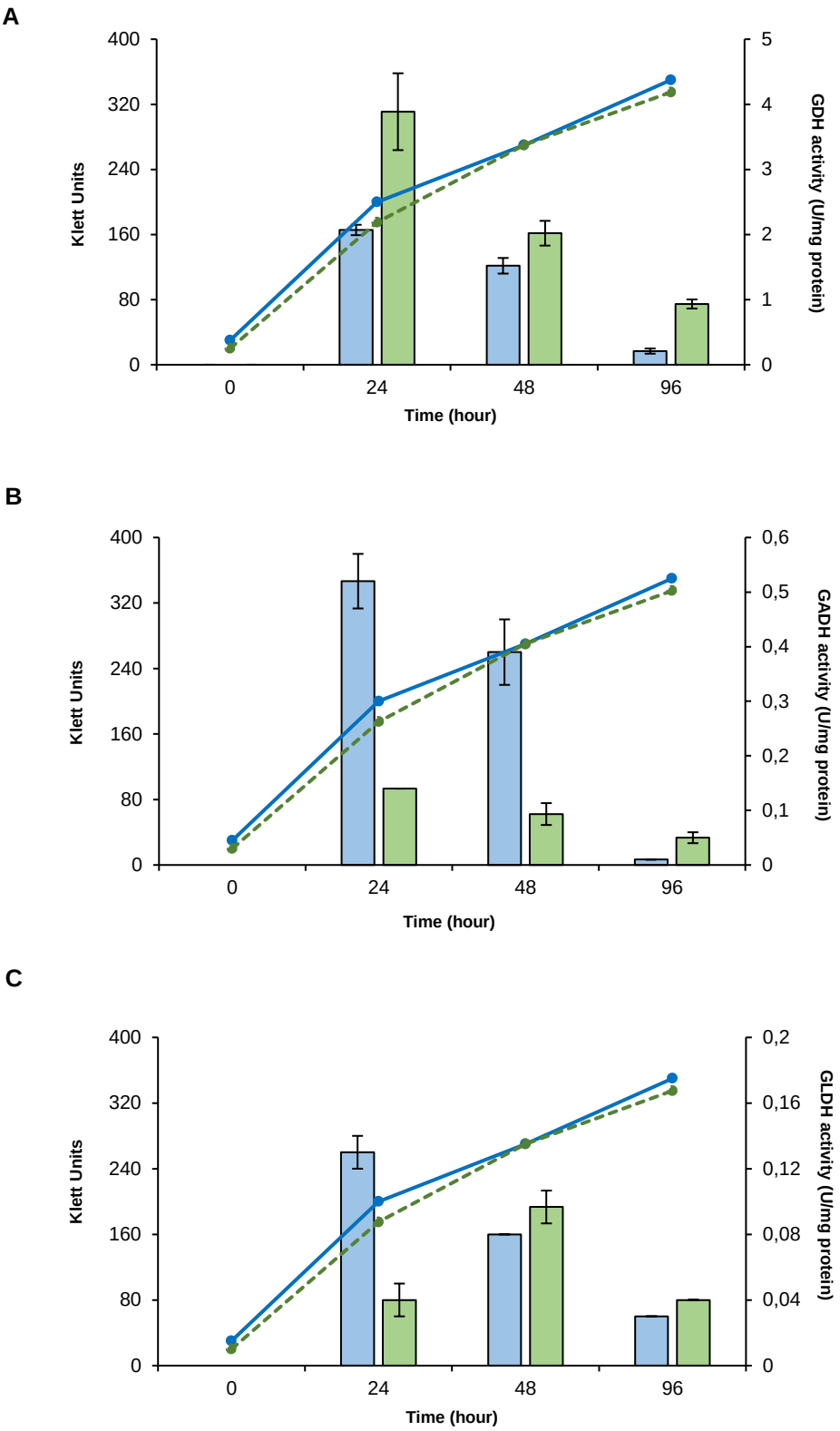

Figure 1. Enzyme activity and growth (expressed in Klett units) of two *Gluconobacter japonicus* strains at three different growth stages (24, 48 and 96 hours). **(A)** Glucose dehydrogenase activity (mGDH); **(B)** Gluconate dehydrogenase activity (GADH); **(C)** Glycerol dehydrogenase activity (GLDH). Enzyme activity represented in bars: NBRC 3271 ( ), CECT 8443 ( ) and cell growth with lines: NBRC 3271 (—); CECT 8443 (--).

Similarly, *G. oxydans* Po5 presented the highest activity of mGDH (Figure 2A) at 24 h, when maximal activity was reached, which was three times higher than in 621H. Then, a clear decrease of the activity was observed in both cases. The GADH activity was only detected at 24 h in both *G. oxydans* strains, with similar values (approximately 0.1 U/mg protein) (Figure 2B). In the case of GLDH, *G. oxydans* strains showed similar activity at 24 h (Figure 2C), later presenting a reduction in the activity. However, in strain Po5, this decrease was more pronounced at 48 h, but an upturn of activity was observed at the end (96 h). Finally, *A. malorum* strains presented a similar evolution of *G. oxydans* strains, although in this case the wild strain (CECT 7742) grew better than the type strain (NBRC 108912) (Figure 3). In this case, the difference in growth between both strains (approximately 90 Klett units) was mainly observed during the first 24 h. After these 24 h, CECT 7742 showed some growth, although with a lower rate, whereas the type strain was not growing. Strain NBRC 108912 showed a very high mGDH activity at 24 h; however, no activity was detected afterwards (Figure 3A). In contrast, CECT 7742 presented less activity but maintained the activity over time (1 U/mg protein at 24 and 48 h. and half at 96 h.). The activity of GADH presented similar evolution as mGDH, although with much lower values. In strain NBRC 108912, GADH activity was only detected at 24 h, and with the highest value, whereas CECT 7742 presented a low and constant activity over time (Figure 3B). Finally, low GLDH activity was observed in both *A. malorum* strains (Figure 3C), although the activity was higher in NBRC 108912. In CECT 7742, residual activity was observed in all the points studied.

The activity of 2KGDH was also studied in all the strains. However, no activity was detected in any of these strains (Table 3).

In the tested strains, evolution of the metabolites derived from D-glucose oxidation was analyzed at the same time points when the enzymatic activity was measured (24, 48 and 96 h). Similar patterns between strains of the same species were obtained according to the consumption and production of the metabolites studied.

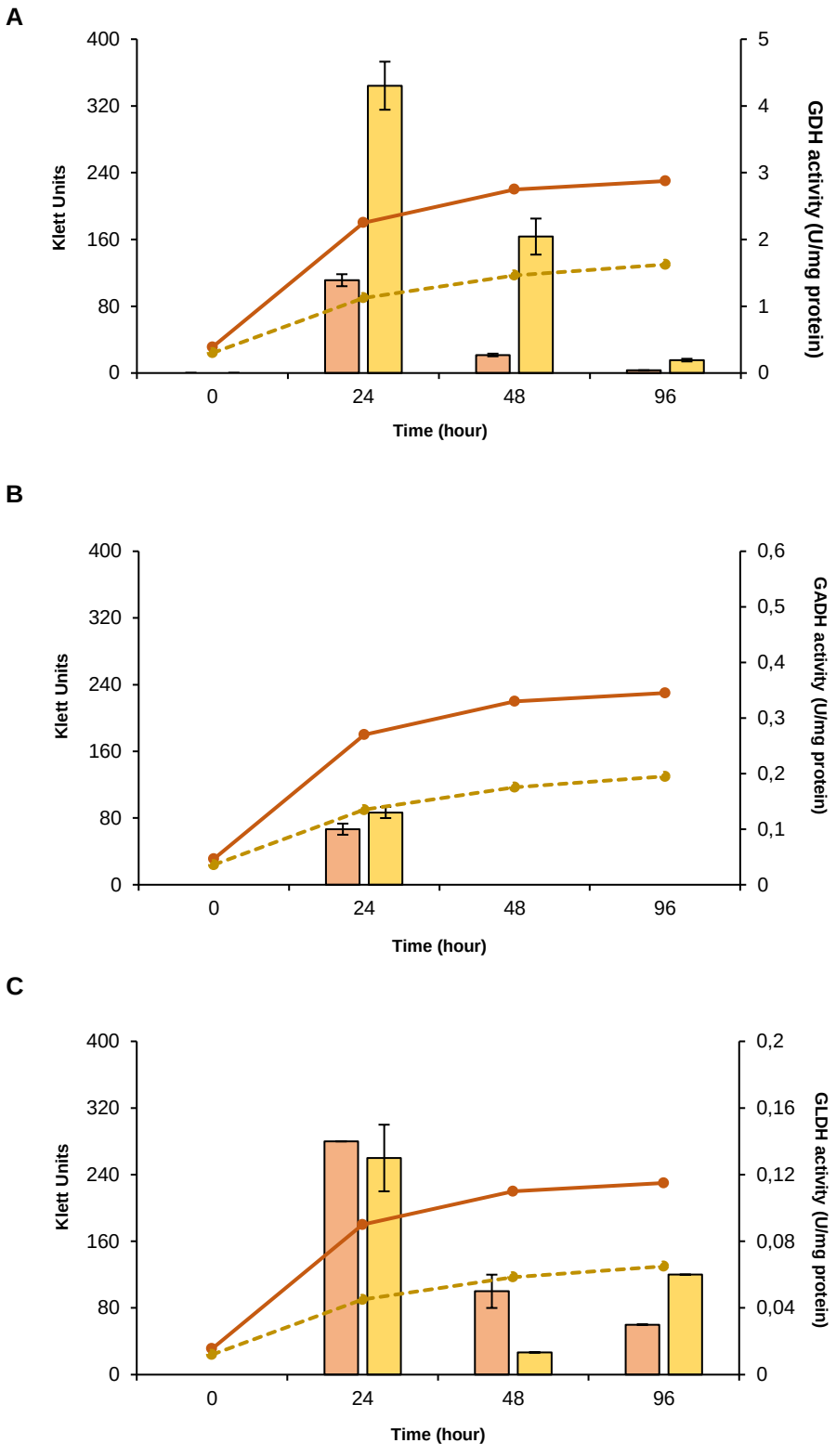

Figure 2. Enzyme activity and growth (expressed in Klett units) of two *Gluconobacter oxydans* strains at three different growth stages (24, 48 and 96 hours). **(A)** Glucose dehydrogenase activity (mGDH); **(B)** Gluconate dehydrogenase activity (GADH); **(C)** Glycerol dehydrogenase activity (GLDH); Enzyme activity represented in bars: 621H ( ), Po5 ( ) and cell growth with lines: 621H (—); Po5 (--).

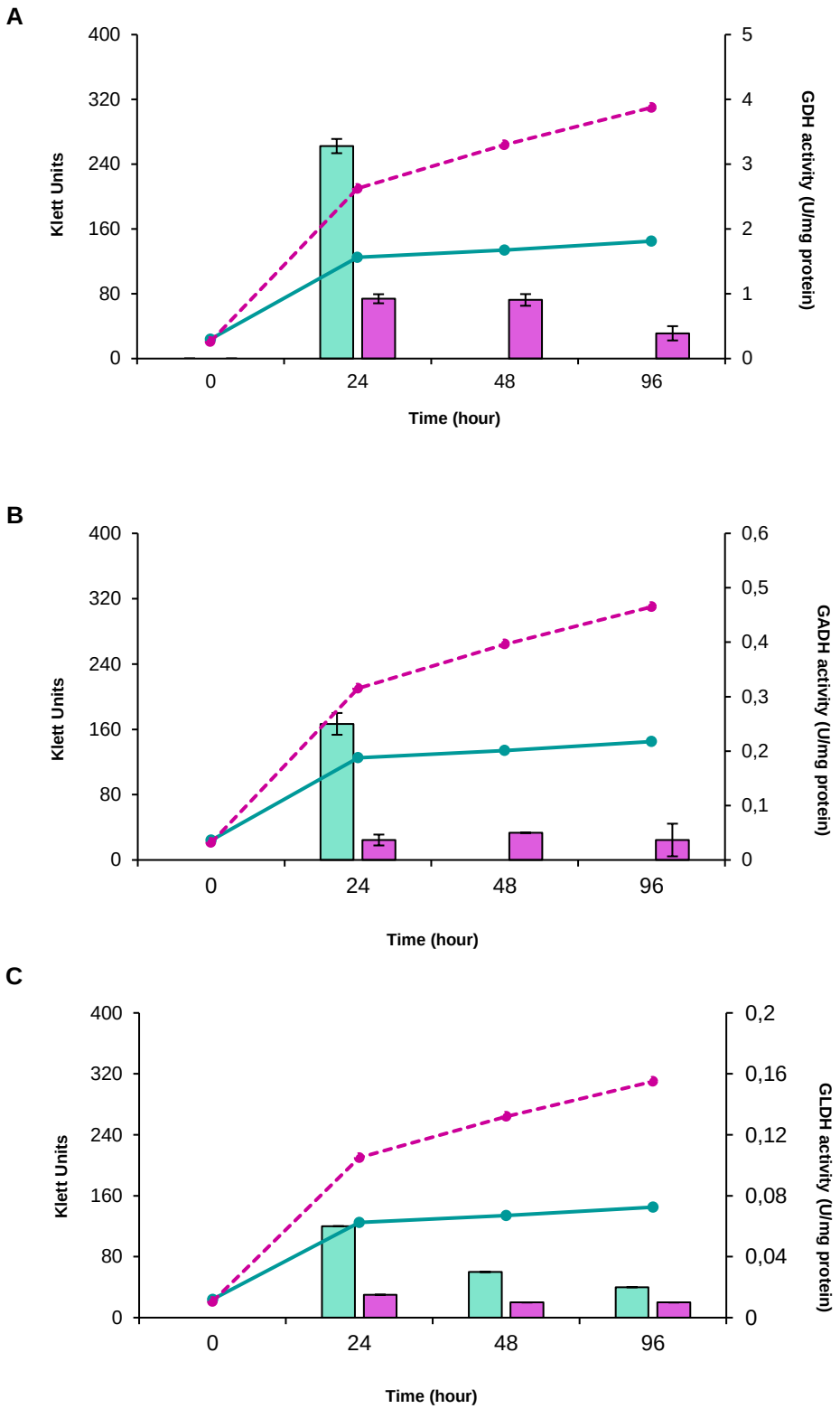

Figure 3. Enzyme activity and growth (expressed in Klett units) of two *Acetobacter malorum* strains at three different growth stages (24, 48 and 96 hours). **(A)** Glucose dehydrogenase activity (mGDH); **(B)** Gluconate dehydrogenase activity (GADH); **(C)** Glycerol dehydrogenase activity (GLDH). Enzyme activity represented in bars: NBRC 108912 (▮), CECT 7742 (▮) and cell growth with lines: NBRC 108912 (—); CECT 7742 (--).

In *G. japonicus* and *G. oxydans* strains, D-glucose was totally exhausted at 24 h, when the maximum accumulation of GA in the medium was observed (Figure 4A, B). Moreover, in *G. japonicus* strains, the depletion of D-glucose appeared to be correlated with the beginning of the oxidation of GA, resulting in the accumulation of 2KGA and 5KGA in the medium. Unlike what happened in strain NBRC 3271, where the initial accumulation of both keto-D-gluconates was similar, in CECT 8443, (Figure 4B) the accumulation of 2KGA occurred before, not detecting 5KGA until 48 h. Both *G. japonicus* strains accumulated more 2KGA than 5KGA, although this difference was really remarkable in the type strain, in which the 2KGA concentration was three times higher than 5KGA. The consumption of GA was not observed in *G. oxydans* strains, and it mostly accumulated in the medium (Figure 4C, D). However, strain 621H produced 5KGA in similar amounts to those obtained with *G. japonicus* NBRC 3271. This accumulation of 5KGA compensated for the lower accumulation of GA in this strain 621H compared with Po5. In the *A. malorum* strains, only NBRC 108912 (Figure 4E) consumed all D-glucose at the first 24 h, whereas CECT 7742 (Figure 4F) consumed the substrate by 48 h. Moreover, after the maximal accumulation of GA (24 h in both *A. malorum* strains), 56% of GA produced was further oxidized in NBRC 108912, whereas only 19% was further oxidized in CECT 7742. CECT 7742 accumulated four times more 2KGA than NBRC 108912.

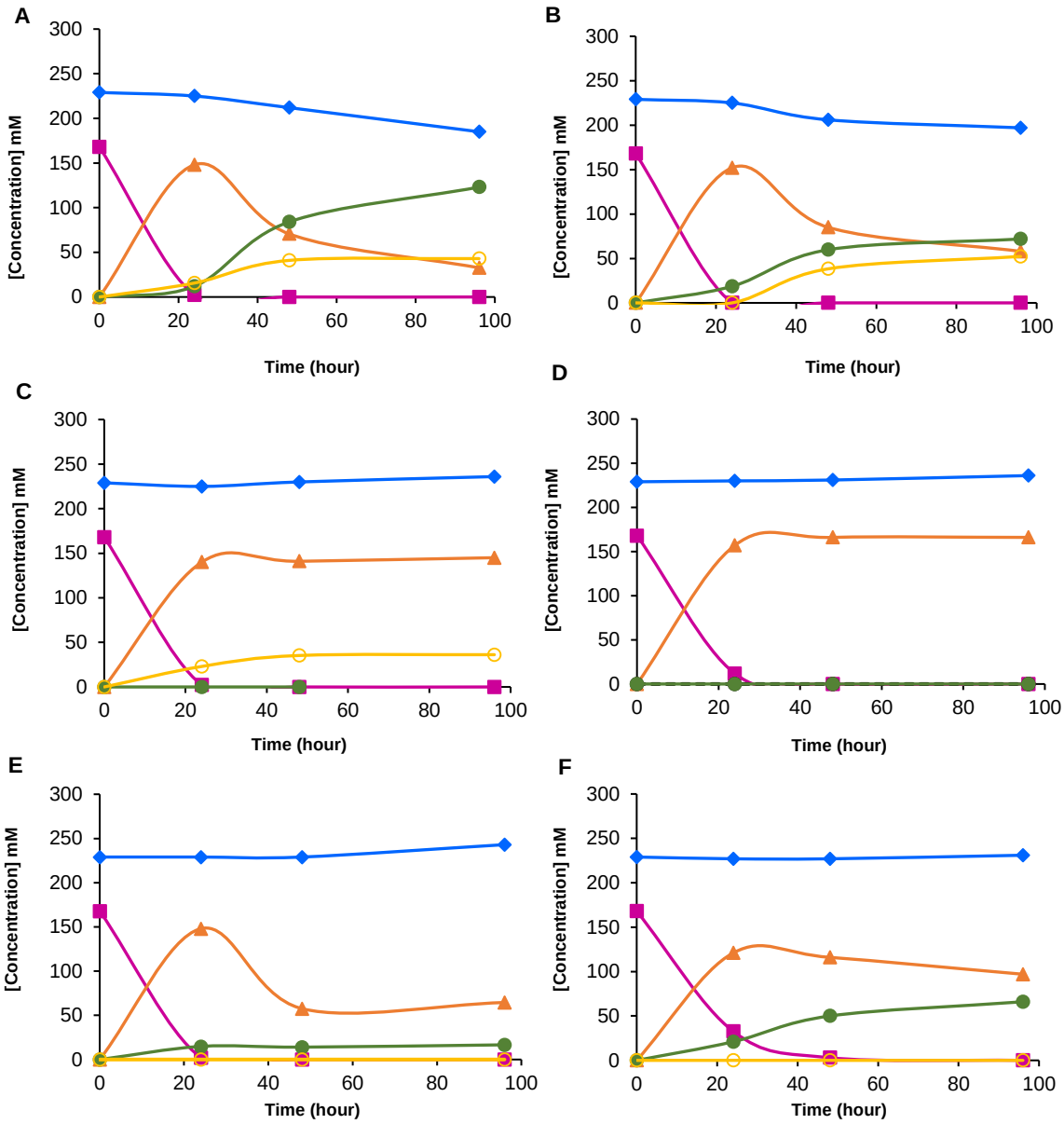

Figure 4. Evolution of metabolites derived from D-glucose oxidation by six different strains of acetic acid bacteria. **(A)** *Gluconobacter japonicus* NBRC 3271; **(B)** *Gluconobacter japonicus* CECT 8443; **(C)** *Gluconobacter oxydans* 621H; **(D)** *Gluconobacter oxydans* Po5: **(E)** *Acetobacter malorum* NBRC 108912; **(F)** *Acetobacter malorum* CECT 7742. D-glucose (■); D-fructose (◆); D-gluconic acid (▲); 2-keto-D-gluconic acid (●); 5-keto-D-gluconic acid (○).

The presence of the genes coding for the measured enzymes (*gcd*, *gnd*L, *sld*A and *kgd*L) was confirmed by the amplification of a fragment of these genes. All primer sequences were designed from already available genome sequences of these three AAB species (see Table 2). In strain NBRC 3271, because two set of genes for GADH and GLDH are present, two sets of primers (*gadh1* and *gadh2*; *gldh1* and *gldh2*) were designed (Table 2). As expected, the presence of the genes for mGDH (*gcd*) and GADH (*gndL*) was confirmed for all the strains (Table 3). However, in strain *G. japonicus* CECT 8443, only one set of primers (*gadh1*) worked for the amplification of *gndL,* and specific primers (*mgdh*) for *gcd* of NBRC 3271 did not work, although amplification was achieved with *G. oxydans* primers. In the case of the GLDH gene, no amplification was obtained in *G. oxydans* Po5 despite presenting activity, and in *G. japonicus* CECT 8443, as in GADH genes, only one set of primers (*gldh2*) worked. Finally, the 2KGDH gene (*kgdL*) was amplified only in *A. malorum* strains, although activity was not detected.

Table 3. Results of PCR analyses of *gcd, gnd*L*, sld*A*,* and *kgd*L genes, and of enzyme activity for the six tested acetic acid bacteria strains.

		gcd		***gnd*L**		***sld*A**		***kgd*L**	
Species	**Strain**	**Activity**	**Gene**	**Activity**	**Gene**	**Activity**	**Gene**	**Activity**	**Gene**
Gluconobacter japonicus	CECT 8443	+	+	+	+ [a]	+	+ [b]	-	n.d
	NBRC 3271	+	+	+	+	+	+	-	n.d
Gluconobacter oxydans	Po5	+	+	+	+	+	-	-	-
	621H	+	+	+	+	+	+	-	-
Acetobacter malorum	CECT 7742	+	+	+	+	+	n.d	-	+
	NBRC 108912	+	+	+	+	+	n.d	-	+

[a] amplified only by primers *gadh1* (Table 2).
[b] amplified only by primers *gldh2* (Table 2).
n.d: not determine.

Phylogenetic trees were constructed using the nucleotide sequences of these genes in these strains in comparison with sequences available in the GenBank Database (Figures 5-7). In all cases, AAB genera were clustered separately according to these gene sequences. In the case of the mGDH gene (Figure 5), two branches were clearly

observed; one branch included the *Komagataeibacter* and *Acetobacter* species and the branch included the *Gluconobacter* and *Asaia* species. In the *Acetobacter* branch, both *A. malorum* enzymes grouped with *A. orleanensis, A. senegalensis* and *A. tropicalis* and were separated from those of *A. pasteurianus, A. pomorum, A. ghanensis, A. syzygii* and *A. aceti*. In the case of the *Gluconobacter* cluster, different species were mixed, and no specific groupings were observed. Our *G. oxydans* enzymes grouped together while our *G. japonicus* strains were separated in different subclusters.

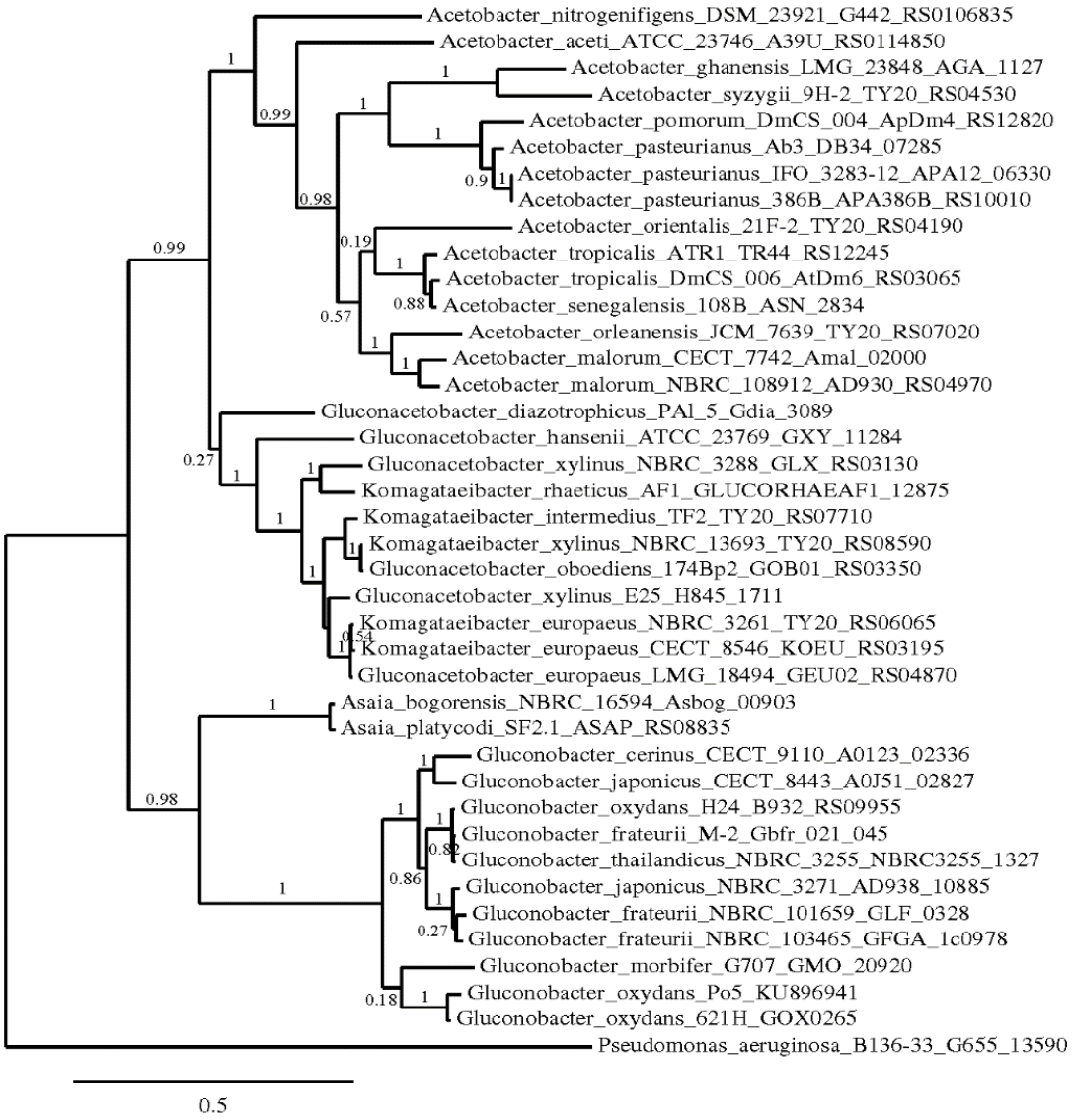

Figure 5. Phylogenetic relationship of *gcd* gene in different species of acetic acid bacteria. The entries of different genotypes include the accession numbers of the GeneBank database sequence. The *gcd* sequence of *Pseudomonas aeruginosa* B136-33 was used as outgroup. The numbers indicate the branch support values.

In the case of the GADH gene (Figure 6), the sequence of one of the genes (the one that was amplified with the set of primers *gadh2*) of *G. japonicus* NBRC 3271 appeared as outgroup. The other sequences were grouped in three clusters, one for *Acetobacter* species, another for *Komagataeibacter* species and the last one for *Gluconobacter* species together with one *Asaia* sequence. As in the mGDH tree, the two *A. malorum* enzymes grouped together. In the *Gluconobacter* cluster, there were two branches; one branch consisted of *Asaia bogorensis* sequence and *gndL* (which was amplified with set of primers *gadh2*) of a strain of *G. oxydans* (DSM 3504). All other sequences grouped together in a common branch.

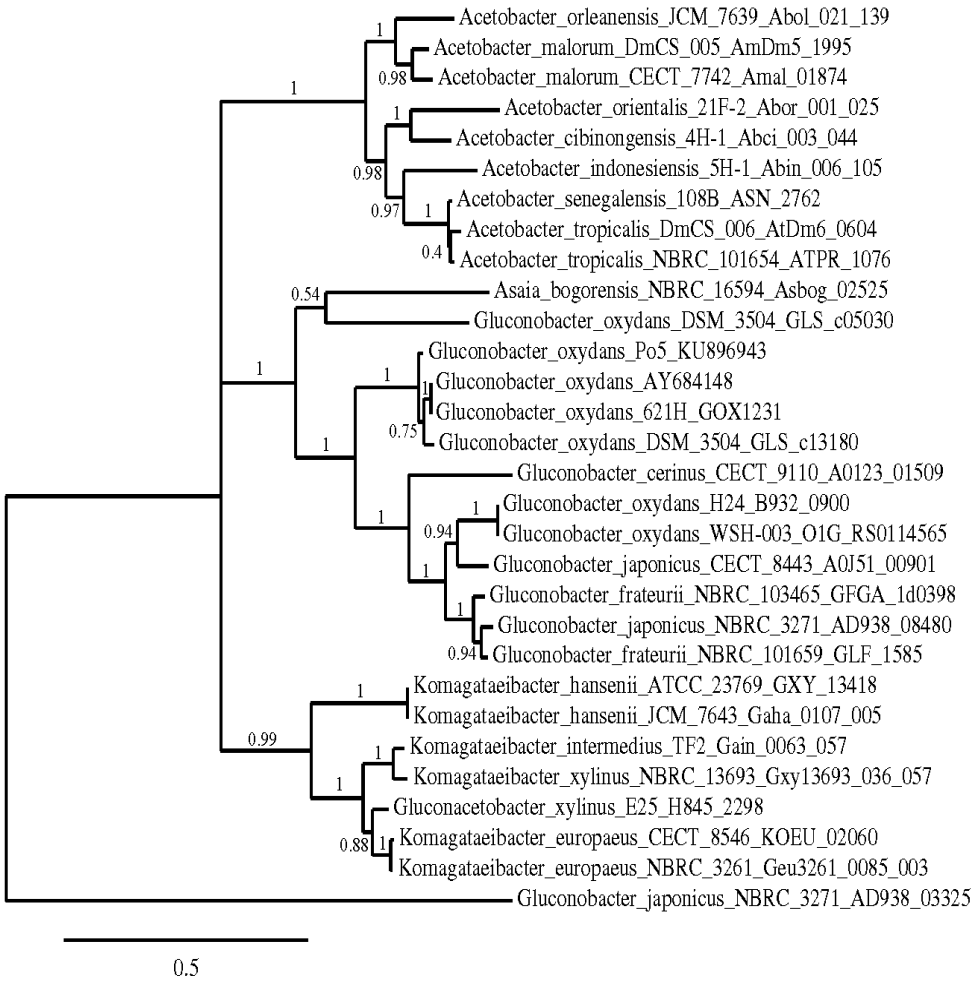

Figure 6. Phylogenetic relationship of *gnd*L gene in different species of acetic acid bacteria. The entries of different genotypes include the accession numbers of the GeneBank database sequence. The sequence of one of the genes coding for GADH in *Gluconobacter japonicus* NBRC 3271 (AD938_03325) was used as outgroup. The numbers indicate the branch support values.

Finally, in the GLDH gene (*sldA*) tree (Figure 7), the sequences were grouped in two clusters; one branch included *Gluconobacter* sequences, and the other branch included *Komagataeibacter* and *Asaia* sequences. Unlike the other genes, in this case, the *Asaia* sequences clustered with *Komagataeibacter* but not with *Gluconobacter*. No *Acetobacter* sequences have been included because this gene has not been described in this genus. In the *Gluconobacter* cluster, three different groups were clearly defined; one cluster was basically *G. oxydans* sequences, while in the other two clusters, the two homologous GLDH genes (that were amplified by the primer sets of *gldh1* and *gldh2*) of *G. japonicus* grouped separately.

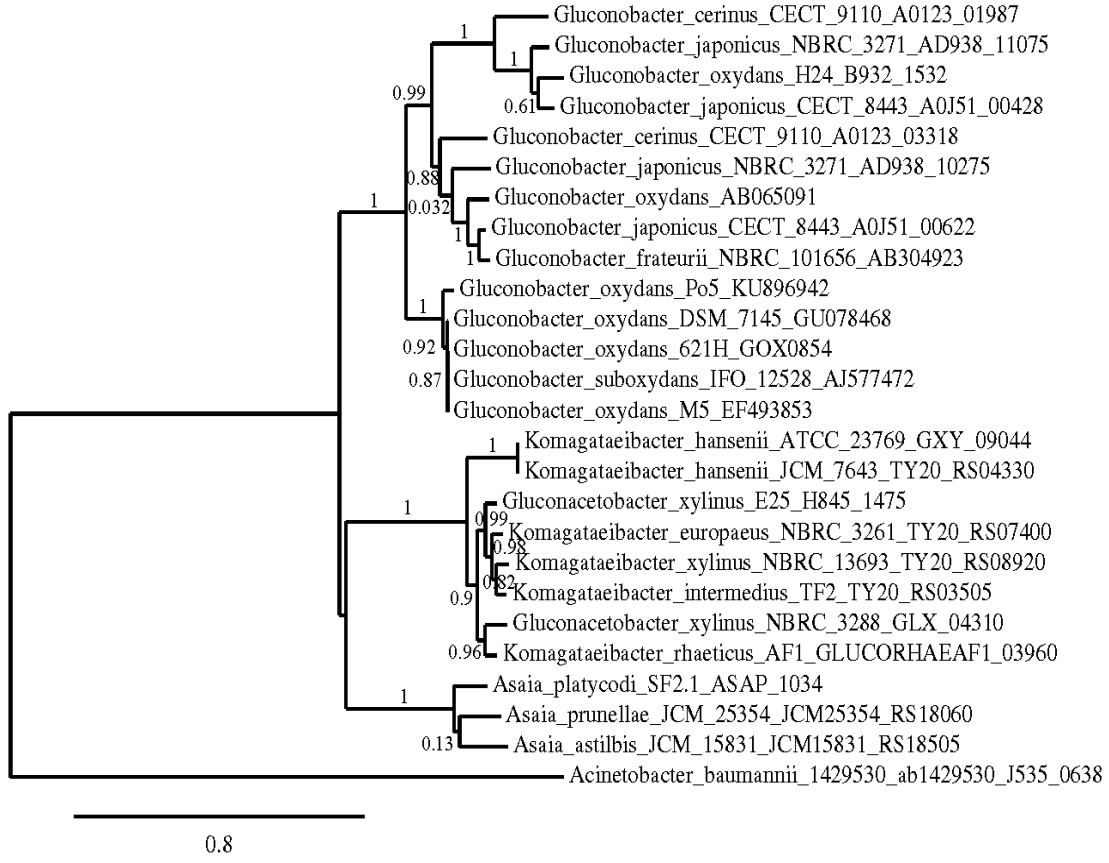

Figure 7. Phylogenetic relationship of *sld*A gene in different species of acetic acid bacteria. The entries of different genotypes include the accession numbers of the GeneBank database sequence. The *sld*A sequence of *Acinetobacter baumannii* 1429530 was used as outgroup. The numbers indicate the branch support values.

4. DISCUSSION

In a previous study (Sainz et al., 2016), we selected three strains of AAB capable of oxidizing D-glucose to GA without consuming D-fructose in a puree of strawberry with the aim of developing a new attractive fermented beverage for consumers preserving the fruit natural sweetness. The following three main requirements were decisive for the selection of these strains: a high production of GA, total consumption of D-glucose and minimal oxidation of D-fructose. Because the consumption of D-glucose and D-fructose and the production of GA were observed to be dependent on the strain and the media, the strains were mainly selected based on their behavior in strawberry puree. The selected strains were *G. japonicus* CECT 8443, *G. oxydans* Po5 and *A. malorum* CECT 7742, depending on the desired final product (final concentration of GA and keto-D-gluconates). Understanding the differences in the production of these compounds in these strains could help control the beverage composition in a more effective and reproducible way.

It has been extensively described that AAB present high numbers of membrane-bound dehydrogenases, classified as quinoproteins and flavoproteins - cytochrome *c* complex, involved in incomplete oxidation of sugars and alcohols to produce the corresponding sugar acids which are accumulated in the medium (Matsushita et al., 1994; Adachi et al., 2007; Matsushita et al., 2004). This feature is essential for industrial applications of these organisms (Meyer et al., 2013). In our study, different AAB strains were collected at different growth phases, and the activity of membrane-bound dehydrogenases involved in D-glucose oxidation and the accumulation of corresponding metabolites were studied. According to the growth of the strains, differences in the maximal population were observed both among species and between strains within the same species, reaching in some cases double the population size. Only *G. japonicus* strains showed identical growth, achieving the highest population of all the studied species. A low biomass formation has been associated with high oxidation rates (Elfari et al., 2005); the more biomass produced, the less D-glucose used for product formation. Krajewski et al. (2010)

explained this low biomass when D-glucose is used as carbon source because most D-glucose is metabolized rapidly to GA and its derivatives in the periplasm, and therefore, it could not be used for biomass formation, just for the generation of proton motive force. In our case, no important differences were observed between the species or strains because D-glucose was completely depleted in all the cases, and the maximal accumulation of GA in the medium was similar, except for strain CECT 7742 belonging to *A. malorum* that accumulated approximately 30 mM less of GA. In this case, the lower accumulation of GA was compensated by a high concentration of 2KGA. Therefore, there was no correlation between low growth and high GA and keto-D-gluconates production, likely because growth in all cases was low, suggesting that the amount of D-glucose used for biomass was only a minor part of the initial D-glucose, and the differences observed in growth were not reflected in metabolite production. This low growth confirms that the oxidation of D-glucose to GA and keto-D-gluconates has a negative effect on the growth rate and the growth yield as stated by Krajewski et al. (2010) for *G. oxydans*. All strains accumulated GA in the medium, being maximal at 24 h, in parallel with the maximal activity of mGDH. This high enzyme activity at the late exponential phase and its subsequent decrease agree with the findings of Matsushita et al. (1980), who described that mGDH activity reached the maximum activity in the mid-to late exponential phase of cultivation and then decreased with progress of growth. Ameyama et al. (1981) observed that the higher formation of this enzyme was achieved at the late exponential phase, between 24 and 30 h, depending on the fermenter used, when AAB grew on a medium containing D-glucose, glycerol and sodium-D-gluconate. The activity levels obtained in this study are consistent with those found in the literature (Ameyama et al., 1981; Matsushita et al., 1987, 1989; Meyer et al., 2013), showing similar or even higher values. Important differences in mGDH activity among strains were observed, and these differences were especially relevant in the case of selected *Gluconobacter* strains with a high activity along the process in comparison to culture collection strains. However, in practically all cases, similar GA concentrations were detected, probably due

to a limitation of substrate (D-glucose) in the media. Moreover, as the first sampling point of activity (24 h) already showed the highest enzyme activity, a similar evolution over time was observed in the enzyme activities between strains.

Regarding GADH, all our tested strains showed GADH activity. Shinagawa et al. (1976) previously reported this activity in the cell free extracts in strains of *Gluconobacter* and *Acetobacter* together with strains from other bacteria (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Serratia marcescens*). In our study, *G. japonicus* NBRC 3271 presented the highest activity of GADH and the highest accumulation of 2KGA, whereas *G. oxydans* strains did not accumulate any 2KGA despite presenting small activity only at 24 h. Strains from different species of the *Gluconobacter* genus are reported to accumulate high concentrations of 2KGA and/or 5KGA from D-glucose or GA without any appreciable assimilation into cells (Sievers and Swings, 2005). Moreover, a sequential accumulation of GA and keto-D-gluconates during the growth of *G. oxydans* 621H and other *Gluconobacter* species on D-glucose media with controlled pH has been described (Weenk et al., 1984), which is in agreement with our results. However, Levering et al. (1988) showed that *G. oxydans* 621H growing in yeast extract medium containing 50 mM D-glucose was able to oxidize quantitatively D-glucose to GA, without the production of 2KGA and 5KGA, similar to our observations with strain Po5. This lack of keto-D-gluconates synthesis in some strains has been associated with the fact that during the first phase of growth on D-glucose in batch cultures, the oxidation of D-glucose by mGDH was so rapid that the respiratory chain becomes saturated. For this reason, the ubiquinone was unable to accept electrons from GADH, resulting in the impossibility of oxidizing the GA in these conditions (Levering et al., 1988). Therefore, strain Po5, with high production of GA but no accumulation of keto-D-gluconates, appeared to be the best strain to obtain and maintain high concentrations of GA in the fermented beverage. In our previous study (Sainz et al., 2016), we tested different media, and this strain was the strain with the highest production of GA and the lowest production of keto-D-gluconates. Diverse studies in *Gluconobacter* strains showed differences in the rate of

2KGA or 5KGA from D-glucose (Weenk et al., 1984; Silberbach et al., 2003; Herrmann et al., 2004; Elfari et al., 2005). The individual product yields vary among different strains and depend also on the media and on the particular conditions used for cultivation (Asai 1968; Olijve and Kok, 1979). GLDH and GADH enzymes compete for the oxidation of GA; therefore, selective expression of either dehydrogenase could increase the production of either of the keto-D-gluconates (Matsushita et al., 2003; Elfari et al., 2005). In our conditions, only *G. japonicus* strains were able to accumulate both keto-D-gluconates. These strains present two genes for GLDH, and strain NBRC 3271 also presents two genes for GADH. However, strain CECT 8443 has only one gene for GADH with a sequence similar to the gene, which was amplified with primers *gadh1* of strain NBRC3271.

Gluconobacter oxydans 621H only accumulated 5KGA. In other studies and culture conditions, 621H exhibited different keto-D-gluconate synthesis profiles, varying from the accumulation of both keto-D-gluconates (Weenk et al., 1984) or no keto-D-gluconates synthesis, confirming that culture conditions are essential for the synthesis of these compounds. The other strain belonging to *G. oxydans*, Po5, did not accumulate any keto-D-gluconate, despite having a similar GLDH activity to 621H. A lack of amplification of the GLDH gene (*sldA*) was observed in this strain (Po5). The 621H *sldA* sequence was used for the design of the primers, and although this gene sequence in both strains is similar (> 96%), there are some nucleotide differences in the region where the reverse primer hybridized (results not shown). In *G. japonicus,* strain NBRC 3271 showed the highest GLDH activity at 24 h and after a decrease, although the 5KGA concentration was increasing until 48 h. Instead, in CECT 8443, the increase in GLDH activity between 24 and 48 h was correlated with the increase in the 5KGA accumulation. *A. malorum* strains presented both activities (GADH and GLDH), but no accumulation of 5KGA was detected. A lack of 5KGA synthesis was expected according to the *A. malorum* description (synthesis of 2KGA and lack of 5KGA synthesis) (Cleenwerck et al., 2002). However, the activity detected in this study together with the accumulation of this

compound by strain CECT 7742 in a previous study (Sainz et al., 2016) appear to confirm that this species or some strains belonging to this species are able to synthesize 5KGA. It has to be emphasized that this previous production of 5KGA was observed in different medium conditions. The absence of the *sldA* sequence in the *A. malorum* genome appears to suggest the possibility that other enzymes for the synthesis of this compound are used. Furthermore, strain NBRC 108912 showed a high decrease in the GA concentration that cannot be only accounted for the oxidation to 2KGA. Nevertheless, the products of D-glucose oxidation have been reported to be assimilated by cytoplasmic reductases during the stationary phase, and then introduced to the pentose-phosphate pathway to produce cell biomass (Saichana et al., 2015). However, this would have as consequence a second phase of growth that was not observed in our case.

A phylogenetic study using the sequences of these three key enzymes for D-glucose oxidation in AAB was performed; in all the cases, trees that showed clear clusters according to the genus were obtained. Gene *gcd* was the one with more sequences available in the GenBank database, allowing for a more reliable study. Based on the *gcd* sequences, the *Acetobacter* and *Komagataeibacter* species seemed to be more closely related, and *Gluconobacter* was more related to *Asaia,* which is different to the findings obtained using the 16S rRNA gene sequence (Yamada et al., 2012). However, this difference should not be surprising because the D-glucose metabolism of these two genera is closer than in the other genera, which have a higher preference for other substrates, such as ethanol. The *Gluconobacter* and *Asaia* genera were reported to develop better in media enriched with sugar (Raspor and Goranovic 2008), with high oxidation activity of sugar and sugar alcohols (D-glucose, GA, D-sorbitol, and glycerol). In addition, Matsutani et al. (2011) claimed that the *Acetobacter* and *Komagataeibacter* species are more closely related to each other than *Gluconobacter* by whole genome level phylogenetic analysis. Therefore, our results agree with this previous work.

For our results, the concentrations of D-glucose and GA show an effective, almost equimolar conversion, which takes place during the first 24 h and is likely to the end of

the exponential phase of growth (Matsushita et al., 1980; Ameyama et al., 1981). At this time, the mGDH activity is the highest during the studied period. The absence of the main substrate makes its activity unnecessary and therefore declines afterwards. It could be assumed that during the first 24 h, the high activity of this enzyme accounts for the full transformation of D-glucose into GA, which occurs in all the species and strains observed. However, the transformation of GA is heavily dependent on the species and the strain (Asai, 1968; Olijve and Kok, 1979; Weenk et al., 1984). Regardless of the presence of the activities of GADH and GLDH in *G. oxydans* natural strain Po5, no further oxidation of GA to keto-D-gluconates was observed. In fact, no production of 5-KGA was detected despite the high activity of GLDH in *G. oxydans,* showing a lack of correlation between the activity and products that could be explained by the lack of specificity of this enzyme (Matsushita et al., 2003). Instead, the absence of GADH activity after 24 h correlated with the lack of 2KGA production in both *G. oxydans* strains. In *G. japonicus* and *A. malorum*, the production of 2KGA was always observed, although no correlation could be found between the activity and the products. However, when activity of GADH was detected after 24 h, albeit it was low, important accumulation of 2KGA in the medium was observed (higher than 50 mM). Comparing between the three selected strains, important differences were observed at the activity level of these enzymes. Both *Gluconobacter* strains (CECT 8443 and Po5) presented a very high activity of mGDH at 24 h, with a further decrease and low activity in the GADH and GLDH but with changes overtime, whereas CECT 7742 presented in the three enzymes a low activity but maintained practically constant throughout all the time.

The possible use of these different strains and species for the production of different concentrations of GA and its derivatives could be achieved through the thorough knowledge of the activity and the expression of the enzymes. However, our results also indicate that the conditions of the process and the composition of the medium are crucial to the final composition of the product because important differences were observed in the synthesis profile of these strains in different media or conditions (Sainz et al., 2016).

Therefore, a next step should be the analysis of the expression of these genes (especially mGDH and GADH) in different conditions to fully understand and control the process of the oxidation of D-glucose by AAB.

ACKNOWLEDGMENTS

This work was supported by the grant AGL2010-22152-C03-02 from the Spanish Ministry of Science and Innovation. F. Sainz holds a FI Fellowship from the Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR), Government of Catalonia.

REFERENCES

Adachi, O., Moonmangmee, D., Toyama, H., Yamada, M., Shinagawa, E., Matsushita, K. (2003). New developments in oxidative fermentation. *Appl. Microbiol. Biotechnol*. 60, 643-653.

Adachi, O., Ano, Y., Toyama, H., Matsushita, K. (2007). "Biooxidation with PQQ- and FAD-Dependent Dehydrogenases," in *Modern Biooxidation Enzymes, Reactions and Applications*, eds. R.D. Schmid and V.B. Urlacher (Weinheim: Wiley-vch Verlag GmbH & Co KGaA).

Ameyama, M., Shinagawa, E., Matsushita, K., Adachi, O. (1981). D-Glucose dehydrogenase of *Gluconobacter suboxydans*: solubilization, purification and characterization. *Agr. Bio. Chem. Tokyo* 45, 851-861.

Asai, T. (1968) Acetic acid bacteria: classification and biochemical activities. Tokyo: University of Tokyo Press.

Asano, T., Yuasa, K., Kunugita, K., Teraji, T., Mitsuoka, T. (1994). Effects of gluconic acid on human faecal bacteria. *Microb. Ecol. Health D.* 7, 247-256.

Asano, T., Yuasa, K., Yoshimura, Y., Takenawa, S., Fukuba, H. (1997). Digestion, absorption and intestinal residue of various gluconic acids in rats. *J. Jp. Soc. Nutr. Food. Sci*. 50, 287–294.

Cañete-Rodríguez, A.M., Santos-Dueñas, I.M., Torija, M.J., Mas, A., Jiménez-Hornero, J.E., García-García, I. (2015). Preparation of a pure inoculum of acetic acid bacteria for the selective conversion of glucose in strawberry purée into gluconic acid. *Food Bioprod. Process*. 96, 35-42.

Cañete-Rodríguez, A.M., Santos-Dueñas, I.M., Jiménez-Hornero, J.E., Torija, M.J., Mas, A., García-García, I. (2016). Revalorization of strawberry surpluses by bio-transformation its glucose content into gluconic acid. *Food Bioprod. Process*. 99, 188-196.

Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol. Biol. Evol.* 17, 540-552.

Cleenwerck, I., Vandemeulebroecke, K., Janssens, D., Swings, J. (2002). Re-examination of the genus *Acetobacter*, with descriptions of *Acetobacter cerevisiae* sp. nov and *Acetobacter malorum* sp. nov. *Int. J. Syst. Evol. Micr*. 52, 1551-1558.

De Ley, J. (1961). Comparative carbohydrate metabolism and a proposal for the phylogenetic relationship of the acetic acid bacteria. *J. Gen. Microbiol.* 24, 31-50.

De Ley, J., and Swings, J. (1984). "Genus *Gluconobacter*," in *Bergey´s Manual of Systematic Bacteriology,* eds. N.R. Kreig and J.G. Holt (USA, Williams and Wilkins), 275-278.

Deppenmeier, U., Hoffmeister, M., Prust, C. (2002). Biochemistry and biotechnological applications of *Gluconobacter* strains. *App. Microbiol. Biotechnol*. 60, 233-242.

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.F., Guindon, S., Lefort, V., Lescot, M., Claverie, J.M., Gascuel, O. (2008). Phylogenyfr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res.* 36, 465-469.

Dereeper, A., Audic, S., Claverie, J.M., Blanc, G. (2010). BLAST-EXPLORER helps you building datasets for phylogenetic analysis. *BMC Evol. Biol.* 10, 1-6.

Dulley, J.R., Grieve, P.A. (1975). A simple technique for eliminating interference by detergents in the Lowry method of protein determination. *Anal. Biochem*. 64, 136–141.

Edgar, R.C. (2004a). MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* 5, 1-19.

Edgar, R.C. (2004b). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res*. 32, 1792-1797.

Elfari, M., Ha, S.W., Bremus, C., Merfort, M., Khodaverdi, V., Herrmann, U., Sahm, H., Görisch, H. (2005). A *Gluconobacter oxydans* mutant converting glucose almost quantitatively to 5-keto-D-gluconic acid. *App. Microbiol. Biotechnol*. 66, 668-674.

Gupta, A., Singh, V.K., Qazi, G.N., Kumar, A. (2001). *Gluconobacter oxydans*: It´s biotechnological applications. *J. Mol. Microbiol. Biotechnol*. 3, 445-456.

Herrmann, U., Merfort, M., Jeude, M., Bringer-Meyer, S., Sahm, H. (2004). Biotransformation of glucose to 5-keto-D-gluconic acid by recombinant *Gluconobacter oxydans* DSM 2343. *App. Microbiol. Biotechnol*. 64, 86-90.

Hidalgo. C., Torija, M.J., Mas, A., Mateo, E. (2013). Effect of inoculation on strawberry fermentation and acetification processes using native strains of yeast and acetic acid bacteria. *Food Microbiol*. 34, 88-94.

Krajewski, V., Simic, P., Mouncey, N.J., Bringer, S., Sahm, H., Bott, M. (2010). Metabolic engineering of *Gluconobacter oxydans* for improved growth rate and growth yield on glucose by elimination of gluconate formation. *Appl. Environ. Microb*. 76, 4369-4376.

Levering, P.R., Weenk, G., Olijve, W., Dijkhuizen, L., Harder, W. (1988). Regulation of gluconate and ketogluconate production in *Gluconobacter oxydans* ATCC 621-H. *Arch. Microbiol*. 149, 534-539.

Lino, T., Suzuki, R., Kosako, Y., Ohkuma, M., Komagata, K., Uchimura, T. (2012). *Acetobacter okinawensis* sp.nov, *Acetobacter papayae* sp.nov, and *Acetobacter persicus* sp.nov; novel acetic acid bacteria isolated from stems of sugarcane, fruits, and a flower in Japan. *J. Gen. App. Microbiol*. 58, 235-243.

Macauley, S., McNeil, B., Harvey, L.M. (2001). The genus *Gluconobacter* and its applications in biotechnology. *Cri. Rev. Biotechnol*. 21, 1-25.

Malimas, T., Yukphan, P., Takahashi, M., Muramatsu, Y., Kaneyasu, M., Potacharoen, W., Tanasupawat, S., Nakagawa, Y., Tanticharoen, M., Yamada, Y. (2009). *Gluconobacter japonicus* sp.nov, an acetic acid bacterium in the *Alphaproteobacteria*. *Int. J. Syst. Evol. Microbiol*. 59, 466-471.

Matsushita, K., Yamada, M., Shinagawa, E., Adachi, O., Ameyama, M. (1980). Membrane-bound respiratory chain of *Pseudomonas aeruginosa* grown aerobically. *J Bacteriol*. 141, 389-392.

Matsushita, K., Ameyama, M. (1982). D-glucose dehydrogenase from *Pseudomonas fluorescens*, membrane-bound. *Methods Enzymol*. 89, 149-154.

Matsushita, K., Nonobe, M., Shinagawa, E., Adachi, O., Ameyama, M. (1987). Reconstitution of pyrroloquinoline quinone-dependent D-glucose oxidase respiratory chain of *Escherichia coli* with cytochrome o oxidase. *J. Bacteriol*. 169, 205-209.

Matsushita, K., Shinagawa, E., Adachi, O., Ameyama, M. (1989). Quinoprotein D-glucose dehydrogenase of the *Acinetobacter calcoaceticus* species respiratory chain: membrane-bound and soluble forms are different molecular species. *Biochemistry* 28, 6276-6280.

Matsushita, K., Toyama, H., Adachi, O. (1994). “Respiratory chains and bioenergetics of acetic acid bacteria,” in *Advances in Microbial Physiology*, eds. A.H. Rose and D.W. Tempest (London, Academic Press Ltd), 247-301.

Matsushita, K., Fujii, Y., Ano, Y., Toyama, H., Shinjoh, M., Tomiyama, N., Miyazaki, T., Sugisawa, T., Hoshino, T., Adachi, O. (2003). 5-Keto-D-Gluconate production is catalyzed by a quinoprotein glycerol dehydrogenase, major polyol dehydrogenase, in *Gluconobacte*r species. *Appl. Environ. Microb*. 69, 1959-1966.

Matsushita, K., Toyama, H., Adachi, O. (2004). “Respiratory chains in acetic acid bacteria: Membrane-bound periplasmic sugar and alcohol respirations,” in *Respiration in Archea and Bacteria Vol 2: Diversity of Prokaryotic Respiratory Systems*, ed. D. Zannoni (The Netherlands, Springer).

Matsutani, M., Hirakawa, H., Yakushi, T., Matsushita, K. (2011). Genome-wide phylogenetic analysis of *Gluconobacter*, *Acetobacter*, and *Gluconacetobacter*. *FEMS Microbiol. Lett.* 315, 122-128.

Merfort, M., Herrmann, U., Bringer-Meyer, S., Sahm, H. (2006). High-yield 5-keto-D-gluconic acid formation is mediate by soluble and membrane-bound gluconate-5-dehydrogenases of *Gluconobacter oxydans*. *App. Microbiol. Biotechnol*. 73, 443-451.

Meyer, M., Schweiger, P., Deppenmeier, U. (2013). Effects of membrane-bound glucose dehydrogenase overproduction on the respiratory chain of *Gluconobacter oxydans*. *App. Microbiol. Biotechnol*. 97, 3457-3466.

Navarro, D., Mateo, E., Torija, M.J., Mas, A. (2013). Acetic acid bacteria in grape must. *Acetic acid bacteria* 2, 19-23.

Olijve, W., Kok, J.J. (1979). Analysis of growth of *Gluconobacter oxydans* in glucose containing media. *Arch. Microbiol*. 121, 283-290.

Prust, C., Hoffmeister, M., Liesegang, H., Wiezer, A., Fricke, W.F., Ehrenreich, A., Gottschalk, G., Deppenmeier, U. (2005). Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydans*. *Nat. Biotechnol*. 23,195-200.

Ramachandran, S., Fontanille, P., Pandey, A., Larroche, C. (2006). Gluconic acid: properties, applications and microbial production. *Food Technol. Biotech*. 44, 185-195.

Raspor, P., Goranovic, D. (2008). Biotechnological applications of acetic acid bacteria. *Crit. Rev. Biotechnol*. 28, 101-124.

Saichana, N., Matsushita, K., Adachi, O., Frébort, I., Frebortova, J. (2015). Acetic acid bacteria: A group of bacteria with versatile biotechnological applications. *Biotechnol. Adv*. 33, 1260-1271.

Sainz, F., Navarro, C., Mateo, E., Torija, M.J., Mas, A. (2016). Comparison of D-gluconic acid production in selected strains of acetic acid bacteria. *Int. J. Food. Microbiol*. 222, 40-47.

Shinagawa, E., Chiyonobu, T., Adachi, O., Ameyama, M. (1976). Distribution and solubilization of particulate gluconate dehydrogenase and particulate 2-ketogluconate dehydrogenase in acetic acid bacteria. *Agr. Biol. Chem*. 40, 475 -483.

Shinagawa, E., Matsushita, K., Toyama, H., Adachi, O. (1999). Production of 5-keto-D-gluconate by acetic acid bacteria is catalyzed by pyrroloquinoline quinone (PQQ)-dependent membrane-bound D-gluconate dehydrogenase. *J. Mol. Catal. B: Enzym*. 6, 341-350.

Sievers, M., and Swings, J. (2005). "Family *Acetobacteriaceae* Gills and De Loy 1980," in *Bergey's Manual of Systematic Bacteriology*, ed. G.M. Garrity, M. Winters, D.B. Searles (Springer, NewYork).

Silberbach, M., Maier, B., Zimmermann, M., Buchs, J. (2003). Glucose oxidation by *Gluconobacter oxydans*: characterization in shaking-flasks, scale-up and optimization of the pH profile. *App. Microbiol. Biotechnol*. 62, 92-98.

Talavera, G., Castresana, J. (2007). Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Syst. Biol*. 56, 564-577.

Toyama, H., Furuya, N., Saichana, I., Ano, Y., Adachi, O., Matsushita, K. (2007). Membrane-bound, 2-keto-D-gluconate-yielding D-gluconate dehydrogenase from "*Gluconobacter dioxyacetonicus*" IFO 3271: molecular properties and gene disruption. *Appl. Environ. Microb*. 73, 6551-6556.

Tsukahara, T., Koyama, H., Okada, M., Ushida, K. (2002). Stimulation of butyrate production by gluconic acid in batch culture of pig cecal digesta and identification of butyrate-producing bacteria. *J. Nutr*. 132, 2229-2234.

Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., Leunissen, J.A. (2007). Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids. Res*. 35, 71-74.

Vegas, C., Mateo, E., González, A., Jara, C., Guillamón, J.M., Poblet, M., Torija, M.J., Mas, A. (2010). Population dynamics of acetic acid bacteria during traditional wine vinegar production. *Int. J. Food Microbiol*. 138, 130-136.

Weenk, G., Olijve, W., Harder, W. (1984). Ketogluconate formation by *Gluconobacter* species. *App. Microbiol. Biotechnol*. 20, 400-405.

Wood, W.A., Fetting, R.A., Hertlein, B.C. (1962). "Gluconic dehydrogenase from *Pseudomonas fluorescens*," in *Methods in Enzymology, vol V,* ed. S.P. Colowick and N.O. Kaplan (New York, Academic Press Inc), 287-291.

Yamada, Y., Yukphan, P., Lan Vu, H.T., Muramatsu, Y., Ochaikul, D., Nakagawa, Y. (2012). Subdivision of the genus *Gluconacetobacter* Yamada, Hoshino and Ishikawa 1998: the proposal of *Komagatabacter* gen. nov, for strains accommodated to the *Gluconacetobacter xylinus* group in the α-*Proteobacteria*. *Ann. Microbiol*. 62, 849-859.

CHAPTER 3

Effect of nitrogen source on the growth of different acetic acid bacteria strains

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In revision in International Journal of Food Microbiology

UNIVERSITAT ROVIRA I VIRGILI
SELECTION AND OPTIMIZATION OF ACETIC ACID BACTERIA FOR D-GLUCONIC ACID PRODUCTION
Florencia Sainz Perez

ABSTRACT

Acetic acid bacteria (AAB) are recognized as fastidious microorganisms due to the problems associated with their isolation and growth on solid media. Furthermore, insufficient information is known about the nutritional requirements of AAB for optimal growth. The aim of this work was to study the effects of different concentrations and sources of nitrogen on the growth of different AAB strains and to establish which nitrogen source best encouraged their growth. Two strains of three species of AAB, *Gluconobacter japonicus*, *Gluconobacter oxydans* and *Acetobacter malorum*, were grown in three different media with diverse nitrogen concentrations (25, 50 100, and 300 mg N/L and 1 g N/L) as a complete solution of amino acids and ammonium. With this experiment, the most favourable medium and the lowest nitrogen concentration beneficial for the growth of each strain was selected. Subsequently, under these conditions, single amino acids or ammonium were added to media individually to determine the best nitrogen sources for each AAB strain. The results showed that nitrogen requirements are highly dependent on the nitrogen source, the medium and the AAB strain. *Gluconobacter* strains were able to grow in the lowest nitrogen concentration tested (25 mg N/L); however, one of the *G. oxydans* strains and both *A. malorum* strains required a higher concentration of nitrogen (100-300 mg N/L) for optimal growth. In general, single nitrogen sources were not able to support the growth of these AAB strains as well as the complete solution of amino acids and ammonium.

Keywords: *Gluconobacter, Acetobacter*, Ammonium, Proline, Glutamine

CHAPTER 3

1. INTRODUCTION

Acetic acid bacteria (AAB) are strictly aerobic microorganisms that are particularly interesting because they are able to quickly and incompletely oxidize a large number of carbohydrates and alcohols, producing an accumulation of organic acids as the final products. This feature makes AAB useful for various biotechnological processes (Deppenmeier et al., 2002; Gullo and Giudici, 2008), such as the production of acetic acid from ethanol; D-gluconic acid, 2-keto-D-gluconic acid and 5-keto-D-gluconic acid from D-glucose; L-sorbose from D-sorbitol; and dihydroxyacetone from glycerol (Gupta et al., 2001; Lino et al., 2012; Prust et al., 2005). Nevertheless, the industrial exploitation of AAB is not fully developed (Mamlouk and Gullo, 2013), mainly due to problems with AAB recovery on solid media and their high mutability (Mas et al., 2007). For these reasons, they are considered fastidious microorganisms. To solve the problem of low AAB culturability, different culture media have been developed to improve AAB isolation from different sources, with D-glucose, ethanol and mannitol as the carbon sources most widely used for the preparation of these enrichment media (Gullo et al., 2006; Mas et al., 2007). However, relatively little new information has become available about the nitrogen and growth factor requirements in AAB since the studies about this topic conducted in the 1950s (Foda and Vaughn, 1953; Raghavendra Rao and Stokes, 1953; Rainbow and Minston, 1953). Raghavendra Rao and Stokes. (1953) reported that the growth factor requirements are critically influenced by the carbon and energy sources present in the medium. These authors also claimed the necessity of using peptone and yeast extract in culture media to ensure a sufficient supply of nitrogen for AAB growth. The problem of using these preparations is that there is no control over the nitrogen composition, and it is not possible to study AAB nitrogen requirements. Previously, Underkofler et al. (1943) reported that the use of a mixture of twenty amino acids can be used instead of hydrolysed casein for *Acetobacter suboxydans* growth, and the study also established pantothenic, nicotinic and *p*-aminobenzoic acids as the factors required for growth of this species. Later, Drysdale and Fleet. (1988) suggested that most AAB are able to grow

using inorganic ammonia as the sole source of nitrogen because they can synthesize all the amino acids from this compound; therefore, there are no essential amino acids for AAB. However, these authors also reported that some amino acids could have a stimulatory or inhibitory effect on the growth of some AAB species, and even earlier studies reported an essential role for some amino acids (Kerwar et al., 1964; Stokes and Larsen, 1945).

In recent years, studies of nitrogen and AAB have mainly focused on ensuring that there was sufficient available nitrogen and appropriate for carrying out the acetification after alcoholic fermentation by yeast. For this reason, different studies have analysed the changes in amino acids during the production of vinegars from different types of raw materials and different acetification conditions (Álvarez-Cáliz et al., 2012; Callejón et al., 2008; Maestre et al., 2008; Valero et al., 2005). The effects of various physico-chemical operations, such as flocculation and filtration, during the stabilization of must and wines (Valero et al., 2005) and the biological ageing of wine (Álvarez-Cáliz et al., 2014) on the availability of nitrogen content for AAB growth have also been studied. Although all these practices are expected to decrease the concentrations of amino acids and vitamins available for the AAB growth, there are others, such as the autolysis of yeasts at the end of the alcoholic fermentation, that have the opposite effect and favour the growth of AAB (Fleet, 2001). However, extreme media, such as wine with a low pH and a high ethanol concentration, could also modify the amino acid requirements of AAB, increasing their nutritional demand (Drysdale and Fleet, 1988). All these studies have demonstrated that AAB growth depends on the substrate used. In the case of wine vinegars, grape musts are rich in arginine and proline; moreover, the latter cannot be used by yeast (Ribéreau-Gayon et al., 2006) and is the major amino acid in wines and one of the amino acids most used by AAB (Álvarez-Cáliz et al., 2012; Callejón et al., 2008; Maestre et al., 2008). Other substrates, such as ethanol or cider, are clearly nitrogen-poor, resulting in the need to add nutrients to favour AAB growth. Therefore, the concentration and type of nitrogen

sources available for AAB growth could be a limiting factor for the best development of a specific process.

In a previous study (Sainz et al., 2016), three natural AAB strains were selected for the production of a new strawberry beverage, which was based on the production of D-gluconic acid from D-glucose to maintain the natural D-fructose from the strawberries in the final product. Two of these strains belong to the *Gluconobacter* genus: CECT 8443, a strain of *Gluconobacter japonicus* isolated from grape must (Navarro et al., 2013) and Po5, a strain of *Gluconobacter oxydans* isolated from wine vinegar (Vegas et al., 2010). The other strain, CECT 7742, belonging to *Acetobacter malorum,* was the only strain isolated from strawberry vinegar (Hidalgo et al., 2013).

Hence, the aim of this study was to determine the nitrogen requirements of these three strains, using the type strain of each species for comparison. For that reason, we first analysed the growth of the six strains in different culture media using a range of nitrogen concentrations to establish the minimum nitrogen concentration for the optimal growth of each strain. Afterwards, in the optimal medium with minimal nitrogen concentration, individual amino acids or ammonium were added to determine the best nitrogen source for each strain.

2. MATERIALS AND METHODS

2.1. Microorganisms

Two strains from three different species (*G. japonicus, G. oxydans* and *A. malorum*) of AAB were used in the study (Table 1). All the strains were initially grown in GY liquid media (5% (w/v) D-glucose and 1% (w/v) yeast extract; Panreac, Barcelona, Spain) at 28 ºC with shaking (125 rpm).

Table 1. Strains used in this study

Species	Strain	Source	Reference
Gluconobacter japonicus	NBRC 3271[T]	*Myrica rubra*	Malimas et al., (2009)
	CECT 8443	Grape Must	Navarro et al. (2013)
Gluconobacter oxydans	621 H	-	(Henneberg, 1987) De Ley . (1961)
	Po5	Vinegar	Vegas et al. (2010)
Acetobacter malorum	NBRC 108912 [T]	Rotting apple	Cleenwerck et al., (2002)
	CECT 7742*	Strawberry vinegar	Hidalgo et al. (2013)

[T] Type strains
[a] This strain has been incorrectly named CECT 7749 in previous studies (Hidalgo et al., 2013 and Sainz et al., 2016).

2.2. Determination of nitrogen requirements

2.2.1. Media used

The effect of the nitrogen source on the growth of the strains was tested in three different media: synthetic medium (SM) prepared according to Riou et al. (1997); yeast nitrogen base medium (YNB; yeast nitrogen base without amino acids (Becton Dickinson & Co, Franklin Lakes, NJ, USA)); and M9 minimal medium (Harwood and Cutting, 1990). For the preparation of M9, a concentrated salt solution (5X) with 64 g/L sodium hydrogen phosphate heptahydrate, 15 g/L monopotassium phosphate and 2.5 g/L sodium chloride was first prepared for a stock solution. Then, to prepare 1 L of the M9 media, 200 mL of this concentrated salt solution was mixed with 2 mL magnesium sulfate (1 M), 0.1 mL calcium chloride (1 M), and different nutrient solutions (sugar, nitrogen, vitamins) and brought to 1 L with distilled water. The three media tested had an initial sugar concentration of 5% (w/v) (2.5% (w/v) D-glucose and 2.5% (w/v) D-fructose), and 10 mL/L vitamins (100X) and 1 mL/L oligo elements (1000X) were added to each medium. The concentrated solution of vitamins (100X) was prepared with 2 g/L myo-inositol; 0.15 g/L calcium pantothenate; 0.025 g/L thiamine hydrochloride; 0.2 g/L nicotinic acid; 0.025 g/L pyridoxine; and 3 mL biotin (100 mg/L). The oligo elements solution (1000X) was comprised of 4 g/L manganese sulfate monohydrate; 4 g/L zinc sulfate heptahydrate; 1

g/L copper sulfate pentahydrate; 1 g/L potassium iodide; 0.4 g/L cobalt chloride hexahydrate; 1 g/L boric acid; and 1 g/L ammonium heptamolybdate.

Different nitrogen concentrations (25, 50, 100, 300 mg N/L and 1 g N/L) were added to media, initially as a complete solution of ammonium and amino acids, taking into consideration all the nitrogen atoms. When the optimal nitrogen concentration was determined for each strain, all the nitrogen was added as a single amino acid or ammonium ions to establish the best nitrogen source for each strain.

Amino acids solutions have been prepared with distilled water at a concentration of 2.5 g N/L and filtered. The amino acids used were: alanine (Ala); arginine (Arg); asparagine (Asn); aspartic acid (Asp); cysteine (Cys); phenylalanine (Phe); γ-Aminobutyric acid (Gaba); glycine (Gly); glutamic acid (Glu); glutamine (Gln); histidine (His); isoleucine (Ile); leucine (Leu); lysine (Lys); methionine (Met); ornithine (Orn); proline (Pro); serine (Ser); threonine (Thr); tryptophan (Trp) and valine (Val).

2.2.2. Growth monitoring

For all experiments, the initial optical density (OD; 600 nm) was adjusted to ca. 0.1. Assays were performed using a microplate reader SpectroStar Nano (BMG LABTECH) at 28 ºC in triplicate. The absorbance was measured continuously for 200 cycles, with stirring at 500 rpm for 80 seconds prior to each reading. For the representation of the growth, the OD was normalized by dividing the values of each strain by its initial OD, so that all graphs began at OD = 1. To compare the different conditions tested, the maximal OD was determined and the maximum growth rate was calculated. The maximum growth rate was the slope obtained in the exponential phase of the OD curve plotted against the time.

2.3. Statistical procedures

Data were analysed using a one-way ANOVA, and significant differences were determined using Tukey´s method ($p < 0.05$). The differences in maximum OD and maximum growth rate for each strain were compared among the different nitrogen

concentrations in the same medium, different media at the same nitrogen concentration, and finally, different nitrogen sources. Values represented with the same letter were not significantly different. All statistical analyses were carried out using SPSS Statistics 23.

3. RESULTS

3.1. Selection of optimal media and nitrogen concentrations

First, the six strains were grown in the three media (YNB, M9 and SM) without the addition of amino acids, ammonium, vitamins or oligo elements to assure that they could not grow without a supply of external nitrogen. No strain was able to grow in these three media (data not shown), confirming that no assimilable nitrogen was available in these media. Then, a complete solution of amino acids and ammonia in different concentrations of nitrogen (25 mg N/L, 50 mg N/L, 100 mg/L, 300 mg N/L and 1 g N/L), vitamins (1X) and oligo elements (1X) was added to the three media to determine the best nitrogen concentration and the best medium for the growth of each AAB strain. In Table 2, the results obtained for the six strains grown in YNB, M9 and SM are shown. In general, *G. japonicus* strains presented the highest growth in all the media tested and in all the concentrations of nitrogen that were used in this study.

In YNB medium, the *G. japonicus* strain CECT 8443 showed better growth than the type strain (LMG 1373) in all the nitrogen concentrations tested; CECT 8443 growth was twelve times higher than the growth of LMG 1373 in nitrogen concentrations of 300 mg N/L and 1 g N/L. High concentrations of nitrogen seemed to be unfavourable for LMG 1373 growth in this medium, but not for strain CECT 8443. Therefore, 25 mg N/L can be considered an optimal concentration for the growth of both strains in this medium. Essentially no differences were observed in maximum growth rates either between strains or among nitrogen concentrations.

Table 2. Maximum OD and growth rate of AAB strains when grown in three different media (YNB, SM, M9) with different nitrogen concentrations (25, 50, 100, 300 mg N/L and 1 g N/L).

			25 mg N/L		50 mg N/L		100 mg N/L		300 mg N/L		1 g N/L	
Species	**Strain**	**Medium**	**Maximal OD**	**Maximal Rate**	**Maximal OD**	**Maximal Rate**	**Maximal OD**	**Maximal Rate**	**Maximal OD**	**Maximal Rate**	**Maximal OD**	**Maximal Rate**
G.japonicus	LMG 1373^{T}	YNB	15.90 ± 0.03^{A,a}	0.11 ± 0.01^{A,a}	10.73 ± 0.03^{A,b}	0.14 ± 0.01^{A,b}	3.84 ± 0.06^{A,c}	0.10 ± 0.00^{A,ac}	4.62 ± 0.05^{A,cd}	0.11 ± 0.01^{A,acd}	4.51 ± 0.03^{A,cd}	0.12 ± 0.01^{A,abd}
		M9	12.51 ± 0.02^{B,a}	0. 13 ± 0.02^{AB,a}	16.87 ± 0.15^{B,b}	0.14 ± 0.03^{AB,a}	20.98 ± 0.14^{B,b}	0.19 ± 0.07^{B,a}	12.84 ± 0.14^{B,ac}	0.18 ± 0.03^{B,a}	11.39 ± 0.13^{B,ac}	0.18 ± 0.03^{B,a}
		SM	21.69 ± 0.01^{B,a}	0.10 ± 0.02^{AB,a}	20.48 ± 0.01^{C,a}	0.13 ± 0.00^{AB,a}	24.95 ± 0.01^{C,ab}	0.09 ± 0.00^{A,b}	29.24 ± 0.01^{C,bc}	0.12 ± 0.01^{A,b}	32.09 ± 0.03^{C,c}	0.13 ± 0.02^{A,b}
	CECT 8443	YNB	40.88 ± 0.11^{A,a}	0.11 ± 0.01^{A,a}	36.18 ± 0.05^{A,ab}	0.12 ± 0.00^{A,a}	35.29 ± 0.08^{A,b}	0.11± 0.00^{A,a}	57.87 ± 0.08^{A,c}	0.14± 0.00^{A,b}	55.57 ± 0.04^{A,c}	0.12 ± 0.00^{A,a}
		M9	7.14 ± 0.03^{B,a}	0.09 ± 0.00^{AB,a}	9.78 ± 0.12^{B,b}	0.10 ± 0.01^{AB,a}	13.97 ± 0.21^{B,c}	0.09 ± 0.00^{AB,a}	14.56 ± 0.20^{B,c}	0.09 ± 0.01^{B,a}	9.73 ± 0.02^{B,b}	0.09 ± 0.01^{B,a}
		SM	11.82 ± 0.21^{C,a}	0.10 ± 0.01^{AB,a}	16.65 ± 0.03^{C,b}	0.10 ± 0.03^{AB,a}	18.23 ± 0.05^{C,b}	0.11 ± 0.03^{AB,a}	16.92 ± 0.14^{B,b}	0.11 ± 0.03^{B,a}	13.91 ± 0.19^{C,b}	0.10 ± 0.02^{B,a}
G.oxydans	621H	YNB	2.84 ± 0.02^{A,a}	0.07 ± 0.02^{A,a}	2.80 ± 0.02^{A,a}	0.05 ± 0.00^{A,a}	4.01 ± 0.02^{A,a}	0.05 ± 0.01^{A,a}	8.41 ± 0.02^{A,b}	0.07 ± 0.00^{A,a}	0.99 ± 0.01^{A,c}	0.05 ± 0.01^{A,a}
		M9	3.16 ± 0.02^{A,a}	0.05 ± 0.00^{AB,a}	3.20 ± 0.02^{AB,a}	0.05 ± 0.00^{A,a}	3.42 ± 0.04^{A,a}	0.05 ± 0.00^{A,a}	2.99 ± 0.02^{B,a}	0.06 ± 0.00^{AB,a}	0.93 ± 0.01^{A,b}	0.03 ± 0.00^{A,a}
		SM	1.92 ± 0.01^{B,a}	0.05 ± 0.01^{AB,a}	4.15 ± 0.01^{B,b}	0.09 ± 0.01^{B,a}	7.55 ± 0.02^{B,c}	0.09 ± 0.02^{B,a}	6.46 ± 0.01^{C,c}	0.05 ± 0.01^{B,a}	6.43 ± 0.02^{B,c}	0.07 ± 0.00^{B,a}
	Po5	YNB	16.07 ± 0.07^{A,a}	0.17 ± 0.02^{A,a}	16.76 ± 0.03^{A,a}	0.17 ± 0.01^{A,a}	16.88 ± 0.17^{A,a}	0.13 ± 0.02^{A,ab}	22.92 ± 0.07^{A,b}	0.07 ± 0.00^{A,b}	7.51 ± 0.01^{A,c}	0.12 ± 0.01^{A,ab}
		M9	12.65 ± 0.00^{B,a}	0.09 ± 0.02^{B,a}	20.53 ± 0.07^{B,b}	0.08 ± 0.01^{B,a}	28.22 ± 0.03^{B,c}	0.11 ± 0.02^{A,a}	27.97 ± 0.07^{C,c}	0.15 ± 0.03^{B,b}	23.30 ± 0.26^{B,b}	0.14 ± 0.03^{AB,b}
		SM	12.84 ± 0.22^{B,a}	0.20 ± 0.02^{A,a}	17.09 ± 0.03^{A,ab}	0.21 ± 0.02^{A,a}	22.16 ± 0.05^{A,bc}	0.20 ± 0.02^{B,a}	20.01 ± 0.05^{B,bcd}	0.17 ± 0.02^{B,ab}	21.20 ± 0.13^{B,bcd}	0.09 ± 0.02^{A,b}
A.malorum	DSM 14337^{T}	YNB	3.45 ± 0.01^{A,a}	0.04 ± 0.00^{A,a}	1.05 ± 0.01^{A,b}	0.04 ± 0.00^{A,ab}	2.02 ± 0.01^{A,c}	0.05 ± 0.01^{A,abc}	1.64 ± 0.04^{A,c}	0.06 ± 0.00^{A,c}	2.46 ± 0.03^{A,c}	0.04 ± 0.00^{A,abc}
		M9	2.89 ± 0.01^{A,a}	0.06 ± 0.00^{AB,a}	5.76 ± 0.02^{B,b}	0.06 ± 0.01^{B,a}	8.88 ± 0.01^{B,c}	0.07 ± 0.01^{AB,a}	10.65 ± 0.01^{B,d}	0.07 ± 0.01^{A,a}	8.74 ± 0.02^{B,c}	0.06 ± 0.01^{B,a}
		SM	0.84 ± 0.02^{B,a}	0.05 ± 0.00^{AB,a}	1.18 ± 0.01^{A,b}	0.05 ± 0.00^{AB,a}	1.44 ± 0.00^{C,c}	0.05 ± 0.00^{AB,a}	1.24 ± 0.00^{A,bd}	0.05 ± 0.00^{AB,a}	1.29 ± 0.00^{C,bcd}	0.05 ± 0.00^{AB,a}
	CECT 7742	YNB	3.10 ± 0.02^{A,a}	0.05 ± 0.01^{A,a}	4.90 ± 0.02^{A,b}	0.08 ± 0.02^{A,b}	6.80 ± 0.03^{A,bc}	0.06 ± 0.01^{A,abc}	10.1 ± 0.00^{A,d}	0.08 ± 0.00^{A,bc}	6.24 ± 0.03^{A,bc}	0.14 ± 0.01^{A,d}
		M9	7.80 ± 0.01^{B,a}	0.08 ± 0.01^{B,a}	9.40 ± 0.05^{B,ab}	0.08 ± 0.01^{A,a}	12.8 ± 0.04^{B,bc}	0.10 ± 0.01^{B,a}	13.0 ± 0.07^{B,cd}	0.08 ± 0.00^{AB,a}	14.1 ± 0.00^{B,d}	0.09 ± 0.01^{B,a}
		SM	5.70 ± 0.01^{C,a}	0.06 ± 0.01^{A,a}	6.18 ± 0.01^{A,a}	0.05 ± 0.01^{B,a}	11.65 ± 0.01^{B,b}	0.08 ± 0.01^{AB,b}	13.62 ± 0.01^{B,bc}	0.08 ± 0.00^{AB,bc}	12.24 ± 0.00^{C,bc}	0.08 ± 0.01^{B,bc}

T: Type strais. Values represented with different letter were significantly different ($p<0.05$). In maximal OD, lower case letters represent differences between concentrations of nitrogen within the same medium, while capital letters represent differences between different media with the same nitrogen concentration. In all the cases the comparison are within the same strain. In maximal rate, the same comparisons were done and differences were marked with italics.

Surprisingly, in the other two media (M9 and SM), the type strain (LMG 1373) presented better growth than CECT 8443, with the only exception being growth in M9 with the addition of 300 mg N/L. However, in both cases, the OD of LMG 1373 was higher than the OD observed for CECT 8443. In the M9 medium, LMG 1373 presented its maximum growth rate with the addition of 100 mg N/L, whereas strain CECT 8443 presented maximum growth at the concentration range between 100 and 300 mg N/L, with very similar values for both the maximal growth and rate. However, higher concentrations (1 g N/L) resulted in a decrease in growth, unlike the pattern that was observed in the YNB medium. Moreover, the maximum growth rates for CECT 8443 were clearly lower than the ones obtained for LMG 1373. Finally, in SM, the maximum growth of LMG 1373 increased as it did the nitrogen concentration, and the growth of this strain showed an opposite trend in YNB. In contrast, CECT 8443 displayed a peak in the maximum OD when grown in SM with an addition of 100 mg/L of N, and the lowest values were observed at both ends of the nitrogen concentration gradient.

Regarding *G. oxydans* strains, 621H grew poorly in all the tested media, especially compared with Po5, which grew well in all media tested (Table 2). In YNB medium, both strains showed the highest maximum OD in this medium with the addition of 300 mg N/L; however, the maximum growth rate was rather low, especially for Po5 compared with the growth rate of this strain at a lower nitrogen concentration. Despite this low rate, the growth of Po5 was three-fold higher than that of 621H. Moreover, strain Po5 also presented satisfactory growth in lower concentrations of nitrogen, but this growth was clearly reduced in presence of the 1 g N/L concentration. At this high nitrogen concentration, no growth was observed in strain 621H. Po5 achieved a higher maximum OD in M9 medium than in YNB media, with 100 mg N/L and 300 mg N/L as the optimal concentrations. In M9 media, a higher concentration of nitrogen also resulted in decreased growth, but this was less pronounced than the decrease observed in YNB medium. Comparing both *G. oxydans* strains, 612H presented a very similar growth between 25 and 300 mg N/L and no growth at 1 g N/L, and its maximal OD was as much

as nine times lower as that of Po5. Finally, both strains presented the same trend in SM medium; less growth was observed with the concentration of 25 mg N/L, and a similar maximum OD was observed between 100 and 1000 mg N/L. The best nitrogen concentration was 100 mg N/L, in which the maximum OD and growth rate were achieved. However, once again, the growth of Po5 was stronger than that of 621H in SM medium, reaching a maximum OD that was between three- and six-fold higher.

Finally, regarding the *A. malorum* strains, although both strains grew poorly in most of the media and nitrogen concentrations, once again, the indigenous strain CECT 7742 grew better than the type strain DSM 14337 (Table 2). In YNB medium, strain DSM 14337 performed better with the concentration of 25 mg N/L, presenting a growth similar to that of CECT 7742. On the other hand, CECT 7742 growth was favoured by higher nitrogen concentrations, with 300 mg N/L as the most optimum nitrogen concentration for this strain in YNB medium. In M9 medium, the growth of both *A. malorum* strains improved when the nitrogen concentration was higher, showing the best growth in the range between 100 and 1000 mg N/L. Moreover, in this medium, the differences between the strains were lower; in general, the difference between them was less than two-fold. Finally, DSM 14337 did not grow in SM media regardless of the nitrogen concentration, while CECT 7742 presented a similar growth pattern and an identical maximum growth rate between 100 and 1000 mg N/L, reaching the maximum OD with the concentration of 300 mg N/L.

After this preliminary study, it was necessary to select the best medium and the lowest nitrogen concentration that was favourable for the growth of each strain. Whenever possible, the same medium and nitrogen concentration were selected for strains belonging to the same AAB species to allow a better comparison between them. For *G. japonicus* strains, YNB media with a concentration of 25 mg N/L was selected. Although this medium was not ideal for the type strain LMG 1373, its growth was sufficient, and this was the best medium for the natural strain CECT 8843. In the case of the *G. oxydans* strains, 621H grew poorly in all media, but the best growth was observed in YNB with

the concentration of 300 mg N/L. Alternatively, Po5 grew well in all media, presenting good growth in YNB with the concentration of both 300 mg N/L and 25 mg N/L. Therefore, both concentrations were chosen for comparison purposes between *G. oxydans* strains, and we extended this comparison to all the *Gluconobacter* strains. For *A. malorum* strains, the best growth was obtained in M9 media; although the nitrogen required in both strains was high, the concentration was fixed at 100 mg N/L.

3.2. Analysis of individual amino acids and ammonium

The second experiment was carried out using the media selected above, but the nitrogen source was added individually as ammonia or as a single amino acid to test the capacity of these strains to grow with a unique nitrogen source as well as to determine the best nitrogen source for each strain. The control medium was the same medium used in the previous experiment (the complete solution of amino acids and ammonium at the same concentration).

In Figure 1, the growth of *G. japonicus* strains LMG 1373 and CECT 8443 in YNB with the different nitrogen sources at 25 mg N/L is shown. In both cases, significant differences in growth were observed between the media with the individual nitrogen sources and the control medium. For LMG 1373, there were some amino acids (Arg, Gaba, His, Lys, Orn and Thr) that essentially did not promote growth of this strain; in contrast, Asn and Gln were the best promoters as sole nitrogen sources and produced two-thirds of the growth obtained by the control. Finally, the addition of ammonium, Ala and Ser presented intermediate effects, permitting growth of approximately half that of the control. On the other hand, CECT 8443, which presented very good growth with the control medium, was not able to recover growth with a unique nitrogen source; not even one-third of the growth obtained by the control medium was observed for any individual nitrogen source. The best sources were Asn and Trp, followed by ammonium, Gln and Met. No growth was observed with the addition of Gaba, His, Orn, Ser or Val. Therefore, for both *G. japonicus* strains, Asn, Gln and ammonium promoted growth while the

presence of Gaba, His and Orn in the medium was not enough to improve the growth of these strains.

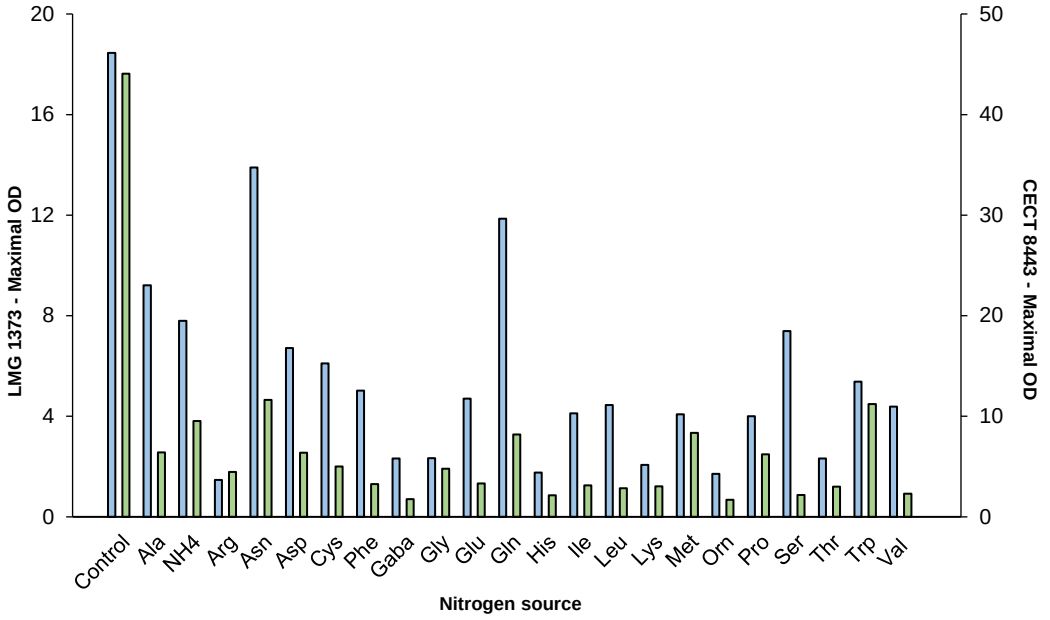

Figure 1. The maximum OD of two *G. japonicus* strains in YNB medium using different single nitrogen sources fixed at 25 mg N/L. As a control, a complete solution of amino acids and ammonium with a final concentration of 25 mg N/L was used. Experiments were carried out in triplicate. LMG 1373 ( ) and CECT 8443 ( ).

As expected based on the previous results, strain 621H did not grow well with the individual nitrogen sources at a concentration of 25 mg N/L (data not shown); therefore, we only considered the results obtained with the concentration of 300 mg N/L (Figure 2). In this case, two amino acids, Gln and His, were the best promoters of 621H growth; using these amino acids as a sole nitrogen sources, 621H was capable of recovering the same or better growth compared with that measured in the control medium. For the remaining amino acids as nitrogen sources, significantly lower growth than that in the control was observed. Furthermore, essentially half of the sources tested were not able to support growth of this strain. For the Po5 strain, we analysed its growth at two nitrogen concentrations (25 and 300 mg N/L).

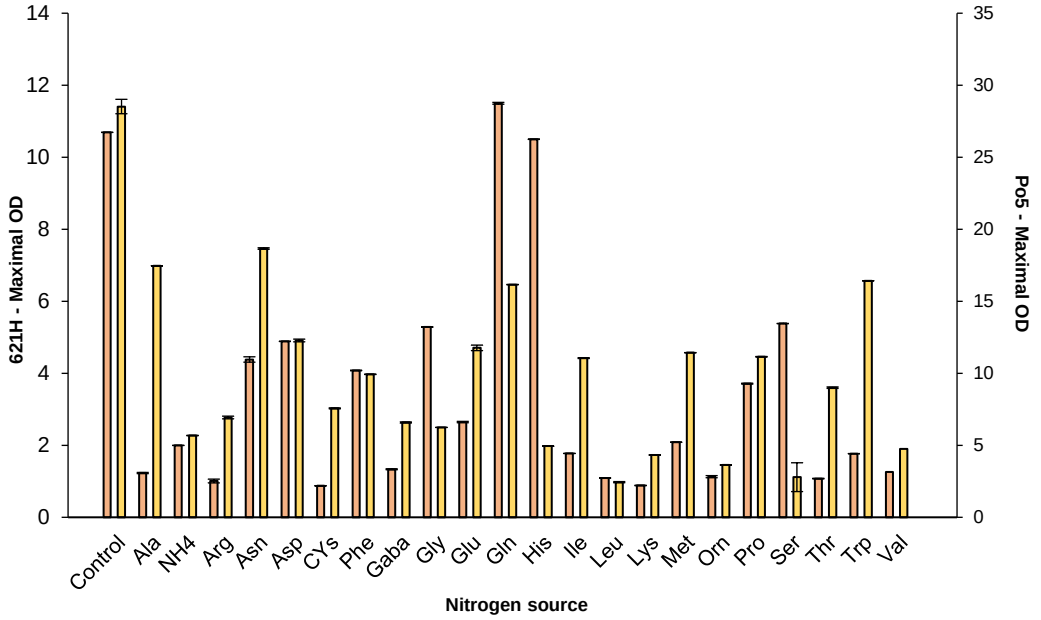

Figure 2. The maximum OD of two *G. oxydans* strains in YNB medium using different single nitrogen sources fixed at 300 mg N/L. As a control, a complete solution of amino acids and ammonium with a final concentration of 300 mg N/L was used. Experiments were carried out in triplicate. 621H ( ) and Po5 ( ).

In the low nitrogen concentration (Figure 3), minor differences from the control were observed, although the growth with only ammonium added was higher. Moreover, in the majority of the nitrogen sources, Po5 was only capable of producing half of the growth of the control. Only His, Lys and Orn were poor nitrogen sources, producing very low growth. Similarly, to the *G. japonicus* strains, for Po5, ammonium and Gln were considered good sources of nitrogen, and His and Orn were poor sources. In the high nitrogen concentration (300 mg N/L) (Figure 2), large differences between the media with the sole nitrogen sources and the control media were highlighted. Curiously, at this nitrogen concentration, ammonium was not a good source of nitrogen, and the sources with the highest maximum OD were Asn, Ala, Gln and Trp. Compared to the other *G. oxydans* strain, the most remarkable observation was that the addition of His supported only low growth, unlike the case for strain 621H but similar to that for the other *Gluconobacter* strains tested, in which this amino acid supported poor growth.

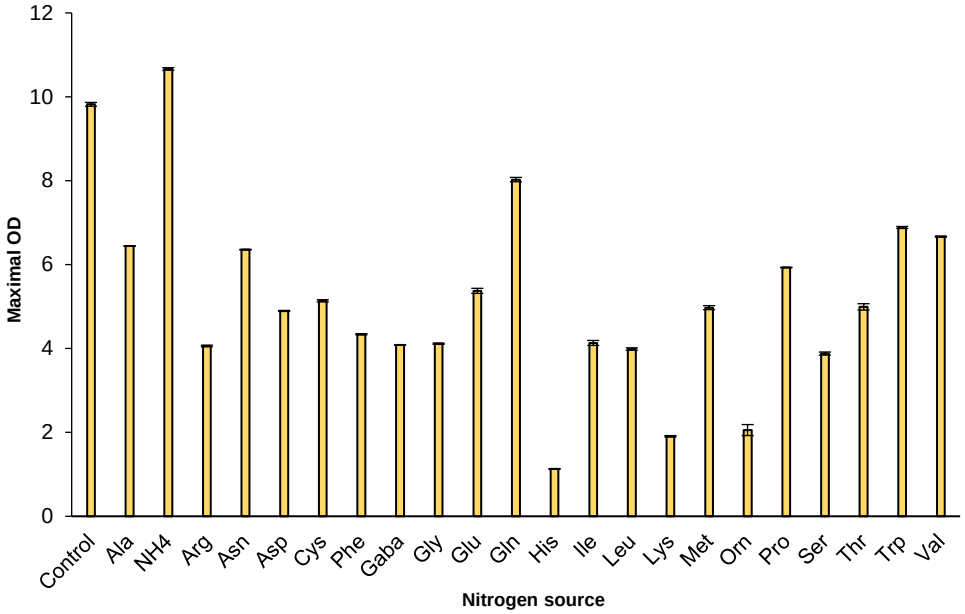

Figure 3. The maximum OD of strain Po5 in YNB medium using different single nitrogen sources fixed at 25 mg N/L. As a control, a complete solution of amino acids and ammonium with a final concentration of 25 mg N/L was used. Experiments were carried out in triplicate.

Finally, the growth response to the different nitrogen sources was similar in both *A. malorum* strains (CECT 7742 and DSM 14337) (Figure 4). Neither the presence of the majority of the amino acids nor the ammonium ion was enough to enhance the growth of these strains. In fact, no growth was observed with these sources, and only five amino acids were able to favour growth of the *A. malorum* strains, Ser, Gln, Glu, Ala and especially Pro. The presence of 100 mg N/L in the medium in the form of Pro increased the growth of both strains to 1.5 times the growth observed in the control medium. For CECT 7742, Ala also enhanced growth.

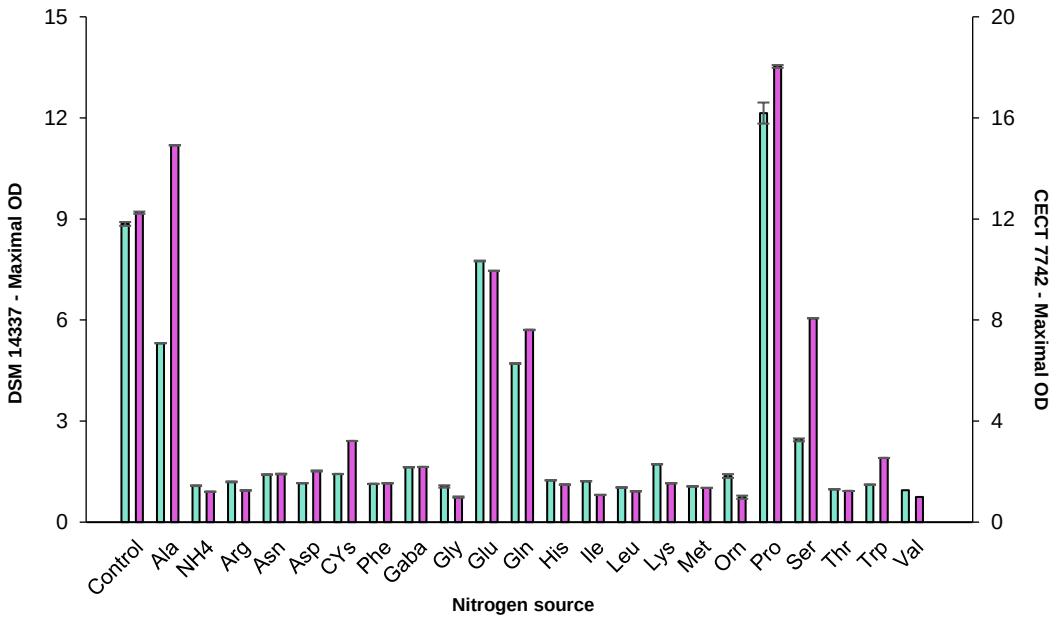

Figure 4. The maximum OD of two *A. malorum* strains in M9 medium using different single nitrogen sources fixed at 100 mg N/L. As a control, a complete solution of amino acids and ammonium with a final concentration of 100 mg N/L was used. Experiments were carried out in triplicate. DSM 14337 (▮) and CECT 7742 (▮).

4. DISCUSSION

Few studies have dealt with the nutrition of AAB; in the 1950s, some studies about the nutritional requirements of AAB were performed (Foda and Vaughn, 1953; Raghavendra Rao and Stokes, 1953; Rainbow and Minston, 1953). More recently, studies have been mainly focused on the consumption of amino acids and ammonium during the acetification process and the differences among the substrates used for the production of wine vinegar (Álvarez-Cáliz et al., 2012, 2014; Callejón et al., 2008; Maestre et al., 2008; Valero et al., 2003). Therefore, the main aim of this work was to determine which nitrogen sources are the best for AAB growth and what is the minimal concentration needed to promote AAB growth. Thus, different media and nitrogen concentrations were tested in two strains of each of three AAB species to determine whether there were different amino acid and nutritional patterns for each strain. Three different media (YNB, M9 and SM) were used, but the carbon source was the same in all cases to prevent changes in the nitrogen utilization due to the carbon source, as described by Raghavendra Rao and Stokes. (1953). In our study, we first worked with a complete

solution of amino acids and ammonium in various media that are commonly used to culture bacteria to determine the best medium and the minimum nitrogen concentration that supported the growth of each strain tested. In the previous studies performed on AAB nutrition, different and sometimes opposing conclusions were made. This can be attributed to the fact that these studies were conducted under different conditions and using different media, strains, etc.; the nitrogen requirements are greatly influenced by the strain used and the growth conditions of the strain (Belly and Claus, 1972), as was observed in the present study. For example, some authors reported that the ammonium ion was a sufficient nitrogen source for AAB growth (Drysdale and Fleet, 1988; Maestre et al., 2008) because AAB could synthesize all amino acids from this compound; other research reported that some amino acids were essential for the growth of some strains or species (Kerwar et al., 1964; Stokes and Larsen, 1945).

Different media were optimal for the tested strains. In general, YNB and M9 were the best media for *Gluconobacter* and *A. malorum* strains, respectively. In fact, in all the media tested, *Gluconobacter* strains grew better than *A. malorum* strains, which, in general, presented poor growth, and the minimum concentrations of nitrogen were high (100 mg N/L), indicating that the *A. malorum* strains had higher nutritional demands. However, the best *A. malorum* growth was supported in M9 medium, which was the simplest medium. M9 only had one component that was not present in the other two media, the sodium hydrogen phosphate. This compound, together with citric acid, has been already used in culture media for *Acetobacter xylinum* to buffer the medium (Hestrin and Schramm, 1954). Another possibility is that some component present in the other two media was inhibitory for these *A. malorum* strains; additional tests should be performed to verify this hypothesis. For the *Gluconobacter* strains, all presented good growth in YNB at 25 mg N/L, except for the strain 621H. This strain grew the worst and needed higher concentrations of nitrogen in the medium. In the case of *G. japonicus*, both strains presented good growth in all media, showing minor nutritional demands and having a higher capacity to adapt to different nitrogen compositions. However, the growth

of the natural strain, CECT 8443, was clearly improved in the YNB medium, while the type strain grew better in the other two media. The YNB medium contains p-aminobenzoic acid, which has been defined as a growth factor for AAB (Underkofler et al., 1943).

After selecting the best medium and minimal nitrogen concentration for the growth of each strain, we tested the growth efficiency of each strain on single nitrogen sources (amino acids or ammonium ion) under the predetermined conditions. In general, different patterns of utilization were observed between strains belonging to the *Gluconobacter* and *Acetobacter* genera. For *Acetobacter* strains, the best nitrogen source was Pro. *Acetobacter* strains are well known to have a preference for ethanol as carbon source and are one of the main players in the transformation of ethanol into acetic acid during vinegar production (De Ley et al., 1984; Raspor and Goranovic, 2008). On the other hand, Pro is the main amino acid found in wine because grapes are rich in this amino acid (Ribéreau-Gayon et al., 2006), and *Saccharomyces cerevisiae* does not use it during alcoholic fermentation because it is an anaerobic process, which avoids proline oxidase activity (Arias-Gil et al., 2007; Bell and Henschke, 2005). For this reason, it is advantageous for *Acetobacter* strains to have their nitrogen requirements met with only Pro. In fact, different studies carried out to study the amino acid consumption during wine acetification have highlighted that Pro is one of the most-consumed amino acids; however, it is normally not fully depleted because its concentration is clearly higher than that of the other amino acids (Álvarez-Cáliz et al., 2012; Callejón et al., 2008; Maestre et al., 2008; Morales et al., 2001). Other good nitrogen sources for the *Acetobacter* strains used in this study were Ala, Glu, Gln and Ser. It is important to note that these strains hardly grew in presence of only ammonium. In fact, there is contradictory information about the use of ammonium sulfate as the sole source of nitrogen by *Acetobacter* strains, but these differences rely on the strain and the carbon source present in the medium (Brown and Rainbow, 1956; Rainbow and Mitson, 1953). Additionally, this statement should be extended to the other AAB genera, as most of the strains used in these

previous studies that were considered *Acetobacter* strains actually belong to the *Gluconobacter* genus. We can confirm this statement with our results because the *G. oxydans* strain Po5 presented this opposite behaviour: full recovery of growth when ammonium is added at 25 mg N/L and very low growth with the addition of 300 mg N/L, probably indicating an inhibition of this compound at high concentrations. On the other hand, O'Sullivan. (1974) reported that some amino acids, such as Thr and homoserine, inhibited growth of *A. aceti* strains, whereas the presence of Met and Ile could reverse this effect. However, in our case, although the presence of Thr did not improve the growth of both *A. malorum* strains, we cannot assert that there was an inhibition of growth by this amino acid, only that this amino acid, similar to Met and Ile, cannot support the growth of these strains as the sole nitrogen source.

In the case of the *G. japonicus* strains, no single amino acid or ammonium ion could replace the complete nitrogen solution because the growth was strongly affected in the presence of sole nitrogen sources, especially for the CECT 8443 strain. Therefore, these strains likely need a more complex nitrogen source to support their growth. In the case of *G. oxydans*, Po5, as mentioned above, can use ammonia as sole nitrogen source at 25 mg/L N, while 621H can use Gln and His at 300 mg N/L as nitrogen sources. In fact, Gln was, in general, a good nitrogen source for all the tested strains (*Acetobacter* and *Gluconobacter* strains) and seemed to have a stimulatory effect on their growth. This was not unexpected because Gln and Glu are the key nitrogen donors for biosynthetic reactions in cells (Merrick and Edwards, 1995). Moreover, the enzymes responsible for the main pathway of nitrogen assimilation, glutamine synthetase and glutamate synthase, were purified and characterized in *G. suboxydans* some years ago (Tachiki et al., 1978), with Gln identified as the specific substrate of the latter one. Nevertheless, the presence of Glu did not present a general improvement in growth; this effect was very evident only in *Acetobacter* strains.

On the other hand, the high growth of the *G. oxydans* strain 621H with His as the sole nitrogen source was surprising, and it seemed to be a specific trait of this strain because

this amino acid was found to be one of the worst growth supporters for the strains tested. In fact, this amino acid has been reported to inhibit the activity of glutamine synthetase (Tachiki et al., 1978), which could explain the low growth observed in the majority of strains. However, previous studies also reported that this amino acid had a stimulatory effect on the growth of a strain of *A. suboxydans* (now renamed as *G. suboxydans*). This study also reported that the only essential amino acid was Val; however, in our study, the presence of this amino acid as an individual source seemed to have a low capacity to support growth in the strains tested. In fact, a great number of amino acids had a very low effect on the growth of these strains, and therefore, these amino acids can be considered as non-essential for these strains. This was especially evident for the growth of the *A. malorum* strains, in which only five amino acids were able to boost their growth in the medium tested.

To summarize, we can conclude that nitrogen requirements for AAB strains are very dependent on the specific strain and the conditions (nitrogen concentration and media); therefore, it is difficult to establish a general protocol for improving AAB growth. Amongst the strains tested in this study, some were able to grow in low concentrations of nitrogen, as low as 25 mg N/L, while others had higher nitrogen demands (100-300 mg N/L). Moreover, most of the strains did not grow well in the presence of single amino acids or ammonium; only Pro seemed to be able to replace the complete nitrogen solution for *A. malorum* strains. However, several other single nitrogen sources could boost the growth of a specific AAB strain or under certain conditions; as a general trend, Gln seemed to be a good nitrogen source for all AAB strains tested. Finally, more tests using combinations of the amino acids that highly impacted the growth could be performed to determine which amino acids are essential to support the growth of each strain.

ACKNOWLEDGEMENTS

This work was supported by grant AGL2010-22152-C03-02 from the Spanish Ministry of Science and Innovation. F. Sainz holds a FI Fellowship from the AGAUR, Government of Catalonia, Spain.

REFERENCES

Álvarez-Cáliz, C., Santos-Dueñas, I.M., Cañete-Rodríguez, A.M., García-Martínez, T., Mauricio, J.C., García-García, I., 2012. Free amino acids, urea and ammonium ion contents for submerged wine vinegar production: influence of loading and air-flow rate. Acetic Acid Bacteria. 1, 1-5.

Álvarez-Cáliz, C., Santos-Dueñas, I.M., García-Martínez, T., Cañete-Rodríguez, A.M., Millán-Pérez, M.C., Mauricio, J.C., García-García, I., 2014. Effect of biological ageing of wine on its nitrogen composition for producing high quality vinegar. Food Bioprod. Process. 92, 291-297.

Arias-Gil, M., Garde-Cerdán, T., Ancín-Azpilicueta, C., 2007. Influence of addition of ammonium and different amino acid concentrations on nitrogen metabolism in spontaneous must fermentation. Food Chem. 103, 1312-1318.

Bell, S.J., Henschke, P.A., 2005. Implications of nitrogen nutrition for grapes, fermentation and wine. Aust. J. Grape Wine Res. 11, 242-295.

Belly, R.T., Claud, G.W., 1972. Effect of amino acids on the growth of *Acetobacter suboxydans*. Arch. Microbiol. 83, 237-245.

Brown, G.D., Rainbow, C., 1956. Nutritional patterns in Acetic Acid Bacteria. J. Gen. Microbiol. 15, 61-69.

Callejón, R.M., Tesfaye, W., Torija, M.J., Mas, A., Troncoso, A.M., Morales, M.L., 2008. HPLC determination of amino acids with AQC derivatization in vinegars along submerged surface acetifications and its relation to the microbiota. Eur. Food Res. Technol. 227, 93-102.

Cleenwerck, I., Vandemeulebroecke, K., Janssens, D., Swings, J., 2002. Re-examination of the genus *Acetobacter*, with descriptions of *Acetobacter cerevisiae* sp. nov and *Acetobacter malorum* sp. nov. Int. J. Syst. Evol. Micr. 52, 1551-1558.

De Ley, J., 1961. Comparative carbohydrate metabolism and a proposal for the phylogenetic relationship of the acetic acid bacteria. J. Gen. Microbiol. 24, 31-50.

De Ley, J., Gillis, M., Swings, J., 1984. Family VI. *Acetobacteraceae*. In: Krieg, N.R., Holt, J.G. (Eds.), Bergey's Manual of Systematic Bacteriology, vol. 1. Williams and Wilkins, Baltimore, MD, pp. 267–278.

Deppenmeier, U., Hoffmeister, M., Prust, C., 2002. Biochemistry and biotechnological applications of *Gluconobacter* strains. Appl. Microbiol. Biot. 60, 233-242.

Drysdale, G.S., Fleet, H., 1988. Acetic Acid Bacteria in winemaking: a review. Am. J. Enol. Viticult. 39, 143-154.

Fleet, G.H., 2001. Wine. In: Doyle, M.P., Beuchat, L.R., Montville, T.J (Eds.), Food Microbiology Fundamentals and Frontiers, ASM Press, Washington, DC, pp. 267-288.

Foda, O.I., Vaughn, R.H., 1953. The nutritional requirements of *Acetobacter melanogenum* and related species. J. Bacteriol. 65, 79-82.

Gullo, M., Caggia, C., De Vero, L., Giudici, P., 2006. Characterization of acetic acid bacteria in "traditional balsamic vinegar". Int. J. Food Microbiol. 106, 209-212.

Gullo, M., Giudici, P., 2008. Acetic acid bacteria in traditional balsamic vinegar: Phenotypic traits relevant for starter cultures selection. Int. J. Food Microbiol. 124, 46-53.

Gupta, A., Singh, V.K., Qazi, G.N., Kumar, A., 2001. *Gluconobacter oxydans*: It´s biotechnological applications. J. Mol. Microbiol. Biotechnol. 3, 445-456.

Harwood, C.R. & Cutting, S.M., 1990. Chemically defined growth media and supplements. In: Harwood, C.R., Cutting, S.M. (Eds.), Molecular biological methods for *Bacillus*, Wiley, Chichester, United Kingdom, pp. 548.

Hestrin, S., Schramm., M., 1954. Synthesis of cellulose by *Acetobacter xylinum*. 2. Preparation of freeze-dried cells capable of polymerizing glucose to cellulose. Biochem. J. 58, 345-352.

Hidalgo, C., Torija, M.J., Mas, A., Mateo, E., 2013. Effect of inoculation on strawberry fermentation and acetification processes using native strains of yeast and acetic acid bacteria. Food Microbiol. 34, 88-94.

Kerwar, S.S., Cheldelin, V.H., Parks, L.W., 1964. Valine-Isoleucine metabolism in *Acetobacter suboxydans* and the inhibition of growth by valine. J. Bacteriol. 88, 179-186.

Lino, T., Suzuki, R., Kosako, Y., Ohkuma, M., Komagata, K., Uchimura, T., 2012. *Acetobacter okinawensis* sp. nov., *Acetobacter papayae* sp. nov., and *Acetobacter persicus* sp. nov.; novel acetic acid bacteria isolated from stems of sugarcane, fruits, and a flower in Japan. J. Gen. Appl. Microbiol. 58, 235-243.

Maestre, O., Santos-Dueñas, I.M., Peinado, R., Jiménez-Ot, C., García-García, I., Mauricio, J.C., 2008. Changes in amino acid composition during wine vinegar production in a fully automatic pilot acetator. Process Biochem. 43, 803-807.

Malimas, T., Yukphan, P., Takahashi, M., Muramatsu, Y., Kaneyasu, M., Potacharoen, W., Tanasupawat, S., Nakagawa, Y., Tanticharoen, M., Yamada, Y., 2009. *Gluconobacter japonicus* sp.nov, an acetic acid bacterium in the *Alphaproteobacteria*. Int. J. Syst. Evol. Microbiol. 59, 466-471.

Mamlouk, M., Gullo, M., 2013. Acetic Acid Bactria: physiology and carbon sources oxidation. Indian J. Microbiol. 54, 377-384.

Mas, A., Torija, M.J., González, A., Poblet, M., Guillamón, J., 2007. Acetic acid bacteria in oenology. Contribution to science. 3, 511-521.

Merrick, M.J., Edwards, R.A., 1995. Nitrogen Control in Bacteria. Microbiol. Rev. 59, 604-622.

Morales, M.L., Tesfaye, W., García-Parrilla, M.C., Casas, J.A., Troncoso, A.M., 2001. Sherry wine vinegar: physicochemical changes during the acetification process. J. Sci. Food Agr. 81, 611-619.

Navarro, D., Mateo, E., Torija, M.J., Mas, A., 2013. Acetic acid bacteria in grape must. Acetic acid bacteria. 2, 19- 23.

O'Sullivan, J., 1974. Growth inhibition of *Acetobacter aceti* by Lthreonine and L-homoserine: the primary regulation of the biosynthesis of amino acids of the aspartate family. J. Gen. Microbiol. 85, 153-159.

Prust, C., Hoffmeister, M., Liesegang, H., Wiezer, A., Fricke, W.F., Ehrenreich, A., Gottschalk, G., Deppenmeier, U., 2005. Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydans*. Nat. Biotechnol. 23, 195-200.

Raghavendra Rao, M.R., Stokes, J.L., 1953. Nutrition of the Acetic Acid Bacteria. J. Bacteriol. 65, 405-412.

Rainbow, C., Mitson, G.W., 1953. Nutritional requirements of Acetic Acid bacteria. J. Gen. Microbiol. 9, 372-375.

Raspor, P., Goranovic, D., 2008. Biotechnological applications of acetic acid bacteria. Crit. Rev. Biotechnol. 28, 101-124.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B., Lonvaud-Funel, A., 2006. Handbook of Enology. The Microbiology of Wine and Vinifications, vol 1, second ed. John Wiley & Sons Ltd, West Sussex, England.

Riou, C., Nicaud, J.M., Barre, P., Gaillardin, C., 1997. Stationary-phase gene expression in *Saccharomyces cerevisiae* during wine fermentation. Yeast. 13, 903-915.

Sainz, F., Navarro, C., Mateo, E., Torija, M.J., Mas, A., 2016. Comparison of D-gluconic acid production in selected strains of acetic acid bacteria. Int. J. Food Microbiol. 222, 40-4

CHAPTER 4

Analysis of ribosomal internal transcribed spacers (16S-23S) in acetic acid bacteria

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SELECTION AND OPTIMIZATION OF ACETIC ACID BACTERIA FOR D-GLUCONIC ACID PRODUCTION
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1. INTRODUCTION

The identification of AAB has gone in parallel with the changes in taxonomy and AAB classification (Guillamón and Mas, 2009). Since their discovery, AAB have been classified with different names and were the subject of several studies and reclassification (Trček and Barja, 2015; Yamada et al., 2012). At first, AAB have been identified to genus and species level by using array of morphological, biochemical and physiological tests (De Ley et al., 1984; Cleenwerck et al., 2008), the problem was that these methods are time consuming and little accurate (Prieto et al., 2007). Nowadays, these phenotypic techniques have been replaced by different molecular techniques based in PCR methods for the differentiation of genera, species and strains of these bacteria (Guillamón and Mas, 2009; Prieto et al., 2007). The application of molecular methods could be the solution for the quick and the accurate identification of AAB (González and Mas, 2011; Trček and Teuber, 2002) in a reasonable period of time (Mas et al., 2007). The molecular techniques used for AAB classification can be divided in two groups, according to the level of determination: those that differentiate at species level and those that differentiate at strain level (Guillamón and Mas, 2009). This work was focused in the study of 16S-23S rDNA internal transcribed spacer (ITS), which is known that exhibits sequences and length variable between species (Barry et al., 1991; Guillamón and Mas, 2009) and conserved sequences because of their functional roles like tRNA and antitermination sequences (Sievers et al., 1996). These characteristics in the ITS sequences showed a higher polymorphism than the 16S rDNA gene (Ruiz et al., 2000). Thereby has been a successfully technique to classify and identify AAB at species level (Ruiz et al., 2000; Trček and Teuber 2002, González and Mas, 2011).

For this reason, the aim of this work was to do an in-depth study of the ITS sequences present in an important number of strains belonging to different genera and species. The strains used were obtained both from different Culture Collections as well as from our own indigenous strains collection.

2. MATERIALS AND METHODS

2.1. Bacteria strains and growth conditions

Acetic acid bacteria (AAB) strains used in this study are listed in Table 1. Out of the 71 strains studied, 55 were obtained from different Culture Collections (Belgian Co-ordinated Collections of Microorganism (BCCM/LMG bacteria collection) and German Collection of Microorganism and cell culture (DSMZ)) and grown according to the provider's specifications. The remaining AAB strains belong to our own AAB strain collection and were isolated from grapes, wines and vinegar over the years. These strains were grown in D-glucose medium (GY: 1% (w/v) yeast extract; 1% (w/v) D-glucose, w/v – Cultimed, Barcelona, Spain) at 28ºC with shaking (125 rpm) for 48 h.

2.2. Analysis of the 16S-23S rRNA Internal Transcribed Spacer (ITS)

2.2.1. Amplification of 16S-23S rRNA ITS

The total DNA from the strains was extracted using the CTAB method (Cetyltrimethylammonium bromide) as described by Ausubel et al. (1992) and modified as in Jara et al. (2008). The conditions for the amplification of 16S-23S rRNA ITS and the primers used were described by Ruiz et al. (2000). Amplified DNA was detected by electrophoresis on a 1,0 % (w/v) agarose gel in 1 x TBE buffer. The gels were stained with ethidium bromide and photographed. The DNA XIV 100 bp ladder (Roche Diagnostics, Mannheim, Germany) was used to estimate the length of the fragments.

Table 1. Strains used in this study with the obtained ITS amplicons length

Species	Strain	Source	Reference	ITS length (bp)
A.aceti	CECT 298	Beechwood shavings of a vinegar plant	Beijerinck (1898)	850 bp
	LMG 1512	Film in fermentor of rice vinegar	Beijerinck (1898). Gosselé et al. (1983b)	850 bp
	LMG 1531	Non-cellulose-producing mutant derived from strain LMG 1530	Li et al. (2014)	850 bp
	LMG 1496	-	Beijerinck (1898). Gosselé et al. (1983b)	850 bp
	DSM 3508	Alcohol turned to vinegar	Beijerinck (1898)	850 bp
A.cerevisiae	LMG 1625	Beer (ale) in storage	Cleenwerck et al. (2002)	850 bp
A. cibinogensis	LMG 21418	Annona montanae	Lisdiyanti et al. (2002)	850 bp
A. estunensis	LMG 1626	Cider	Carr (1958). Lisdiyanti et al. (2001)	850 bp
A.indonesiensis	LMG 19824	Annona muricata	Lisdiyanti et al. (2002)	850 bp
A.lovaniensis	LMG 1579	Sewage on soil	Lisdiyanti et al. (2002)	800bp
A.malorum	LMG 1746	Rotting apple	Cleenwerck et al. (2002)	850 bp
	NBRC 108912	Rotting apple	Cleenwerck et al. (2002)	850 bp
	CECT 7749	Strawberry vinegar	Hidalgo et al. (2013)	850 bp
	Am21	Grape	Valera et al. (2011)	850 bp
	Am23	Grape	Valera et al. (2011)	850 bp
	1.16	Must Grape	Navarro et al. (2013)	850 bp
A.nitrogenifigens	LMG 23498	Kombucha tea	Dutta and Gachhui (2006)	850 bp
A.orientalis	LMG 21417	Canna hybrida	Lisdiyanti et al. (2002)	850 bp
A. orleanesis	LMG 1583	Beer	Lisdiyanti et al. (2001)	850 bp
	LMG 1282	-	-	850 bp

A. pasteurianus	LMG 1635	Ditch water	Visser´t Hooft (1925)	850 bp
	DSM 2347	-	Hansen (1879). Beijerinck and Folpmers (1916)	850 bp
	LMG 1605	Vinegar brews	Hansen (1879). Beijerinck and Folpmers (1916). Gosselé et al. (1983b)	850 bp
	DSM 46617	-	Hansen (1879) Beijerinck and Folpmers (1916)	850 bp
	1.18	Grape must	Navarro et al. (2013)	850 bp
	Lz70	Grape	Valera et al. (2011)	850 bp
	Lz75	Grape	Valera et al. (2011)	850 bp
A.pomorum	LMG 18848	Cider vinegar	Sokollek et al. (1998)	850 bp
A.syzygii	LMG 21419	Syzygium malaccense	Lisdiyanti et al. (2002)	850 bp
A.tropicalis	LMG 1663	Fermenting putrified meat sample	Lisdiyanti et al. (2001)	850 bp
	Lz26	Grape	Valera et al (2011)	850 bp
Ga. azotocaptans	LMG 21311	Coffea arabica L., rhizosphere	Fuentes-Ramírez et al. (2001)	800 bp
Ga.diazotrophicus	LMG 7603	Saccharum officinarum, root	Yamada et al. (1997)	850 bp
Ga.liquefaciens	DSM 5603	Dried fruit (*Diospyros* sp.)	Asai (1935). Yamada et al. (1997)	800 bp
	LMG 1347	-	-	800 bp
Ga. johannae	DSM 13595	Rhizosphere of coffee plants	Fuentes-Ramírez et al. (2001)	800 bp
Ga.sacharii	LMG 19747	Sugar cane, leaf sheath	Franke et al. (1999)	800 bp
G.cerinus	LMG 1368	Prunus sp. (cherry)	Asai (1935). Asai and Shoda (1958). Yamada and Akita (1984). Katsura et al. (2002)	820 bp / 700 bp
	CECT 9110	Grape Must	Navarro et al. (2013)	800 bp/ 700 bp
	1.20	Grape Must	Navarro et al. (2013)	800 bp/ 700 bp
	1.4	Grape Must	Navarro et al. (2013)	800 bp/ 700 bp
	1.9	Grape Must	Navarro et al. (2013)	800 bp/ 700 bp
G.frateurii	LMG 1365	Fragaria ananassa	Mason and Claus 1989	820 bp / 700 bp
G.japonicus	CECT 8443	Grape Must	Navarro et al. (2013)	800 bp / 700 bp
	Gj1	Grape	Valera et al. (2011)	820 bp / 700 bp
	Gj2	Grape	Valera et al. (2011)	820 bp / 700 bp
	Lz52	Grape	Valera et al. (2011)	820 bp / 700 bp
	LMG 1373	*Myrica rubra*, fruit	Malimas et al. (2009)	820 bp / 700 bp
	1.13	Grape must	Navarro et al. (2013)	800 bp/ 700 bp

G.oxydans	DSM 2343	Descendent of ATCC 621	De Ley (1961). Gosselé et al. (1983a). Mason and Claus, (1989)	820 bp/ 700 bp
	CECT 360	Amstel beer	De Ley (1961). Mason and Claus, (1989)	820 bp / 700 bp
	DSM 7145	Beer	De Ley (1961). Mason and Claus, (1989)	820 bp / 700 bp
	DSM 3503	-	De Ley (1961). Mason and Claus. (1989)	820 bp / 700 bp
	LMG 1484	-	.	820 bp / 700 bp
	LMG 1414	Grapes	Ameyama (1975). Malimas et al. (2008)	820 bp / 700 bp
	LMG 1408	Beer	De Ley (1961). Mason and Claus. (1989)	820 bp / 700 bp
	Po5	Vinegar	Vegas et al. (2010)	820 bp / 700 bp
G.thailandicus	LMG 23137	Indian cork tree, flower	Tanasupawat et al. (2004)	820 bp / 700 bp
K.europaeus	DSM 6160	-	Sievers et al. (1992)	850 bp
	DSM 2004	-	Brown (1886)	870 bp
	LMG 18494	Red wine vinegar	Sievers et al. (1992)	800 bp
K.hansenii	LMG 1524	Vinegar	Gosselé et al. (1983a)	800 bp
	DSM 5602	Vinegar	Gosselé et al. (1983a)	800 bp
K.intermedius	LMG 18909	Kombucha tea	Boesch et al. (1998)	900 bp
K. nataicola	LMG 1536	Nata de coco	Lisdiyanti et al. (2006)	850 bp
K.oboediens	LMG 18849	Red wine vinegar fermentation	Sokollek et al. (1998)	850 bp
K. sacharivorans	LMG 1582	Beet juice	Lisdiyanti et al. (2006)	850 bp
K. maltiaceti	LMG 1529	Malt vinegar	Slapšak et al. (2013) Yamada et al. (2014)	800 bp
K. swingsii	LMG 22125	Organic apple juice	Dellaglio et al. (2005) Yamada et al. (2012)	900 bp
K. xylinus	DSM 2325	-	Brown (1886)	900 bp

2.2.2. Sequencing of ITS amplicons

The ITS amplicons directly obtained from the previous PCR were sequenced by Macrogen Inc. (Seoul, South Korea) using an ABI3730 XL automatic DNA sequencer. When in a sample more than one band in the ITS amplification was observed, a new electrophoretic gel with a higher concentration of agarose (1.5% (w/v)) was prepared

and the different amplicons were separated and excised. Each DNA fragments was purified using a QIAquick Gel Extraction Kit (Quiagen, Netherlands) and re-amplified using the above protocol. Finally, the samples were sequenced by Macrogen Inc. (Seoul, South Korea) using an ABI3730 XL automatic DNA sequencer. Sequences were compared with those in the GenBank databases.

2.2.3. ITS cloning and sequencing

Some of the samples with more than one ITS amplicon were cloned into pGEM-T Easy vector (Promega, MA, USA) in accordance with the manufacture´s instruction to determine the frequency of each type of amplicons. This vector is a high-copy-number vector containing a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide allows identification of recombinants by blue/white screening on indicator plates. White colonies were selected randomly from LB plates supplemented with ampicillin (100 µg/ mL), IPTG (0.5 mM) and X-gal (80 µg/ mL) and each colony was grown in LB liquid medium. Plasmid DNA was extracted from the clones using NucleoSpin Plamid (Macherey Nagel) kit, in accordance with manufature´s instruction. A total of 12 clones for *G. japonicus* strains and 10 clones for the others strains tested were screened by digestion with the restriction endonuclease E*co*RI (Roche) and restriction fragments were visualized in 1% (w/v) agarose gels to determine the size of the insert in each clone. The insert of some clones were sequenced by Macrogen Inc, and their sequences were compared with those in the GenBank databases.

3. RESULTS

Genomic DNA was used as the PCR template to amplify the ITS region of 70 strains of 33 different species of AAB (Table 1). The electrophoresis patterns generated by the strains tested in this work showed that *Acetobacter* and *Komagataeibacter* strains

present a single amplicon. However, multiple PCR products were obtained by *Gluconobacter* strains (Figure 1).

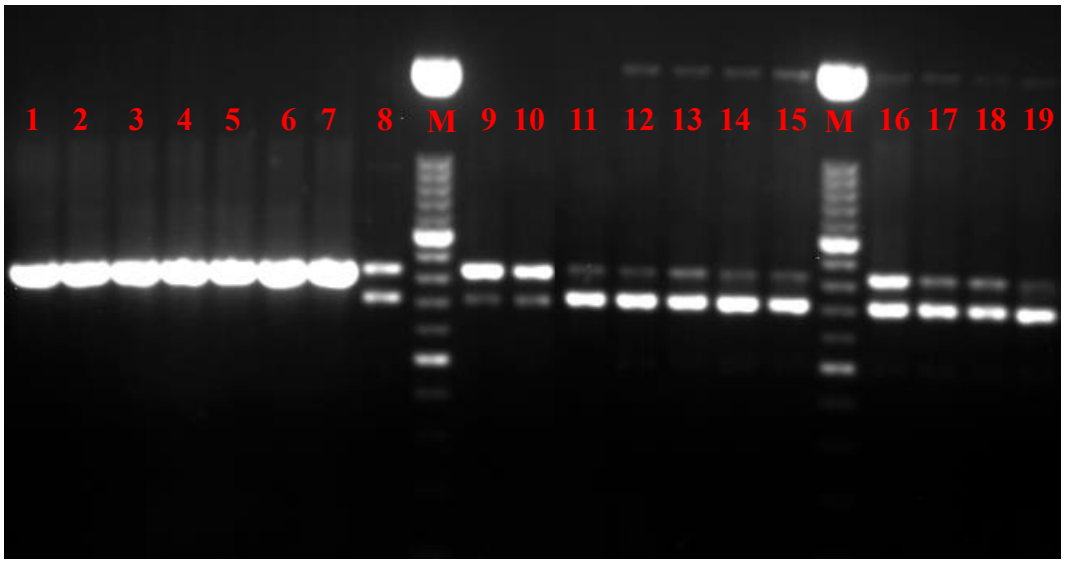

Figure 1. Electrophoretic patterns of 16S-23S rRNA ITS region in different AAB strains. Lanes: M, 100 bp DNA ladder; 1) LMG 1512^T; 2) LMG 1625^T; 3) LMG 1746^T; 4) CECT 7749; 5) LMG 21417^T; 6) DSM 2347^T; 7) LMG 21419^T; 8) DSM 7145^T; 9) Po5; 10) LMG 1408^T; 11) LMG 1368^T; 12) CECT 9110; 13) LMG 23137^T; 14) LMG 1365^T; 15) LMG 1373^T; 16) Gj2; 17) Gj1; 18) CECT 8443; 19) Lz52.

Among the bands obtained, one of them, often the smallest band, used to have a higher band intensity. Amplicons from some *Gluconobacter* strains were sequenced directly from the PCR product (total ITS amplification) or after the separation in electrophoresis gel and purification of each band (individual ITS amplicons). The sequences obtained were compared with those of the Databases to identify the different strains at species level (Table 2). In most strains, the sequences obtained from total ITS amplification were clear and corresponded to the expected *Gluconobacter* species. However, in three strains (LMG 1368; LMG 1484; LMG 23137), it was not possible to obtain a clean sequence and in other three strains (Gj2, LMG 1408 and Po5), the sequence was misleading and showed the highest homology with some *Acetobacter* species. These three stains together with strain DSM 7145 were those that presented a higher intensity in the biggest band or at least, similar intensity in both bands.

Table 2. Homology of the electrophoretic bands obtained from selected *Gluconobacter* strains with multiple PCR products. The results are expressed as homology of the sequences from direct PCR product (complete ITS) and each separated fragment.

	LMG 1373^T	**CECT 8443**	**Gj1**	**Gj2**	**DSM 7145^T**	**LMG 1408**	**DSM 3503**	**DSM 2343**	**LMG 1484**	**Po5**	**LMG 23137^T**	**LMG 1368**	**CECT 9110**	**1.20**
Complete ITS	100% *G.frateurii*	99.8% *G.frateurii*	100% *G.frateurii*	100% *A.malorum*	98% *G.oxydans*	99.3% *A.aceti*	99.8% *G.oxydans*	100% *G.oxydans*	Low quality sequence	97.6% *A.malorum*	Low quality sequence	Low quality sequence	98.2% *G.cerinus*	98.8% *G.cerinus*
Fragment 800-820 bp	99.3% *A.malorum*	99.3 % *A.malorum*	99.7% *A.malorum*	100% *A.malorum*	93% *K.naitaicola* / *K.europaeus*	99.4% *A.aceti*	99.8% *A.malorum*	Low quality sequence	100% *K.hansenii*	99.5% *A.malorum*	99.8% *A.aceti* /*K.oboediens*	99.8% *A.aceti* /*K.oboediens*	Low quality sequence	99.4% *A.malorum*
Fragment 700-720 bp	100 % *G.frateurii*	99.8% *G.frateurii*	100% *G.frateurii*	100% *G.frateurii*	100% *G.oxydans*	99.8% *G.oxydans*	100% *G.oxydans*	100% *G.oxydans*	99.8% *G.oxydans*	97.3% *G.oxydans*	99,6% *G.frateurii* / *G.oxydans* /*G.thailandicus*	100% *G.cerinus*	99.2% *G.cerinus*	100% *G.cerinus*

When the sequences obtained from the individual ITS amplicons was used, clear sequences were obtained for all the strains. Out of the fragments with different lengths, the biggest fragment had 800-820 bp and the smallest fragment, 700-720 bp. In all the tested strains, the sequences of the 800-820 bp amplicon showed higher percentage similarity with other AAB species different from the expected *Gluconobacter* species. This amplicon in the *G. japonicus* strains presented 99-100% of identity with *A. malorum*. Instead, the sequences from *G. oxydans* strains had high similarities with those of *A. malorum, A. aceti*, *K. europaeus* and *K. hansenii*. In LMG 23137 (*G. thailandicus*) and LMG 1368 (*G. cerinus*) this fragment was similar with *A. aceti* or *K. oboediens*; instead for the strain *G. cerinus* 1.20 the highest homology was with *A. malorum*. The sequences of the smallest fragment showed 99-100% homology with the corresponding species of each strain. As example, the alignment of the two amplicons obtained in the strain Po5 is shown in Figure 2.

In some strains from those with more than one ITS amplicon, the PCR product was cloned using the kit pGEM-T Easy Vector and were digested using the enzyme *Eco*RI. To determine the frequency of each amplicon. The number of clones was variable according to the strains, but for all of them we could recover between 10 and 12. Five different lengths were obtained of 820, 773, 752, 720 and 700 bp (Table 3). The 820 bp had the highest homology with *Acetobacter* genus, mostly *A. malorum*, except for DSM 2343 and LMG 23137 where the highest homology was with *Komagataeibacter* genus. The 773 bp fragment was only present in two strains (LMG 1484 and LMG 1408) and had the highest homology with *A. aceti* or *K. hansenii*. The 752 and 720 bp fragments were only found once each and had the identity of *Ga. liquefaciens* and *A. pasterianus*, respectively. Finally, the 700 bp fragment had the same sequence identity as the species of origin. This fragment was the most abundant in the analyzed plasmids, except for LMG 1408 where no *Gluconobacter* sequences were found.

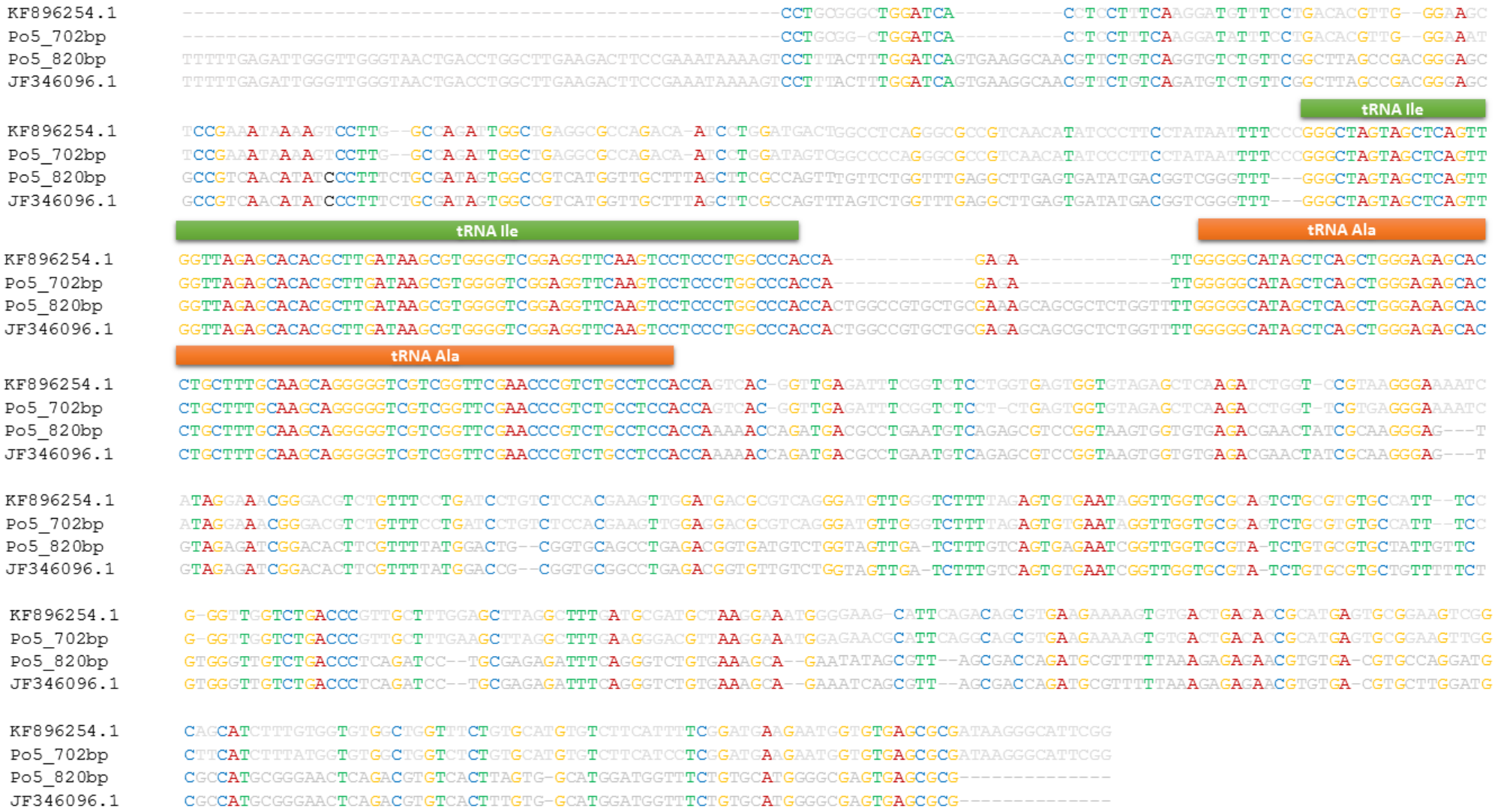

Figure 2. Aligment of the amplicons obtained in the strain Po5. KF896254.1 = *G.oxydans* and JF346096.1 = *A.malorum.*

Table 3. Homology of the sequences from the PCR products cloned from *Gluconobacter* strains.

Species	Strain	Total plasmid	Length of the fragment inserted					Identity (%)				
			820 bp	773 bp	752 bp	720bp	700 bp	820 bp	773 bp	752 bp	720bp	700 bp
G. japonicus	LMG1373	12	4	–	–	–	8	99 % *A.malorum*	–	–	–	100% *G. frateurii*
	CECT 8443	12	7	–	–	–	5	99% *A. malorum*	–	–	–	99.6% *G. frateurii*
G. oxydans	621 H	10*	1	–	–	–	8	99% *A. malorum*	–	–	–	99% *G. oxydans*
	Po5	10	4	–	–	–	6	99% *A. malorum*	–	–	–	97% *G. oxydans*
	DSM 2343	10*	1	–	–	2	6	99.5 *K. xylinus*	–	–	100% *A. pasteurianus*	99% *G. oxydans*
	DSM 3503	10	5	–	–	–	5	99% *A. malorum*	–	–	–	99% *G. oxydans*
	LMG 1484	10	–	5	2	–	3	–	99.4% *A. aceti* / 100% K.hansenii	100% *Ga. liquefaciens*	–	99% *G. oxydans*
	LMG 1408	10*	3	6	–	–	–	97.3% *A. pasteurianus*	98% *A. aceti*	–	–	–
G.thailandicus	LMG 23137	10*	1	–	–	–	8	99% *K. europaeus*	–	–	–	
G.cerinus	CECT 9110	10*	3	–	–	–	5	99.4% *A. malorum*	–	–	–	

* Some sequences were not found in the databases or were low quality sequences.

4. DISCUSSION

In this work, the amplification of the 16S-23S rRNA ITS was studied in a high number of strains. Surprisingly, in all *Gluconobacter* strains, more than one band was obtained when this region was amplified. No previous AAB studies had observed this polymorphism in the ITS amplification, probably because when total ITS amplification was sequenced, in most cases, a correct assignation of species was done. It is important to highlight that if different ITS sequences are present in the genome, PCR amplification may run into the problem of preferential amplification of one operon over the others. Therefore, the most abundant operon could be overrated, minimizing the possibility of amplify minor sequences, which could explain why in AAB multiple ITS were not detected before. However, in our study, some strains were incorrectly identified by direct sequencing of ITS due to the fact that the fragment that was preferentially amplified was the non-specific ITS, confirming that a misleading identification could happen although in low frequency. Furthermore, the results of strains DMS 7145 and LMG 1408 were surprising; these strains are the same, specifically the type strain of *G. oxydans*, but obtained from different Culture Collections, yet the results obtained were different. This fact confirmed the PCR's randomness when two different fragments that can be amplified are present in the genome.

Heterogeneity of the ITS region sequences has been reported in several species (Barry et al., 1991; Gurtler, 1999; Acinas et al, 2004; Milyutina et al., 2004). Boyer et al. (2001) analyzed the ITS amplicons from *Cyanobacteria* species and observed two bands for an isolate of *Calothrix parietina* and three bands for an isolate of *Scytonema hyalinum*. The authors suggested that the isolates had multiple rRNA operons since some of them contained the sequences coding for two tRNA molecules but others had no tRNA features. rRNA operons are normally present in multiple copies in the bacterial genome (from 1 or 2 operons in members of Archaea and several eubacterial genera to 6-10 in some members of Proteobacteria and gram-positive eubacteria), and major heterogeneities among operons have been reported to be due to the type and number

of tRNA genes present (Antón et al., 1998). Nevertheless, in our case, all the ITS amplicons presented the two tRNA present in the AAB ITS sequences, $tRNA^{Ala}$ and $tRNA^{Ile}$, and practically identical sequence. On the other hand, in bacteria of the genus *Acinetobacter*, ITS copies with different length and sequences were also observed (Maslunka et al., 2014, 2015). According to these authors, the intergenomic insertion/deletion (indels) occurring during horizontal transfer events from other species of *Acinetobacter* or unrelated bacterial genera were the responsible for these differences. Various studies share this suggestion of transfers between species and attribute it to horizontal transfer (Cao et al, 2009; Milyutina et al, 2004; Gurtler and Barrie, 1995). However, in our case, the higher length of some ITS copies are not the result of a fragment insertion. The resulting sequence has 97-100% similarity with the ITS of different AAB species and the ITS of these species is very different from the expected ITS in these strains (the one of *Gluconobacter* species), sharing only about 50% of similarity. Therefore, different hypothesis can explain our results. Horizontal transfer could be the first one. According to Lawrence (1999), essential genes, present in all organisms, are less probably to suffer a successful transfer; in contrast not functional genes under weak selection could be benefit from horizontal transfer (Dutta and Pan, 2002). Another option is that these regions are included in plasmids. Various studies reported the presence of plasmids in AAB (Fukaya et al., 1985; Prust et al, 2005; Tonouchi et al., 2003). According to Azuma et al. (2009), *K. xylinus* has a plasmid with a gene with high similarity with *A. pasteurianus*, enhancing the idea of the relation between plasmids and genetic instability of AAB.

The results obtained in this work remain for us doubtful and uncertain because so far, some *Gluconobacter* strains have been totally sequenced and no non-specific ITS sequence has been detected. Therefore, as it is the first time that multiple bands in the ITS amplification of AAB has been observed, it is necessary to further analyze this aspect to understand what happens and why only happens in *Gluconobacter* strains.

Furthermore, these results may challenge the use of the ITS region as a good tool for identification of AAB strains.

Due to these results, we decided to sequence the whole genome of our three selected strains together with strain CECT 9110 (*G. cerinus*), to detect the non-specific ITS in the genome of the *Gluconobacter* strains and verify unequivocally its presence. We selected these strains because they would allow us to confirm the observed results but also to have a valuable information about the selected strains with a potential industrial application. The results of whole genome sequencing of the *G. oxydans* strain Po5 have not been published due to the high number of sequences belonging to this species already available.

REFERENCES

Acinas, S.G., Marcelino, L.A., Klepac-Ceraj, V., Polz, M.F., 2004. Divergence and Redundancy of 16S rRNA Sequences in Genomes with Multiple rrn Operons. J. Bacteriol. 186, 2629-2635.

Ameyama, M., 1975. *Gluconobacter oxydans* subsp. *sphaericus*, new subspecies isolated from grapes. Int. Syst. Bacteriol. 25, 365-370.

Antón, A.I., Martínez-Murcia, A.J., Rodríguez-Valera, F., 1998. Sequence diversity in the 16S-23S intergenic spacer region (ISR) of the rRNA operon in representatives of the *Escherichia coli* ECOR collection. J. Mol. Evol. 47, 62-72.

Asai, T., 1935. Taxonomic studies on acetic acid bacteria and allied oxidative bacteria isolated from fruits. A new classification of the oxidative bacteria. J. Agr. Chem. Soc. Jpn. 11, 499-513, 610-620, 674-708.

Asai, T., Shoda, K., 1958. The taxonomoy of *Acetobacter* and allied oxidative bacteria. J.Gen. Appl. Microbiol. 4, 289-311.

Ausubel, F. M., Brent, R., Kingston, R.E., Moore, D. D., Seidman, J. G., Smith, J. A., Struh, K., 1992. Short Protocols in Molecular Biology: Jonh Willey and Sons Inc, London.

Azuma, Y., Hosoyama, A., Matsutani, M, Furuya, N., Horikawa, H., Harada, T., Hirakawa, H., Kuhara, S., Matsushita, K., Fujita, N., Shirai, M., 2009. Whole-genome analyses reveal genetic instability of *Acetobacter pasteurianus*. Nucleic Acids Res. 37, 5768-83.

Barry, T., Colleran, G., Glennon, M., Dunican, L.K., Gannon, F., 1991. The 16s/23s Ribosomal Spacer Region as a Target for DNA Probes to Identify Eubacteria. PCR Methods Appl. 1, 51-56.

Beijerinck, M.W., 1898. Uber die Arten der Essigbakterien. Zentralblatt für Bakteriologie, Parasitenkunde. Infektionskrankheiten und Hygiene, 2Abt. 4, 209-216.

Beijerinck, M.W., Folpmers, T., 1916. Verslagen van de gewone vergadering der wis- en naturkundige afdeeling. K Akad Wet, Amsterdam, 18, 1198-1200.

Boesch, C., Trček, J., Sievers, M., Teuber, M., 1998. *Acetobacter intermedius* sp. nov. Syst. Appl. Microbiol. 21, 220–229.

Boyer, S.L., Flechtner, V.R., Johansen, J.R., 2001. Is the 16S-23S rRNA Internal Transcribed Spacer Region a Good Tool for Use in Molecular Systematics and Population Genetics? A Case Study in *Cyanobacteria*. Mol. Biol. Evol. 18, 1057-1069.

Brown, A.J., 1886. XLIII- On an acetic ferment which forms cellulose. J.Chem. Soc. Trans. 49, 432-439.

Cao, B., Wang, M., Liu, L., Zhou, Z., Wen, S., Rozalski, A., Wang, L., 2009. 16S-23S rDNA internal transcribed spacer regions in four Proteus species. J. Microbiol. Methods. 77, 109-118.

Carr, J. G., 1958. *Acetobacter estunense* nov. spec. An addition to Frateur's ten basic species. Antonie van Leeuwenhoek J. Microbiol. Serol. 24, 157–160.

Cleenwerck, I., Vandemeulebroecke, K., Janssens, D., Swings, J., 2002. Re-examination of the genus Acetobacter, with descriptions of *Acetobacter cerevisiae* sp. nov. and *Acetobacter malorum* sp. nov. Int. J. Syst. Evol. Microbiol. 52, 1551-1558.

Cleenwerck, I., and De Vos, P., 2008. Polyphasic taxonomy of acetic acid bacteria: An overview of currently applied methodology. Int. J. Food Microbiol. 125, 2-14.

De Ley, J., 1961. Comparative carbohydrate metabolism and a proposal for the phylogenetic relationship of the acetic acid bacteria. J. Gen. Microbiol. 24, 31-50.

De Ley, J., Gossele, F., Swings, J., 1984. Genus I *Acetobacter*. In: Krieg, N.R., Holt, J.G., (Eds.), Bergey's Manual of Systematic Bacteriology, vol. 1, William & Wilkins, Baltimore, Maryland USA, pp 268-274.

Dellaglio, F., Cleenwerck, I., Felis, G.E., Engelbeen, K., Janssens, D., Marzotto, M., 2005. Description of *Gluconobacter swingsii* sp. nov. and *Gluconobacter rhaeticus* sp. nov., isolated from Italian apple fruit. Int. J. Syst. Evol. Microbiol. 55, 2365- 2370.

Dutta, C., and Pan, A., 2002. Horizontal gene transfer and bacterial diversity. J. Biosci. 27, 27-33.

Dutta, D., Gachhui, R., 2006. Novel nitrogen-fixing *Acetobacter nitrogenifigens* sp. nov., isolated from Kombucha tea. Int. J. Syst. Evol. Micr. 56, 1899-1903.

Franke, I.H., Fegan, M., Hayward, C., Leonard, G., Stackebrandt, E., Sly, L.I., 1999. Description of *Gluconacetobacter sacchari* sp. nov., a new species of acetic acid bacterium isolated from the leaf sheath of sugar cane and from the pink sugar-cane mealy bug. Int. J. Syst. Bacteriol. 49, 1681-1693.

Fuentes-Ramírez, L.E., Bustillos-Crystales, R., Tapia-Hernández, A., Jimenéz- Salgado, T., Wang, E.T., Martínez-Romero, E., Caballero-Mellado, J., 2001. Novel nitrogen-fixing acetic acid bacteria, *Gluconacetobacter johannae* sp. nov. and *Gluconacetobacter azotocaptans* sp. nov., associated with coffee plants. Int. J. Syst. Evol. Microbiol. 51, 1305-1314.

Fukaya, M., Tayama, K., Okumura, H., Masai, H., Uozumi, T., Beppu, T., 1985. Improved Transformation Method for *Acetobacter* with Plasmid DNA. Agr. Biol. Chem. 49, 2091-2097.

González, A., Mas., A., 2011. Differentiation of acetic acid bacteria based on sequence analysis of 16S-23S rRNA gene internal transcribed spacer sequences. Int. J. Food Microbiol. 147, 217-222.

Gosselé, F., Swings, J., Kersters, K., De Ley, J., 1983a. Numerical analysis of phenotypic features and protein gel electrophoregram of *Gluconobacter Asai* 1935 emend. mut. char. Asai, Iizuka and Komagata 1964. Int. J. Syst. Bacteriol. 33, 65-81.

Gosselé, F., Swings, J., Kersters, K., Pauwels, P., De Ley, J., 1983b. Numerical analysis of phenotypic features and protein gel electrophoregrams of a wide variety of *Acetobacter* strains. Proposal for the improvement of the taxonomy of the genus *Acetobacter* Beijerinck 1898, 215. Syst. Appl. Microbiol. 4, 338-368.

Guillamón, J., Mas, A., 2009. Acetic Acid Bacteria. In: Konig, H et al., (Eds.), Biology of Microorganism on Grapes, in Must and Wine. Springer-Verlag, Berlin.

Gurtler, V., Barrie, H.D., 1995. Typing of *Staphylococcus aureus* strains by PCR amplification of variable-length 16S-23S rDNA spacer regions: characterization of spacer sequences. Microbiology. 141, 1255-1265.

Gurtler, V., 1999. The role of recombination and mutation in 16S-23S rDNA spacer rearrangements. Gene. 238, 241-252.

Hansen, E. C.,1879. *Mycoderma aceti* (Kütz) Pasteur et *Myc. pasteurianus*. Compte Rendu des travaux du Laboratoire Carlsberg, 1, 96–100.

Hidalgo, C., Torija, M.J., Mas, A., Mateo, E., 2013. Effect of inoculation on strawberry fermentation and acetification processes using native strains of yeast and acetic acid bacteria. Food Microbiol. 34, 88-94.

Katsura, K., Yamada, Y., Uchimura, T., Komagata, K., 2002. *Gluconobacter asaii* Mason and Claus 1989 is a junior subjective synonym of *Gluconobacter cerinus* Yamada and Akita 1984. Int. J. Syst. Evol. Microbiol. 52, 1635-1640.

Lawrence, J.G., 1999. Gene transfer, speciation, and the evolution of bacterial genomes. Curr. Opin. Microbiol. 2, 519-523.

Li, L., Wieme, A., Spitaels, F., Balzarini, T., NUnes, O.C., Manaia, C.M., Van Landschoot, A., De Vuyst, L., Cleenwerck, I., Vandamme, P., 2014. *Acetobacter sicerae* sp.nov., isolated from cider and kefir, and identification of species of the genus *Acetobacter* by dnaK, GroEL and rpoB sequences analysis. Int. J. Syst. Evol. Microbiol. 64, 2407-2415.

Lisdiyanti, P., Kawasaki, H., Seki, T., Yamada, Y., Uchimura, T., Komagata, K., 2001. Identification of *Acetobacter* strains isolated from Indonesian sources, and proposals of *Acetobacter syzygii* sp. nov., *Acetobacter cibinongensis* sp. nov. and *Acetobacter orientalis* sp. nov. J. Gen. Appl. Microbiol. 47, 119-131.

Lisdiyanti, P., Kawasaki, H., Widyastuti, Y., Saono, S., Seki, T., Yamada, Y., Uchimura, T. Komagata, K., 2002. *Kozakia baliensis* gen. nov., sp. nov., a novel acetic acid bacterium in the α -Proteobacteria. Int. J. Syst. Evol. Microbiol. 52, 813-818.

Lisdiyanti, P., Navarro, R.R., Uchimura, T., Komagata, K., 2006. Reclassification of *Gluconobacter hansennii* strains ans proposals of *Gluconobacter saccharivorans* sp. nov. and *Gluconacetobacter nataicola* sp. nov. Int. J. Syst. Evol. Microbiol. 56, 2101-2111.

Malimas, T., Yukphan, P., Takahashi, M., Muramatsu, Y., Kaneyasu, M., Potacharoen, W., Tanasupawat, S., Nakagawa, Y., Tanticharoen, M., Yamada, Y., 2008. *Gluconobacter sphaericus* (Ameyama 1975) comb. nov., a brown pigment-producing acetic acid bacterium in the α -Proteobacteria. J. Gen. Appl. Microbiol. 54, 211-220.

Malimas, T., Yukphan, P., Takahashi, M., Muramatsu, Y., Kaneyasu, M., Potacharoen, W., Tanasupawat, S., Nakagawa, Y., Tanticharoen, M., Yamada, Y., 2009. *Gluconobacter japonicus* sp. nov., an acetic acid bacterium in the α -Proteobacteria. Int. J. Syst. Evol. Microbiol. 59, 466-471.

Mas, A., Torija, M.J., González, A., Poblet, M., Guillamón, J., 2007. Acetic acid bacteria in oenology. Contribution to science. 3, 511-521.

Maslunka, C., Gifford, B., Tucci, J., Gurtler, V., Seviour, R.J., 2014. Insertions or deletions (indels) in the *rrn* 16S-23S rRNA gene internal transcribed spacer region (ITS) compromise the typing and identification of strains within the *Acinetobacter calcoaceticus-baumannii* (Abc) complex and closely related members. Plos One. 9, e105390.

Maslunka, C., Gurtler, V., Seviour, R., 2015. Unusual features of the sequences of copies of the 16S-23S rRNA internal transcribed spacer regions of *Acinetobacter bereziniae*, *Acinetobacter guillouiae* and *Acinetobacter baylyi* arise from horizontal gene transfer events. Microbiology. 161, 322-329.

Mason, L.M., Claus, G.W., 1989. Phenotypic characteristics correlated with deoxyribonucleic acid sequence similarities for three species of *Gluconobacter*: *G. oxydans* (Henneberg 1897) De Ley 1961, G. frateurii sp. nov. And G. asaii sp. nov. Int. J. Syst. Bacteriol. 39, 174-184.

Milyutina, I.A., Bobrova, V.K., Matveeva, E., Schaad, N.W., Troitsky, A.V., 2004. Intragenomic heterogeneity of the 16S rRNA – 23S rRNA internal transcribed spacer among *Pseudomonas syringae* and *Pseudomonas fluorescens* strains. FEMS Microbiol. Lett. 239, 17-23.

Navarro, D., Mateo, E., Torija, M.J., Mas, A., 2013. Acetic acid bacteria in grape must. Acetic Acid Bacteria. 2, 19-23.

Prieto, C., Jara, C., Mas, A., Romero, J., 2007. Application of molecular methods for analysing the distribution and diversity of acetic acid bacteria in Chilean vineyards. Int. J. Food Microbiol. 115, 348-355.

Prust, C., Hoffmeister, M., Liesegang, H., Wiezer, A., Fricke, W.F., Ehrenreich, A., Gottschalk, G., Deppenmeier, D., 2005. Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydans*. Nat. Biotechnol. 23, 195-200.

Ruiz, A., Poblet, M., Mas, A., Guillamón, J. M., 2000. Identification of acetic acid bacteria by RFLP of PCR-amplified 16S rDNA and 16S-23S rDNA intergenic spacer. Int. J. Syst. Evol. Microbiol. 50, 1981-1987.

Sievers, M., Lorenzo, A., Gianotti, S., Boesch, C., Teuber, M., 1996. 16-23S ribosomal RNA spacer regions of *Acetobacter europaeus* and *A. xylinum*, tRNA genes and antitermination sequences. FEMS Microbiol. Lett.142, 43-48.

Sievers, M., Sellmer, S., Teuber, M., 1992. *Acetobacter europaeus* sp-nov a main component of industrial vinegar fermenters in Central-Europe. Syst. Appl. Microbiol. 15, 386-392.

Slapšak, N., Cleenwerck, I., De Vos, P., Trček J., 2013. *Gluconacetobacter maltaceti* sp.nov., a novel vinegar producing acetic acid bacterium. Syst. Appl. Microbiol. 36, 17-21.

Sokollek, S.J., Hertel, C., Hammes, W.P., 1998. Description of *Acetobacter odoediens* sp. nov. and *Acetobacter pomorum* sp. nov., two new species isolated from industrial vinegar fermentations. Int. J. Syst. Bacteriol. 49, 935-940.

Tanasupawat, S., Thawai, C., Yukphan, P., Moonmangmee, D., Itoh, T., Adachi, O. Yamada, Y., 2004. *Gluconobacter thailandicus* sp. nov., an acetic acid bacterium in the α -Proteobacteria. J. Gen. Appl. Microbiol. 50, 159-167.

Tonouchi, N., Sugiyama, M., Yokozeki, K., 2003. Construction of a Vector Plasmid for Use in *Gluconobacter oxydans*. Biosci. Biotechnol. Biochem. 67, 211-213.

Trček, J., and Teuber, M., 2002. Genetic and restriction analysis of the 16S-23S rDNA internal transcribed spacer regions of the acetic acid bacteria. FEMS Microbiol. Lett. 208, 69-75.

Trček, J., Barja, F., 2015. Updates on quick identification of acetic acid bacteria with focus on the 16S-23S rrna gene internal transcribed spacer and the analysis of cell protein by MALDI-TOF mass spectrometry. Int. J. Food Microbiol. 196, 137-144.

Valera, M.J., Laich, F., González, S., Torija, M.J., Mateo, E., Mas, A., 2011. Diversity of acetic acid bacteria present in healthy grapes from the Canary Islands. Int. J. Food Microbiol. 151, 105-112.

Vegas, C., Mateo, E., Gonzalez, A., Jara, C., Guillamon, J.M., Poblet, M., Torija, M.J., Mas, A., 2010. Population dynamics of acetic acid bacteria during traditional wine vinegar production. Int. J. Food Microbiol. 138, 130–136.

Visser´t Hooft, F., 1925. Biochemische Onderzoekingen over het geslacht Acetobacter. Dissertation, Technical University, Delft, Meinema.

Yamada, Y., Akita, M., 1984. *Gluconobacter cerinus*. In: Validation of the Publication of New Names and New Combinations Previously Effectively Published Outside the IJSB, List no. 16. Int. J. Syst. Bacteriol. 34, 503–504.

Yamada, Y., Hoshino, K., Ishikawa, T., 1997. The phylogeny of acetic acid bacteria based on the partial sequences of 16S ribosomal RNA: the elevation of the subgenus *Gluconoacetobacter* to the generic level. Biosci. Biotechnol. Biochem. 61,1244–1251.

Yamada, Y., Yukphan, P., Lan Vu, H.T., Muramatsu, Y., Ochaikul, D., Nakagawa, Y., 2012. Subdivision of the genus *Gluconacetobacter* Yamada, Hoshino and Ishikawa 1998: the proposal of *Komagatabacter* gen. nov., for strains accommodated to the *Gluconacetobacter xylinus* group in the α-Proteobacteria. Ann. Microbiol. 62, 849-859

Yamada, Y., 2014. Transfer of *Gluconobacter kakiaceti*, *Gluconacetobacter medelliensis* and *Gluconobacter maltaceti* to the genus *Komagataeibacter* as *Komagataeibacter kakiaceti* comb. nov., *Komagataeibacter medellinensis* comb. nov., and *Komagataeibacter maltaceti* comb. nov. Int. J. Syst. Evol. Microbiol. 64, 1670-1672.

CHAPTER 5

Draft genome sequence of *Acetobacter malorum* CECT 7742, a strain isolated from strawberry vinegar

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Genome Announcement 4(3):e00620-16

UNIVERSITAT ROVIRA I VIRGILI
SELECTION AND OPTIMIZATION OF ACETIC ACID BACTERIA FOR D-GLUCONIC ACID PRODUCTION
Florencia Sainz Perez

Acetobacter malorum was proposed as new species in 2002 (1) after a polyphasic study of 34 *Acetobacter* strains. This strain was isolated in rotten apples in Ghent (Belgium) and initially classified as *Acetobacter pasteurianus*. The genus *Acetobacter* is included in the group of acetic acid bacteria (AAB), which are Gram-negative bacteria from the family *Acetobacteraceae*. AAB are aerobic microorganisms that are responsible for vinegar production (2). Its main characteristic is the incomplete oxidation of a wide range of carbohydrates and alcohols yielding the corresponding ketones, aldehydes, and acids that are left in the media (3). The biotechnological industry has taken advantage of this capacity to recover some compounds, such as intermediaries in the production of vitaminC(L-sorbose) and miglitol (antidiabetic drug, after amino-L-sorbose), although these have mostly been applied to members of the genus *Gluconobacter* (4, 5).

After the initial description of *A. malorum*, this species has been found in other environments, generally associated with fruits but also with the processing of these fruits. *A. malorum* has been isolated in rotten grapes from Australia (6), must from healthy grapes from Tarragona, Spain (7), and in fermented grape musts in the Canary Islands (8). *A. malorum* was also isolated from fermented persimmon juices (9) and fermented milk (10). The present strain of *A. malorum* was isolated from strawberry vinegar and has been used as starter culture for the production of different fruit vinegars (11–13). The identification of *A. malorum* is difficult due to the high sequence homology with *Acetobacter cerevisiae* when using the 16S sequence analysis. The use of the internal transcribed spacer (ITS) 16S-23S rRNA coding region has provided conclusive differentiation for both of them (6, 14). The polymorphism in this region has allowed the development of specific TaqMan probes that can be used routinely to differentiate these two species by a culture-independent quantitative PCR technique (15).

The strain *A. malorum* CECT 7742 has provided excellent results in the production of D-gluconic acid from D-glucose without the oxidation of D-fructose, which has been used for the production of new strawberry beverages based on the presence of D-fructose as a sweetener and being free of D-glucose (16).

Genomic DNA was extracted according to the cetyltrimethylammonium bromide (CTAB) method (17). For whole-genome sequencing, the Genome Analyzer Ion Torrent PGM (Thermo Fisher Scientific, Madrid, Spain) was used. Preparation of shotgun libraries was performed according to the protocols of the manufacturers and resulted in 5,149,025 reads (256 bp).

The genome of *A. malorum* CECT 7742 consists of a chromosome with 4.04 Mb and an overall G_C content of 56.78%. The genome was assembled in 331 contigs from 927,367 reads using the software MIRA 4.9.5_2 (18). Prokka (19) was used for automatic annotation and gene detection. The genome harbored 6 rRNA genes, 64 tRNA genes, 3,416 protein-coding genes with predicted functions, and 649 genes coding for hypothetical proteins. Among them, 189 genes encoded dehydrogenases, including membrane PQQ-dependent glucose dehydrogenase and flavin adenine dinucleotide (FAD)-dependent gluconate-2-dehydrogenase and 2-keto-D-gluconate dehydrogenase, responsible for the synthesis of D-gluconic acid and its further oxidation to 2-keto-D-gluconic acid and 2,5-di-keto-Dgluconic acid, respectively.

NUCLEOTIDE SEQUENCE ACCESSION NUMBERS

This whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession no. LVHD00000000. The version described in this paper is version LVHD01000000.

FUNDING INFORMATION

This work was funded by the Spanish Ministry of Science and Innovation (AGL2010-22152-C03-02). Florencia Sainz is the recipient of an FI fellowship from the Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR) of Catalonian Government.

REFERENCES

1. Cleenwerck I, Vandemeulebroecke K, Janssens D, Swings J. 2002. Reexamination of the genus *Acetobacter*, with descriptions of *Acetobacter cerevisiae* sp. nov. and *Acetobacter malorum* sp. nov. Int J Syst Evol Microbiol 52:1551–1558. http://dx.doi.org/10.1099/00207713-52-5-1551.

2. Guillamón JM, Mas A. 2011. Acetic acid bacteria, p 227–255. *In* Carrascosa AV, Muñoz R, González R (ed.). Molecular wine microbiology. Elsevier, Inc., London United Kingdom.

3. Adachi O, Moonmangmee D, Toyama H, Yamada M, Shinagawa E, Matsushita K. 2003. New developments in oxidative fermentation. Appl Microbiol Biotechnol 60:643–653. http://dx.doi.org/10.1007/s00253-002 -1155-9.ç

4. Deppenheimer U, Hoffmeiter M, Prust C. 2002. Biochemistry and biotechnological application of *Gluconobacter* strains. Appl Microbiol Biotechnol 60:233–242. http://dx.doi.org/10.1007/s00253-002-1114-5.

5. MacauleyS, McNeil B, Harvey LM. 2001. The genus *Gluconobacter* and its applications in biotechnology. Crit Rev Biotechnol 21:1–25. http:// dx.doi.org/10.1080/20013891081665.

6. Mateo E, Torija MJ, Mas A, Bartowsky EJ. 2014. Acetic acid bacteria isolated from grapes of South Australian vineyards. Int J Food Microbiol 178:98–106. http://dx.doi.org/10.1016/j.ijfoodmicro.2014.03.010.

7. Navarro D, Mateo E, Torija MJ, Mas A. 2013. Acetic acid bacteria in grape must. Acetic Acid Bacteria 2:, 19 –23. http://dx.doi.org/10.4081/ aab.2013.s1.e4.

8. Valera MJ, Laich F, González SS, Torija MJ, Mateo E, Mas A. 2011. Diversity of acetic acid bacteria present in healthy grapes from the Canary Islands. Int J Food Microbiol 151:105–112. http://dx.doi.org/10.1016/ j.ijfoodmicro.2011.08.007.

9. Hidalgo C, Mateo E, Mas A, Torija MJ. 2012. Identification of yeast and acetic acid bacteria isolated from the fermentation and acetification of persimmon (*Diospyros kaki*). Food Microbiol 30:98 –104. http:// dx.doi.org/10.1016/j.fm.2011.12.017.

10. Ogawa S, Tachimoto H, Kaga T. 2010. Elevation of ceramide in *Acetobacter malorum* S24 by low pH stress and high temperature stress. J Biosci Bioeng 109:32–36. http://dx.doi.org/10.1016/j.jbiosc.2009.07.007.

11. Hidalgo C, Torija MJ, Mas A, Mateo E. 2013. Effect of inoculation on strawberry fermentation and acetification processes using native strains of yeast and acetic acid bacteria. Food Microbiol 34:88 –94. http:// dx.doi.org/10.1016/j.fm.2012.11.019.

12. Ubeda C, Callejón RM, Hidalgo C, Torija MJ, Mas A, Troncoso AM, Morales ML. 2011. Determination of major volatile compounds during elaboration of fruit vinegars by headspace gas chromatography-mass spectrometry method. Food Res Int 44:259 –268. http://dx.doi.org/ 10.1016/j.foodres.2010.10.025.

13. Callejón RM, Ubeda C, Hidalgo C, Mas A, Troncoso AM, Morales ML. 2015. Changes of free amino acids during the alcoholic fermentation of strawberry and persimmon. Int J Food Microbiol 50:48–54.

14. González A, Mas A. 2011. Differentiation of acetic acid bacteria based on sequence analysis of 16S-23S rRNA gene internal transcribed spacer sequences. Int J Food Microbiol 147:217–222. http://dx.doi.org/10.1016/ j.ijfoodmicro.2011.04.005.

15. Valera MJ, Torija MJ, Mas A, Mateo E. 2013. *Acetobacter malorum* and *Acetobacter cerevisiae* identification and quantification by real-time PCR with TaqMan-MGB probes. Food Microbiol 36:30–39. http://dx.doi.org/ 10.1016/j.fm.2013.03.008.

16. Sainz F, Navarro D, Mateo E, Torija MJ, Mas A. 2016. Comparison of D-gluconic acid production among selected strains of acetic acid bacteria. Int J Food Microbiol 222:40 – 47. http://dx.doi.org/10.1016/ j.ijfoodmicro.2016.01.015.

17. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. 1992. Short protocols in molecular biology. John Wiley & Sons, London, United Kingdom.

18. Chevreux B, Pfisterer T, Drescher B, Driesel AJ, Müller WEG, Wetter T, Suhai S. 2004. Using the miraEST assembler for reliable and automated mRNA transcript assembly and SNP detection in sequenced ESTs. Genome Res 14:1147–1159. http://dx.doi.org/10.1101/gr.1917404.

19. Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30:20168 – 22069. http://dx.doi.org/10.1093/bioinformatics/ btu153.

CHAPTER 6

Draft genome sequences of *Gluconobacter cerinus* CECT 9110 and *Gluconobacter japonicus* CECT 8443, acetic acid bacteria isolated from grape must

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Genome Announcement 4(3):e00621-16

UNIVERSITAT ROVIRA I VIRGILI
SELECTION AND OPTIMIZATION OF ACETIC ACID BACTERIA FOR D-GLUCONIC ACID PRODUCTION
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The species *Gluconobacter cerinus* (1) was reclassified to *G. cerinus* in 1984 (2) after protein electrophoretic profiles analysis. This species was studied due to the production of ketofructose (3) and some particularities in lipid composition (4, 5). Instead, the species *Gluconobacter japonicus* was proposed in 2009 after DNA hybridization within the genus *Gluconobacter* (6). The type strain had been isolated from the fruits of *Myrica rubra* in Japan. The genus *Gluconobacter* is included in acetic acid bacteria (AAB) and has preference for sugary substrates. AAB are Gram-negative bacteria from the family *Acetobacteraceae*. AAB are aerobic microorganisms that are the main bacteria responsible for vinegar production but also for other biotechnological applications (7). AAB produce the corresponding ketones, aldehydes, and acids from the incomplete oxidation of a wide range of carbohydrates and alcohols. The oxidized products can be recovered from media (8). The biotechnological industry has taken advantage of this capacity of *Gluconobacter* species to recover some compounds, such as intermediaries, in the production of vitaminC(L-sorbose) and miglitol (antidiabetic drug, after amino-L-sorbose) (9, 10).

Both species have been described in grapes or grape musts. *G. cerinus* was isolated in grape musts from Spain (11) and rotten grapes in Australia (12), whereas *G. japonicus* has been recovered in Spain, both in grape musts (11) as well as in fermented musts (13). *G. japonicus* was also detected in kefir grains by culture independent methods (denaturing gradient gel electrophoresis PCR [DGGE-PCR]) (14). The present strains were isolated in our experimental cellar in Tarragona, Spain (11).

Our interest in these strains was for their use in the production of gluconate from D-glucose without the oxidation of D-fructose (15). This transformation is sought for use in the production of new strawberry beverages based on the presence of D-fructose as a sweetener and free of D-glucose because it is converted into D-gluconic acid or the corresponding gluconates (16–18).

Genomic DNA was extracted according to the cetyltrimethylammonium bromide (CTAB) method (19). For whole-genome sequencing, the Genome Analyzer Ion Torrent PGM

(Thermo Fisher Scientific, Madrid, Spain) was used. Preparation of shotgun libraries was performed according to the protocols of the manufacturers and resulted in 5,149,025 reads (256 bp).

The genomes consisted of a chromosome with 3.66 Mb and an overall G_C content of 55.68% for *G. cerinus*, and 3.50 Mb and a G_C content of 56.28% for *G. japonicus*. The genomes were assembled in 45 contigs from 1,529,910 reads (*G. cerinus*) and 50 contigs from 1,831,761 reads (*G. japonicus*) using the software MIRA 4.9.5_2 (20). Prokka (21) was used for automatic annotation and gene detection. The genome harbored 2 rRNA genes, 49 tRNA genes, 2,616 protein-coding genes with predicted functions, and 786 genes coding for hypothetical proteins for *G. cerinus*, and 2 rRNA genes, 53 tRNA genes, 2,594 protein-coding genes with predicted functions, and 645 genes coding for hypothetical proteins for *G. japonicus*.

Among the identified genes, 122 and 132 encoded dehydrogenases in *G. cerinus* and *G. japonicus*, respectively, including membrane PQQ-dependent glucose dehydrogenase, flavin adenine dinucleotide (FAD)-dependent gluconate-2-dehydrogenase, and PQQ-dependent sorbitol dehydrogenase. Both strains have two genes encoding PQQ-dependent sorbitol dehydrogenase.

NUCLEOTIDE SEQUENCE ACCESSION NUMBERS

This whole-genome shotgun project of *G. cerinus* CECT 9110 and *G. japonicus* CECT 8443 has been deposited at DDBJ/EMBL/GenBank under the accession numbers LUTU00000000 and LVHE00000000, respectively. The versions described in this paper are versions LUTU01000000 for *G. cerinus* CECT 9110 and LVHE01000000 for *G. japonicus* CECT 8443.

ACKNOWLEDGMENTS

This work was supported by grant AGL2010-22152-C03-02 from the Spanish Ministry of Science and Innovation. F. Sainz is a recipient of an AGAUR Fellowship (Government of Catalonia).

REFERENCES

1. Asai T. 1935. Taxonomic studies on acetic acid bacteria and allied oxidative bacteria isolated from fruits. A new classification of the oxidative bacteria. J Agric Chem Soc Jpn 11:674–708.

2. Yamada Y, Akita M. 1984. An electrophoretic comparison of enzymes in strains of *Gluconobacter* species. J Gen Appl Microbiol 30:115–126. http://dx.doi.org/10.2323/jgam.30.115

3. England S, Avigad G. 1965. 5-keto-D-fructose. II. Patterns of formation and of associated dehydrogenase activities in *Gluconobacter cerinus*. J Biol Chem 240:2297–2301.

4. Tahara Y, Yamada Y, Kondo K. 1975. Occurrence of phosphatidylcholine in *Gluconobacter cerinus*. Agr Biol Chem 39:2261–2262.

5. Tahara Y, Kameda M, Yamada Y, Kondo K. 1976. A new lipid; the ornithine and taurine-containing “cerilipin.” Agr Biol Chem 40:243–244.

6. Malimas T, Yukphan P, Takahashi M, Muramatsu Y, Kaneyasu M, Potacharoen W, Tanasupawat S, Nakagawa Y, Tanticharoen M, Yamada Y. 2009. *Gluconobacter japonicus* sp. nov., an acetic acid bacterium in the *Alphaproteobacteria*. Int J Syst Evol Microbiol 59:466–471. http://dx.doi.org/10.1099/ijs.0.65740-0

7. Guillamón JM, Mas A. 2011. Acetic acid bacteria, p 227–255. *In* Carrascosa AV, Muñoz R, González R (ed.), Molecular wine microbiology. Elsevier, Inc., London, United Kingdom.

8. Adachi O, Moonmangmee D, Toyama H, Yamada M, Shinagawa E, Matsushita K. 2003. New developments in oxidative fermentation. Appl Microbiol Biotechnol 60:643–653. http://dx.doi.org/10.1007/s00253-002-1155-9

9. Deppenmeier U, Hoffmeister M, Prust C. 2002. Biochemistry and biotechnological application of *Gluconobacter* strains. Appl Microbiol Biotechnol 60:233–242. http://dx.doi.org/10.1007/s00253-002-1114-5

10. Macauley S, McNeil B, Harvey LM. 2001. The genus *Gluconobacter* and its applications in biotechnology. Crit Rev Biotechnol 21:1–25. http:// dx.doi.org/10.1080/20013891081665.

11. Navarro D, Mateo E, Torija MJ, Mas A. 2013. Acetic acid bacteria in grape must. Acetic Acid Bacteria 2:e419-23. http://dx.doi.org/10.4081/ aab.2013.s1.e4.

12. Mateo E, Torija MJ, Mas A, Bartowsky EJ. 2014. Acetic acid bacteria isolated from grapes of South Australian vineyards. Int J Food Microbiol 178:98–106. http://dx.doi.org/10.1016/j.ijfoodmicro.2014.03.010

13. Valera MJ, Laich F, González SS, Torija MJ, Mateo E, Mas A. 2011. Diversity of acetic acid bacteria present in healthy grapes from the Canary Islands. Int J Food Microbiol 151:105–112. http://dx.doi.org/10.1016/j.ijfoodmicro.2011.08.007

14. Miguel MGDCP, Cardoso PG, Lago LdA, Schwan RF. 2010. Diversity of bacteria present in milk kefir grains using culture-dependent and cultureindependent methods. Food Res Int 43:1523–1528. http://dx.doi.org/10.1016/j.foodres.2010.04.031

15. Sainz F, Navarro D, Mateo E, Torija MJ, Mas A. 2016. Comparison of D-gluconic acid production among selected strains of acetic acid bacteria. Int J Food Microbiol 222:40 – 47. http://dx.doi.org/10.1016/j.ijfoodmicro.2016.01.015

16. Cañete-Rodríguez AM, Santos-Dueñas IM, Torija-Martínez MJ, Mas A, Jiménez-Hornero JE, García-García I. 2015. Preparation of a pure inoculum of acetic acid bacteria for the selective conversion of glucose in strawberry purée into gluconic acid. Food Bioprod Process 96:35– 42. http://dx.doi.org/10.1016/j.fbp.2015.06.005

17. Cañete-Rodríguez AM, Santos-Dueñas IM, Jiménez-Hornero JE, Torija-Martínez MJ, Mas A, García-García I. 2016. An approach for estimating the maximum specific growth rate of *Gluconobacter japonicus* in strawberry purée without cell concentration data. Biochem Eng J 105: 314–320. http://dx.doi.org/10.1016/j.bej.2015.10.005

18. Ordóñez JL, Sainz F, Callejón RM, Troncoso AM, Torija MJ, García- Parrilla MC. 2015. Impact of gluconic fermentation of strawberry using acetic acid bacteria on amino acids and biogenic amines profile. Food Chem 178:221–228. http://dx.doi.org/10.1016/j.foodchem.2015.01.085

19. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. 1992. Short protocols in molecular biology. John Wiley & Sons, London, United Kingdom.

20. Chevreux B, Pfisterer T, Drescher B, Driesel AJ, Müller WEG, Wetter T, Suhai S. 2004. Using the miraEST assembler for reliable and automated mRNA transcript assembly and SNP detection in sequenced ESTs. Genome Res 14:1147–1159. http://dx.doi.org/10.1101/gr.1917404

21. Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30:2068–2069. http://dx.doi.org/10.1093/bioinformatics

GENERAL DISCUSSION

UNIVERSITAT ROVIRA I VIRGILI
SELECTION AND OPTIMIZATION OF ACETIC ACID BACTERIA FOR D-GLUCONIC ACID PRODUCTION
Florencia Sainz Perez

A large amount of fruits and vegetables is wasted due the excess of crops and the lack of quality standards (size, form, color…) required by the market. This waste is an economic and environmental challenge, which must be solved. One solution has been the production of different products like jams, preserves, juices, etc. However, although there is a great demand for this type of products, the market is already saturated for most of the fruits and vegetables that can go to this market. Therefore, a possible alternative is to develop new products such as beverages or vinegars. In addition, consumers are interested in new tailored products, with high quality and added values that might also provide health benefits. Thus, the industry should combine these two current targets and invest in the development of new products that can satisfy both needs, consumers and producers. A possibility is to transform these surpluses of raw materials by fermentation. The fermentation process is known to offer numerous benefits that include the improvement of sensory characteristics, acceptability, nutritional value and safety of foods (Malo and Urquhart, 2016).

Spain is one of the largest world producers of strawberries in the world and the first in Europe. Furthermore, strawberries are easily perishable and large amounts are wasted, which converts this fruit in a very good candidate for the development of new fermented products. In this thesis we hypothesized that appropriate starter cultures would be able to produce a new non-alcoholic fermented beverage with surplus strawberry by oxidizing D-glucose to D-gluconic acid. The proposed beverage would be a mixture of two products obtained from different strawberry fermentations. The main one was the selective oxidation of D-glucose into D-gluconic acid by acetic acid bacteria (AAB), without fermenting D-fructose, where this thesis is focused. The idea of this “gluconated strawberry juice” was to keep the natural sweetness of strawberry, increasing the acceptability of the product. The second one, already developed in a previous project, dealt with the production of a strawberry vinegar, which added to the “gluconated strawberry juice” would contribute to the microbial stability of the product, unstable because of the presence of D-fructose.

AAB would be involved in both processes. AAB are well known due to their ability to carry out oxidative fermentation of a wide range of alcohols and sugars, resulting in the accumulation of near-quantitative oxidative products in the medium. In this thesis, different *Gluconobacter* and *Acetobacter* strains were tested to perform this selective oxidation of D-glucose to D-gluconic acid. Initially, *Gluconobacter* strains were expected to be the best candidates for this process. *Gluconobacter* strains are known to prefer sugars rather than ethanol, unlike what happens in *Acetobacter* strains (Raspor and Goranovic, 2008). However, this does not mean that *Acetobacter* strains are not able to oxidize D-glucose to D-gluconic acid. In fact, some authors noted that the *Acetobacter* strains could also produce high concentration of D-gluconic acid, although not as much as *Gluconobacter* strains. Furthermore, we had a single strain isolated from strawberry in a previous project. This strain was classified as *Acetobacter malorum*. Therefore, for all these reasons, we incorporated *Acetobacter* strains. All the strains tested were capable of oxidizing D-glucose into D-gluconic acid. However, other features, like complete exhaustion of D-glucose and further oxidation of this acid to keto-D-gluconic acids was strain and medium dependent. All the strains tested preferred D-glucose rather than D-fructose, although some of them also consumed a part of the D-fructose present. To select the best strains, different media and conditions were tested, but due to the high influence of these parameters for the development of the process, we focused the strain selection based on the data obtained in strawberry concentrated puree medium. Three strains were selected: *G. japonicus* CECT 8443, *G. oxydans* Po5 and *A. malorum* CECT 7742. These strains were isolated for our research group from different natural sources: grapes, wine vinegar and strawberry vinegar, respectively (Navarro et al., 2011; Vegas et al., 2010; Hidalgo et al., 2013). These strains were the focus of the study in all the chapters of this thesis, usually compared to the type strain of each species.

Under optimal conditions, high yields of D-gluconic acid can be produced. Most of the studies about D-glucose oxidation focused on the optimization of medium composition, and process conditions. Medium pH seemed to be one of the most important factor for

D-glucose oxidation. Diverse authors suggested different pHs for a better selective production of D-gluconic or keto-D-gluconic acids and recommended maintaining constant the pH of the medium throughout the process. According to Weenk et al. (1984) and Beschkov and Velizarov (1995), when the pH was not controlled, the D-glucose was quantitatively oxidized to D-gluconic acid and no production of keto-D-gluconic acids was detected. In our work, the medium pH was adjusted to pH 3.3 at the beginning of the process (in one experiment the pH was also adjusted to 4.8), but, then, it was allowed to freely evolve throughout the process. However, although as expected the pH decreased in all the cases, we were able to detect the production of keto-D-gluconic acids in most of the strains and conditions. Essentially, only Po5 strain showed a very low concentration of these products. According to the literature, we should assume that if the pH remained constant during the process, greater concentrations of these keto-D-gluconic acids would have been obtained. In the case of pH 4.8, calcium carbonate was used to adjust the pH. The addition of calcium carbonate at the beginning of the process was described to increase the keto-D-gluconic acids formation in the medium (Stadler-Szöke et al., 1980; Weenk et al., 1984) and also to speed up their production (Beschkov and Velizarov, 1995), especially of 5KGA, which was the main product observed. This statement was confirmed in our study since the processes with addition of calcium carbonate presented higher concentration of keto-D-gluconic acids than the processes carried out at pH 3.3. Different initial pHs were tested because Silberbach et al. (2003) described that each stage of the process had an optimum pH: pH 5.5 for cell growth; pH 4.8 for complete D-glucose oxidation and pH 3.15 for 2KGA oxidation to 2,5KGA. However, in our case, the D-gluconic acid production was higher at pH 3.3 whereas the formation of keto-D-gluconic acids was higher at pH 4.8, as above mentioned, resulting in a lower concentration of D-gluconic acid. Therefore, we can conclude that it is difficult to establish the optimum pH for the selective production of a particular product, but it does not seem necessary the pH control to obtain a high concentration of D-gluconic acid. However, for implementation at industrial scale, it would be absolutely necessary

to establish the particular conditions of the process if pH control could prevent that D-glucose oxidation progresses towards the production of keto-D-gluconic acids, diminishing the concentration of D-gluconic acid present in the medium.

Production of D-gluconate by AAB is inversely related to biomass formation or, in other terms, high levels of D-gluconic acid in the medium imply low growth of AAB (Olijve and Kok, 1979; Deppenmeier et al., 2002; Elfari et al., 2005). Furthermore, when D-glucose and D-gluconic acid oxidation occurred at pH values of 3.5 - 4.0, like in our study, the pentose phosphate pathway is inhibited (Olijve and Kok, 1979) and AAB are unable to use these substrates present in the medium as a source of assimilable carbon, and thus, to form cell mass (Kostner et al., 2013). This was confirmed in our study when growth and membrane-bound glucose dehydrogenase (mGDH) activity was compared between both strains of *G.oxydans* (Po5 and 621H). Po5 presented lower growth, but three times higher activity of mGDH than the strain 621H. In fact, the latter was described as a good strain for industrial purposes because it had a high substrate oxidation due to very active membrane-bound dehydrogenases, despite the disadvantage of low biomass production (Kostner et al., 2015). In our case, although Po5 was also well qualified for industrial production, because it had more active membrane-bound dehydrogenases, the industrial application would be restricted by its poor biomass yield. Therefore, if it were possible to boost the AAB growth, the productivity of the process would increase and also its economic yield.

In AAB, the membrane-bound dehydrogenases seemed to compete with the NADH dehydrogenases for channeling electrons in the respiratory chain. A recent work (Kotsner et al., 2015) suggested that the activity of the NADH dehydrogenase could be a key factor to control the biomass formation and the activity of membrane-bound dehydrogenases. Two enzymes would have a great influence in the growth of *G. oxydans*: type II NADH dehydrogenase (NDH-2) and triose-phosphate isomerase (TPI). In fact, when an additional copy of the *ndh* gene was introduced in the strain 621H, this strain showed an important rise of growth and a sharp decrease of the activity of

membrane-bound dehydrogenases. Therefore, a compromise between both parameters is required for selection as starter cultures for industrial purposes. In our case, from the three selected strains, the most interesting was the strain CECT 8443 of *G. japonicus*, because this strain showed a high growth yield and a high activity of membrane-bound dehydrogenases, especially in mGDH. However, in this case, similar to what happened with CECT 7742, this high biomass was linked to the use of different carbon sources throughout the process. Initially, they used D-glucose to grow, but when depleted, these strains continued to grow using D-gluconic acid and even keto-D-gluconic acids. Therefore, the strain selection would be also determined by the kind of product to be elaborated. In other words, the selection would be in terms of maintaining all the D-gluconic acid formed or of obtaining a less acidic product and therefore the decrease of D-gluconic acid would be due to oxidation to keto-D-gluconic acids. In our study, no direct relationship was observed between the activity of keto-D-gluconic acid dehydrogenases (GADH and GLDH) and the product content present in the medium: Strains Po5 and CECT 8443 presented similar dehydrogenase activities but quite different pattern of products. Po5 essentially did not synthesize keto-D-gluconic acids, and, thus, D-gluconic acid remained constant throughout the process, whereas CECT 8443 oxidized part of the accumulated D-gluconic to the formation of both keto-D-gluconic acids. The synthesis of the keto-D-gluconic acids was also strain and medium dependent (Asai, 1968; Olivje and Kok, 1979). GLDH and GADH enzymes compete for the oxidation of D-gluconic acid, therefore a selective expression of either dehydrogenase could increase the production of either of the keto-D-gluconic acids (Elfari et al., 2005; Matsushita et al., 2003). More extreme would be the case of CECT 7742, because in some conditions (see Chapter 1), this strain was capable of totally oxidizing the D-gluconic acid to keto-D-gluconic acids and even a part of these keto-D-gluconic acids, which can result in a sharp increase of the pH, with a clear increase of the microbial instability. This could discourage the use of this strain as starter for this process.

Moreover, *A. malorum* CECT 7742 produced 5KGA accumulation in some conditions tested in this thesis, although according to this species description (Cleenwerck et al., 2002), *A. malorum* strains are unable to produce 5KGA. However, no DNA sequence similar to the *gldh* gene described in other species was detected when its whole genome was sequenced. Therefore, another enzyme different from GLDH and able to produce 5KGA should be present at least in some strains of *A. malorum*.

In our study, the activity of mGDH, GADH and GLDH was, in general, higher at 24 hours and decreased over time. However, it would be interesting to study what happened during these first 24 hours to have a more precise evolution of the activity of these dehydrogenases. Summarizing the results obtained in the two studies related with D-gluconic acid production by AAB, the medium and the conditions of the process, together with the strain, were crucial to determine the composition of the final product. Two *Gluconobacter* strains, CECT 8443 and Po5 seemed to be the best strains to carry out this selective oxidation of D-glucose. Nevertheless, the desired final composition of the product would determine which strain is the most appropriate in each case.

The nutritional requirements are a crucial point to determine if a strain could be good candidate to be used at industrial level. In AAB, very few studies have been focused on their nutritional requirements, and the ones done were quite old, around the fifties (Foda and Vaughn, 1953; Raghavendra Rao and Stokes, 1953; Rainbow and Minston, 1953). Lately, studies about the nitrogen consumption patterns of AAB in different processes and conditions have been performed (Valero et al., 2003; Maestre et al., 2008; Callejón et al., 2008; Álvarez-Cáliz et al., 2012, 2014). In our study, we have focused in the nitrogen requirements of the three previously selected strains. To carry out this study, we have used as base medium different laboratory media commonly used to grow bacteria (YNB, M9 and SM). Our first intention was to do this study also in media with addition of ethanol/acetic acid. However, the bad and poorly reproducible growth of AAB in most of these media made us to discard them and to focus only in the abovementioned media. As expected, strains had different nitrogen requirements although some patterns

can be drawn. *Gluconobacter* strains grew better in YNB, and most of them had low nitrogen needs, they could grow correctly in YNB with 25 mg N/L, when a complete solution of ammonium and amino acid was used. However, 621H had higher nitrogen requirements, and therefore, we can assume that if this strain has been described as good for industrial applications (Prust et al., 2005; Wei et al., 2014; Kostner et al., 2015), our selected *Gluconobacter* strains may be even better prepared for this purpose. Low nitrogen requirement is a good feature for a candidate to be selected as industrial starter culture. Instead, *A. malorum* strains grew better in M9 minimal medium, and they needed higher nitrogen concentration (100 mg N/L). In general, all strains tested preferred a complete nitrogen solution. However, some single amino acids could supply all the nitrogen requirements of some strains. This was especially interesting in *Acetobacter* strains since Pro could support the growth even better than complete nitrogen solution. This is an advantage for these strains to carry out wine vinegar acetification, since Pro is the main amino acid in wines (Castor and Archer, 1956; Ough and Stashak, 1974; Ribéreau-Gayon et al., 2006), so AAB have a great availability of this amino acid for the acetification. Moreover, in studies about nitrogen consumption during acetification, it has been reported that Pro is the main amino acid consumed in surface acetifications (Callejón et al., 2008). In fact, the CECT 7742 strain was selected during strawberry surface acetification (Hidalgo et al., 2013). In *Gluconobacter* strains, there was not a single source capable of replacing the complete nitrogen solution, especially in our indigenous strains. However, there were some sources such as Gln, ammonium, Ala, Asn, especially for Po5. Strain CECT 8443 growth was strongly affected in the presence of sole nitrogen sources. Additionally, the amino acid consumption during superficial strawberry D-gluconic fermentations of strawberry was analyzed with the three selected strains and the most consumed amino acids were Gln, Ala and Trp, confirming that this nitrogen sources are good nitrogen sources for these strains (Ordoñez et al., 2015). Different results were obtained when this D-gluconic fermentation was carried out using submerged conditions, without consumption of nitrogen or even and a slight increase in

nitrogen content at the end of the process was observed (Ordoñez et al., 2016). This could be due to the fast rate of submerged processes, AAB only needed few hours to consume all the D-glucose, without AAB growth, which had only a catalytic function (Ordoñez et al., 2016).

Thus, we could conclude that the nitrogen requirements for AAB were very dependent on the specific strain and the process conditions. Nevertheless, in general, our selected strains had low nitrogen requirements, what made them good candidates for industrial processes. Strain CECT 7742 seemed to be better prepared for acetification, especially for strawberry vinegar, and *Gluconobacter* strains (CECT 8443 and Po5) for D-gluconic oxidations. In fact, CECT 7742 and CECT 8443 had been already used as starter cultures in semi-industrial conditions for the elaboration of strawberry vinegar and strawberry gluconated juices, respectively (Hidalgo et al., 2010, 2013; Cañete-Rodríguez et al., 2015, 2016). Therefore, this confirmed that *Acetobacter* strains were better for processes involving oxidation of ethanol whereas *Gluconobacter* strains preferred sugary environments.

As it has already been mentioned several times throughout this thesis, the strawberry beverage proposed was the combination of two different based products, one of them with presence of D-fructose. Therefore, both products must be microbiologically stable, so in the final products, the levels of microorganisms must be low or absent. Our group has developed generic and specific TaqMan-MGB probes and primers for identification and quantification of both yeast as well as AAB by Real-time PCR (Hierro et al., 2006; Andorrà et al., 2011; González et al., 2006; Torija et al., 2010; Valera et al., 2013). For the AAB control in the proposed strawberry beverage, we had available a specific probe for *A. malorum* (Valera et al., 2013), then we can monitor the strain CECT 7742 during acetification, and a generic probe for *Gluconobacter* (Torija et al., 2010). Although initially, this probe, designed in the 16S rDNA gene, was described as specific of *G. oxydans*, the high number of closely related species recently described in the *Gluconobacter* genus, reduced the specificity of this probe that can be used as genus

specific (Valera et al., 2013). However, after a PCR enrichment using primers designed in the ITS region of *G. oxydans* (Torija et al., 2010), it was possible to selectively amplify this species and therefore, it was possible to differentiate it from *G. japonicus* (Valera et al., 2013). Thus, we needed to design a specific TaqMan-MGB probe for the detection of *G. japonicus*. Due to the high similarity of all *Gluconobacter* species in the 16S rDNA gene, we used the 16S-23S rRNA gene ITS region for the probe design. The problem arose when we amplified the ITS region in different *G. japonicus* strains available in our AAB collection, and multiple amplicons were obtained. Our surprise increased when we observed these multiple amplicons in all the sequences of *Gluconobacter* strains tested and instead, a unique amplicon in strains belonging to *Acetobacter* and *Gluconacetobacter* genera. Analysis of these amplicons by sequencing showed additional bands with high similarity to diverse species of the *Acetobacter* genus. Different assumptions could be made to try to elucidate these results: horizontal transfer between the species, presence of this region in a plasmid or DNA contamination during the amplifications. In order to confirm the presence of this “exogenous” ITS copy in the *Gluconobacter* strains, the whole genome sequencing of our three selected strains (CECT 7742, CECT 8443, Po5) together with a *G. cerinus* strain (CECT 9110) isolated from grape must (Navarro et al., 2013) was done. According to our results, the three *Gluconobacter* strains sent for complete genome sequencing presented a ITS with high similarity to *A. malorum*. Unluckily, in any of the *Gluconobacter* strains, the *A. malorum* ITS fragment was found in the complete genome. However, with the extraction method used, it was very probably that the possible plasmids could be lost, for this reason, a hypothetical presence of this “exogenous” ITS in a plasmid could still not be discarded. Therefore, further analysis should be performed to elucidate this possibility. Although the analysis of the complete genome of our selected strains was not an initial task in the project of thesis, to have all this genomic information about our selected strains gives us valuable data to better understand their physiological features and different

performances. Moreover, we have completely sequenced a strain of *G. cerinus*, that, to our knowledge, it is the first whole genome available of this species in the Databases.

So, we can conclude that indeed it is possible to selectively oxidize the D-glucose to D-gluconic acid using selected AAB. Moreover, we have been capable of selecting different starter strains that offer us the possibility of elaborating different products according to the final D-gluconic acid concentration desired. Transfer of this knowledge to industry remains a "pending" task as it happens often with academic research. The three teams that were involved in the project (the Universities of Sevilla, Cordoba and Rovira i Virgili) have demonstrated that a new healthy beverage can be produced at low cost from perishable and surplus substrates. It is now up to the industry and the knowledge transfer offices from Universities to find the appropriate tools to produce it.

REFERENCES

Álvarez-Cáliz, C., Santos-Dueñas, I.M., Cañete-Rodríguez, A.M., García-Martínez, T., Mauricio, J.C., García-García, I., 2012. Free amino acids, urea and ammonium ion contents for submerged wine vinegar production: influence of loading and air-flow rate. Acetic Acid Bacteria. 1, 1-5.

Álvarez-Cáliz, C., Santos-Dueñas, I.M., García-Martínez, T., Cañete-Rodríguez, A.M., Millán-Pérez, M.C., Mauricio, J.C., García-García, I., 2014. Effect of biological ageing of wine on its nitrogen composition for producing high quality vinegar. Food Bioprod. Process. 92, 291-297.

Andorrà, I., Monteiro, M., Esteve-Zarzoso, B., Albergaria, H., Mas A., 2011. Analysis and direct quantification of *Saccharomyces cerevisiae* and *Hanseniaspora guilliermondii* populations during alcoholic fermentation by fluorescence in situ hybridization, flow cytometry and quantitative PCR. Food Microbiol. 28, 1483-1491.

Asai, T., 1968. Acetic Acid Bacteria: Classification and Biochemical Activities. University of Tokyo Press, Tokyo.

Beschkov, V., Velizarov, S., Peevva, L., 1995. Some kinetic aspects and modelling of biotransformation of D-glucose to Keto-D-Gluconate. Bioprocess Biosyst. Eng. 6, 301-305.

Callejón, R.M., Tesfaye, W., Torija, M.J., Mas, A., Troncoso, A.M., Morales, M.L., 2008. HPLC determination of amino acids with AQC derivatization in vinegars along submerged surface acetifications and its relation to the microbiota. Eur. Food Res. Technol. 227, 93-102.

Cañete-Rodríguez, A.M., Santos-Dueñas, I.M., Torija-Martinez, M.J., Mas, A., Jiménez-Hornedo, J.E., García-García, I., 2015. Preparation of a pure inoculum of acetic acid bacteria for the selective conversion of glucose instrawberry purée into gluconic acid. Food Bioprod. Process. 96, 35-42.

Cañete-Rodríguez, A.M., Santos-Dueñas, I.M., Jiménez-Hornedo, J.E., Torija-Martinez, M.J., Mas, A., García-García, I., 2016. Revalorization of strawberry surpluses bybio-transforming its glucose content into gluconic acid. Food Bioprod. Process. 99, 188-196.

Castor, J.G.B. and Archer, T.E., 1956. Amino acids in musts and wines; proline, serine and threonine. Am. J. Enol. Vitic. 7, 19-25.

Cleenwerck, I., Vandemeulebroecke, K., Janssens, D., Swings, J., 2002. Re-examination of the genus *Acetobacter*, with descriptions of *Acetobacter cerevisiae* sp. nov. And *Acetobacter malorum* sp. nov. Int. J. Syst. Evol. Microbiol. 52, 1551-1558.

Deppenmeier, U., Hoffmeister, M., Prust, C., 2002. Biochemistry and biotechnological applications of *Gluconobacter* strains. Appl. Microbiol. Biotechnol. 60, 233-242.

Elfari, M., Ha, S.W., Bremus, C., Merfort, M., Khodaverdi, V., Herrmann, U., Sahm, H., Görisch, H., 2005. A *Gluconobacter oxydans* mutant converting glucose almost quantitatively to 5-keto-D-gluconic acid. Appl. Microbiol. Biotechnol. 66, 668-674.

Foda, O.I., Vaughn, R.H., 1953. The nutritional requirements of *Acetobacter melanogenum* and related species. J. Bacteriol. 65, 79-82.

González, A., Hierro, N., Poblet, M., Mas, A., Guillamón, J.M., 2006. Enumeration and detection of acetic acid bacteria by real-time PCR and nested PCR. FEMS Microbiol. Lett. 254, 123–128

Hidalgo, C., Mateo, E., Cerezo, A.B., Torija, M.J., Mas, A., 2010. Technological process for production of persimmon and strawberry vinegars. International Journal of Wine Research. 2, 55-61.

Hidalgo, C., Torija, M.J., Mas, A., Mateo, E., 2013. Effect of inoculation on strawberry fermentation and acetification processes using native strains of yeast and acetic acid bacteria. Food Microbiol. 34, 88-94.

Hierro, N., Esteve-Zarzoso, B., González, A., Mas, A., Guillamón, J.M., 2006. Real-time quantitative PCR (QPCR) and reverse transcription-QPCR for detection and enumeration of total yeast in wine. Appl. Environ. Microbiol. 72, 71488-7155.

Kostner, D., Luchterhand, B., Junker, A., Volland, S., Daniel, R., Buchs, J., Liebl, W., Ehrenreich, A., 2015. The consequence of an additional NADH dehydrogenase paralog on the growth of *Gluconobacter oxydans* DSM 3504. Appl. Microbiol. Biotechnol. 99, 375-386.

Maestre, O., Santos-Dueñas, I.M., Peinado, R., Jiménez-Ot, C., García-García, I., Mauricio, J.C., 2008. Changes in amino acid composition during wine vinegar production in a fully automatic pilot acetator. Process Biochem. 43, 803-807.

Malo, P.M., Urquhart, E.A., 2016. Fermented Foods: Use of Starter Cultures. In: Caballero, B., Finglas, P.M., Toidrá, F. (Eds.), Encyclopedia of Food and Health, Elsevier, United Kingdom, pp. 681-685.

Matsushita, K., Fujii, Y., Ano, Y., Toyama, H., Shinjoh, M., Tomiyama, N., Miyazaki, T., Sugisawa, T., Hoshino, T., Adachi, O., 2003. 5-Keto-D-gluconate production is catalyzed by a quinoprotein glycerol dehydrogenase, major polyol dehydrogenase, in *Gluconobacter* species. Appl. Environ. Microbiol. 69, 1959-1966.

Navarro, D., Mateo, E., Torija, M.J., Mas, A., 2013. Acetic acid bacteria in grape must. Acetic Acid Bact. 2, 19-23.

Olijve, W., Kok, J.J., 1979. Analysis of growth of *Gluconobacter oxydans* in glucose containing media. Arch. Microbiol. 121**,** 283-290.

Ordóñez, J.L., Sainz, F., Callejón, R.M., Tronocoso, A.M., Torija, M.J., García-Parrilla, M.C., 2015. Impact of gluconic fermentation of strawberry using acetic acid bacteria on amino acids and biogenic amines profile. Food Chem. 178, 221-228.

Ordóñez, J.M., Cañete-Rodríguez, A.M., Callejón, R.M., Santos-Dueñas, M.I., Troncoso, A.M., García-García, I., García-Parrilla, C., 2016. Effect of gluconic acid submerged fermentation of strawberry purée on amino acids and biogenic amines profile. J. Food Process. Pres. doi:10.1111/jfpp.12787.

Ough, C.S., Stashak, R.M., 1974. Further studies on proline concentration in grapes and wines. Am. J. Enol. Vitic. 25, 7-12.

Prust, C., Hoffmeister, M., Liesegang, H., Wiezer, A., Fricke, W.F., Ehrenreich, A., Gottschalk, G., Deppenmeier, U., 2005. Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydans*. Nat. Biotechnol. 23,195-200.

Raghavendra Rao, M.R., Stokes, J.L., 1953. Nutrition of the Acetic Acid Bacteria. J. Bacteriol. 65, 405-412.

Rainbow, C., Mitson, G.W., 1953. Nutritional requirements of Acetic Acid bacteria. J. Gen. Microbiol. 9, 372-375.

Raspor, P., Goranovic, D., 2008. Biotechnological applications of acetic acid bacteria. Crit. Rev. Biotechnol. 28, 101-124.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B., Lonvaud-Funel, A., 2006. Handbook of Enology. The Microbiology of Wine and Vinifications, vol 1, second ed. John Wiley & Sons Ltd, West Sussex, England.

Silberbach, M., Maier, B., Zimmermann, M., Buchs, J., 2003. Glucose oxidation by *Gluconobacter oxydans*: characterization in shaking-flasks, scale-up and optimization of the pH profile. Appl. Microbiol. Biotechnol. 62, 92-98.

Stadler-Szöke, A., Nyeste, L., Holló, J., 1980. Studies on factors affecting gluconic acid and 5-keto-d-gluconic acid formation by *Acetobacter*. Acta. Aliment. 9, 155-172.

Torija, M. J., Mateo, E., Guillamón, J. M., Mas, A., 2010. Identification and quantification of acetic acid bacteria in wine and vinegar by TaqMan-MGB probes. Food Microbiol. 27, 257-265.

Valera, J.M., Torija, M.J., Mas, A., Mateo, E., 2013. *Acetobacter malorum* and *Acetobacter cerevisiae* identification and quantification by Real-Time PCR with TaqMan-MGB probes. Food Microbiol.36, 30-39.

Valero, E., Millán, C., Ortega, J.M., Mauricio, J.C., 2003. Concentration of amino acids in wine after the end of fermentation by *Saccharomyces cerevisiae* strains. J. Sci. Food Agr. 83, 830-835.

Vegas, C., Mateo, E., González, A., Jara, C., Guillamón, J.M., Poblet, M., Torija, M.J., Mas, A., 2010. Population dynamics of acetic acid bacteria during traditional wine vinegar production. Int. J. Food Microbiol. 138, 130-136.

Weenk, G., Olijve, W., Harder, W., 1984. Ketogluconate formation by *Gluconobacter* species. Appl. Microbiol. Biotechnol. 20, 400-405.

Wei, L., Zhu, D., Zhou, J., Zhang, J., Zhu, K., Du., Hua, Q., 2014. Revealing in vivo glucose utilization of *Gluconobacter oxydans* 621H Δ*mgdh* strain by mutagenesis. Microbiol. Res. 169, 469-475.

UNIVERSITAT ROVIRA I VIRGILI
SELECTION AND OPTIMIZATION OF ACETIC ACID BACTERIA FOR D-GLUCONIC ACID PRODUCTION
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GENERAL CONCLUSIONS

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- It is possible to produce a new non-alcoholic fermented beverage with surplus strawberry using AAB by oxidizing D-glucose to D-gluconic acid.

- All the strains of *Gluconobacter* and *Acetobacter* genera tested were able to oxidize D-glucose to D-gluconic acid. However, only some strains did not consume D-fructose.

- Strains Po5 and CECT 8443, belonging to *Gluconobacter* genus, were the most appropriate ones for the selective oxidation of D-glucose to D-gluconic acid, without fermenting D-fructose. The strain Po5 produced higher accumulation of D-gluconic acid in most of the media tested. In contrast, CECT 8443 could oxidize D-gluconic acid to 2KGA and 5KGA. Thus, the desired concentration of D-gluconic acid in the final product would be main criteria for the selection of the strain

- The *A. malorum* strain CECT 7749, the only one isolated from strawberry, was better prepared to carry out the acetification than for D-gluconic production, since in some conditions, it completely oxidized the D-gluconic acid produced.

- The selected strains belonging to *Gluconobacter* showed a high mGDH activity and low activity in GADH and GLDH, whereas the *A. malorum* strain presented low activity in the three enzymes.

- Nitrogen requirements were different among the species of AAB tested, being lower for the *Gluconobacter* strains. The addition of a complete solution of amino acids and ammonium encouraged ABB growth better than the use of single nitrogen sources. However, in *A. malorum* strains proline could be as efficient as the complete solution of amino acids.

- Further studies are necessary to elucidate the presence of non-specific ITS in *Gluconobacte*r strains and to determine if the use of the amplification of ITS 16S-23S rDNA remains, by itself, as the most appropriate technique for the identification of AAB at species level.

APPENDIXES

UNIVERSITAT ROVIRA I VIRGILI
SELECTION AND OPTIMIZATION OF ACETIC ACID BACTERIA FOR D-GLUCONIC ACID PRODUCTION
Florencia Sainz Perez

APPENDIXES INDEX

UNIVERSITAT ROVIRA I VIRGILI
SELECTION AND OPTIMIZATION OF ACETIC ACID BACTERIA FOR D-GLUCONIC ACID PRODUCTION
Florencia Sainz Perez

1. CULTURE MEDIA

1.1. GYC medium (D-glucose Yeast Calcium carbonate)

GYC is a general medium to grow AAB.

1.1.1. Liquid medium:

D-glucose	100 g/L
Yeast extract	10 g/L

1.1.2. Solid medium:

D-glucose	100 g/L
Yeast Extract	10 g/L
CaCO3	20 g/L
Agar	15 g/L

Calcium carbonate is used to detect acid production. When acid is produced, a halo is formed around the colony. The medium is autoclaved at 121ºC for 15 min. Once the medium is warm, natamicine (100 mg/L) can be added to avoid yeast growth.

1.2. YPD medium (Yeast Extract Peptone Dextrose)

D-glucose	20 g/L
Peptone	20 g/L
Yeast Extract	10 g/L

This medium can be made as a liquid or as a solid by adding 15 g/L of agar. The medium is autoclaved at 121ºC for 15 min.

1.3. Potato medium

Glycerol	20 g/L
D-glucose	5 g/L
Polypeptone	10 g/L
Yeast extract	10 g/L
Potato extract	100 mL

The medium is autoclaved at 121ºC for 15 min.

1.4. LB medium

Tryptone	10 g/L
Yeast extract	5 g/L
Sodium chloride	10 g/L

This medium can be liquid or solid by adding 15 g/L of agar. The medium is autoclaved at 121ºC for 15 min. For Blue/White screening after autoclaving, the medium is supplemented with 500µl of ampicillin (100mg/ml), 500µl of IPTG (50mg/mL) and 1mL of X-gal (40mg/mL) sterile filtered

2. FERMENTATIVE MEDIA

2.1. Minimal Medium (MM)

YNB (without amino acids)	1.7 g/L
D-glucose 30 g/L	30 g/L
D-fructose 40 g/L	40 g/L

Adjust pH 3.3. This medium is autoclaved at 121ºC for 15 min.

2.2. Synthetic Medium or must (SM)

This medium is prepared according to Riou et al. (1997), however the sugar concentration is modified.

D-glucose	30.0 g/L
D-fructose	40.0 g/L
Malic acid	5.0 g/L
Citric acid	0.5 g/L
L-Tartaric acid	3.0 g/L
Potassium Dihydrogen Phosphate	0.75 g/L
Potassium sulfate	0.5 g/L
Magnesium sulfate heptahydrate	0.25 g/L
Calcium Chloride Dehydrate	0.16 g/L
Sodium chloride	0.2 g/L
Ammonium chloride	0.46 g/L

Autoclaved at 121ºC for 15 min.

When is cold add:

Vitamins (100X)	10.0 mL/L
Oligo elements (1000X)	1.0 mL/L
Complete solution amino acids	10.0 mL/L

Adjust the pH 3.3. Complete the volume to 1 L and filter.

Vitamins solution (100x)

Myo-inositol	2.0 g/L
Calcium pantothenate	0.15 g/L
Thiamine hydrochloride	0.025 g/L
Nicotinic acid	0.2 g/L
Pyridoxine	0.025 g/L
Biotine (100 mg.l-1)	3.0 mL/L

The solution is sterilized by filtration and is stored at -20 ºC.

Oligo elements (1000x)

Manganese(II) sulfate monohydrate	4 g/L
Zinc Sulfate Heptahydrate	4.0 g/L
Copper(II) Sulfate Pentahydrate	1.0 g/L
Potassium Iodide	1.0 g/L
Cobalt(II) Chloride Hexahydrate	0.4 g/L
Boric Acid	1.0 g/L
Ammonium Heptamolybdate	1.0 g/L

The solution is sterilized by filtration and stored at 4 ºC.

APPENDIX 1

Complete solution of AA (100X) in Na_2CO_3 2%

Tyrosine	1.95 g/L	Heat up 100ºC
Tryptophan	17.42 g/L	70 ºC
Isoleucine	3.25 g/L	70 ºC
Aspartic acid	4.42 g/L	
Glutamic acid	11.96 g/L	
Arginine	36.79 g/L	
Leucine	4.81 g/L	Increase temperature
Threonine	7.54 g/L	
Glycine	1.82 g/L	
Glutamine	49.92 g/L	
Alanine	14.56 g/L	
Valine	4.42 g/L	
Methionine	3.12 g/L	
Phenylalanine	3.77 g/L	
Serine	7.8 g/L	
Histidine	3.38 g/L	
Lysine	1.69 g/L	
Cysteine	2.08 g/L	
Proline	59.93 g/L	

The solution is sterilized by filtration and is stored at -20 ºC.

2.3. YNB medium (YNB)

D-glucose	25g/L
D-fructose	25g/L
YNB (without amino acids)	1.7g/L

Autoclaved at 121ºC for 15 min and stored at room temperature.

2.4. Minimal medium (M9)

5X Solution salt	200 mL/L
Distilled water	452 mL

Autoclaved at 121ºC for 15 min. After autoclaving, swirl to mix and stored at room temperature to cool.

Then, add:

Magnesium sulfate (1M)	2 mL /L
Calcium chloride	0.1 mL /L
Concentrated sugar solution*	250 mL

*(20% sugar (10% D-glucose (w/v) + 10% D-fructose (w/v))).

Solution salt (5X)

Sodium Monohydrogen Phosphate Heptahydrate	64 g/L
Potassium Dihydrogen Phosphate	15 g/L
Sodium chloride	2.5 g/L

3. NITROGEN REQUIREMENT STUDY

3.1. Selection of the best media and nitrogen concentrations

The strains are grown in GY medium, with shaking at 28ºC. Forty mL of each medium tested (YNB, M9 and SM) are placed in 50 mL falcons. The media are prepared with the different nitrogen concentrations. The following volumes of the different stock solutions are used

	25 mg N/L	**50 mg N/L**	**100 mg N/L**	**300 mg N/L**	**1 g N/L**
Ammonium chloride	0.04 g/L	0.08 g/L	0.15 g/L	0.46 g/L	1.53 g/L
Vitamins (100X)	10 mL/L	10 mL/L	10 mL/L	10 mL/L	10 mL/L
Oligo elements (1000X)	1 mL/L	1 mL/L	1 mL/L	1 mL/L	1 mL/L
Complete solution amino acids	0.833 mL/L	1.66 mL/L	3.33 mL/L	10 mL/L	33.33 mL/L

For all the conditions an initial OD of 0.1 is taken.

The microplate is prepared by adding 250 µL of medium control to each well. The absorbance is measured continuously for 200 cycles, with stirring at 500 rpm for 80 seconds prior to each reading.

APPENDIX 1

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		25	25	25	25	25	25	25	25	25	*B*	
C		50	50	50	50	50	50	50	50	50	*B*	
D		100	100	100	100	100	100	100	100	100	*B*	
E		300	300	300	300	300	300	300	300	300	*B*	
F		1	1	1	1	1	1	1	1	1	*B*	
G		*B*	*B*	*B*	*B*	*B*	*B*	*B*	*B*	*B*	*B*	
H												

Green: YNB control medium with 25, 50, 100, 300 mg N/L and 1 g N/L
Blue: M9 control medium with 25, 50, 100, 300 mg N/L and 1 g N/L
Orange: SM control medium with 25, 50, 100, 300 mg N/L and 1 g N/L
B: blank

3.2. Analysis of individual amino acids and ammonium

After the selection of the best medium and the lowest nitrogen concentration that was advantageous for AAB growth, the addition of a single amino acid or ammonium as the only nitrogen source is analyzed.

The strains grow in GY media, with shaking at 28ºC. The control medium is prepared as mentioned above and the selected medium is prepared in two falcons (final volume 40 mL). In this case, only vitamins and oligoelements are added.

For all the conditions an initial OD of 0.1 is taken.

In the tubes, the corresponding volume of the amino acid solution or ammonia (2.5 g/L) is added to have the final concentration of nitrogen selected (25, 50, 100, 300 mg N/L or 1 g N/L) and the final volume (3 mL) was completed with the addition of the medium.

The microplate is prepared by adding 250 µL of medium control to each well. The absorbance is measured continuously for 200 cycles, with stirring at 500 rpm for 80 seconds prior to each reading.

	1	2	3	4	5	6	7	8	9	10	11	12
A					Asn	Asp	Cys					
B		Ala	NH_4	Arg	Asn	Asp	Cys	Phe	Gaba	Gly	Glu	
C		Ala	NH_4	Arg	Asn	Asp	Cys	Phe	Gaba	Gly	Glu	
D	Gln	Ala	NH_4	Arg	Gln	Trp	Val	Phe	Gaba	Gly	Glu	Val
E	Trp	His	Ile	Leu	Gln	Trp	Val	Pro	Ser	Cont*	Thr	
F		His	Ile	Leu	Lys	Met	Orn	Pro	Ser	Cont	Thr	
G		His	Ile	Leu	Lys	Met	Orn	Pro	Ser	Cont	Thr	
H		*B*	*B*	*B*	Lys	Met	Orn	*B*	*B*	*B*	*B*	

*Contr= control medium

4. DETERMINATION OF SUGAR CONCENTRATION

The residual amount of D-glucose and D-fructose was determined using an enzymatic kit (Boeringher Mannheim).

D-Glucose and D-fructose are phosphorylated to D-glucose-6-phosphate (G-6-P) and D-fructose-6-phosphate (F-6-P) by the enzyme hexokinase (HK) and adenosine-5'-triphosphate (ATP) with the simultaneous formation of adenosine-5'-diphosphate (ADP) (1,2).

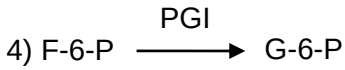
$$\text{1) D-glucose + ATP} \xrightarrow{\text{HK}} \text{G-6-P + ADP}$$

$$\text{2) D-fructose + ATP} \xrightarrow{\text{HK}} \text{F-6-P + ADP}$$

In the presence of the enzyme glucose-6-phosphate dehydrogenase (G6PDH), G-6-P is oxidized by nicotinamide-adenine dinucleotide phosphate (NADP) to D-gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (3).

$$\text{3) G-6-P + } NADP^+ \xrightarrow{\text{G6P-DH}} \text{D-gluconate-6-phosphate + NADPH + } H^+$$

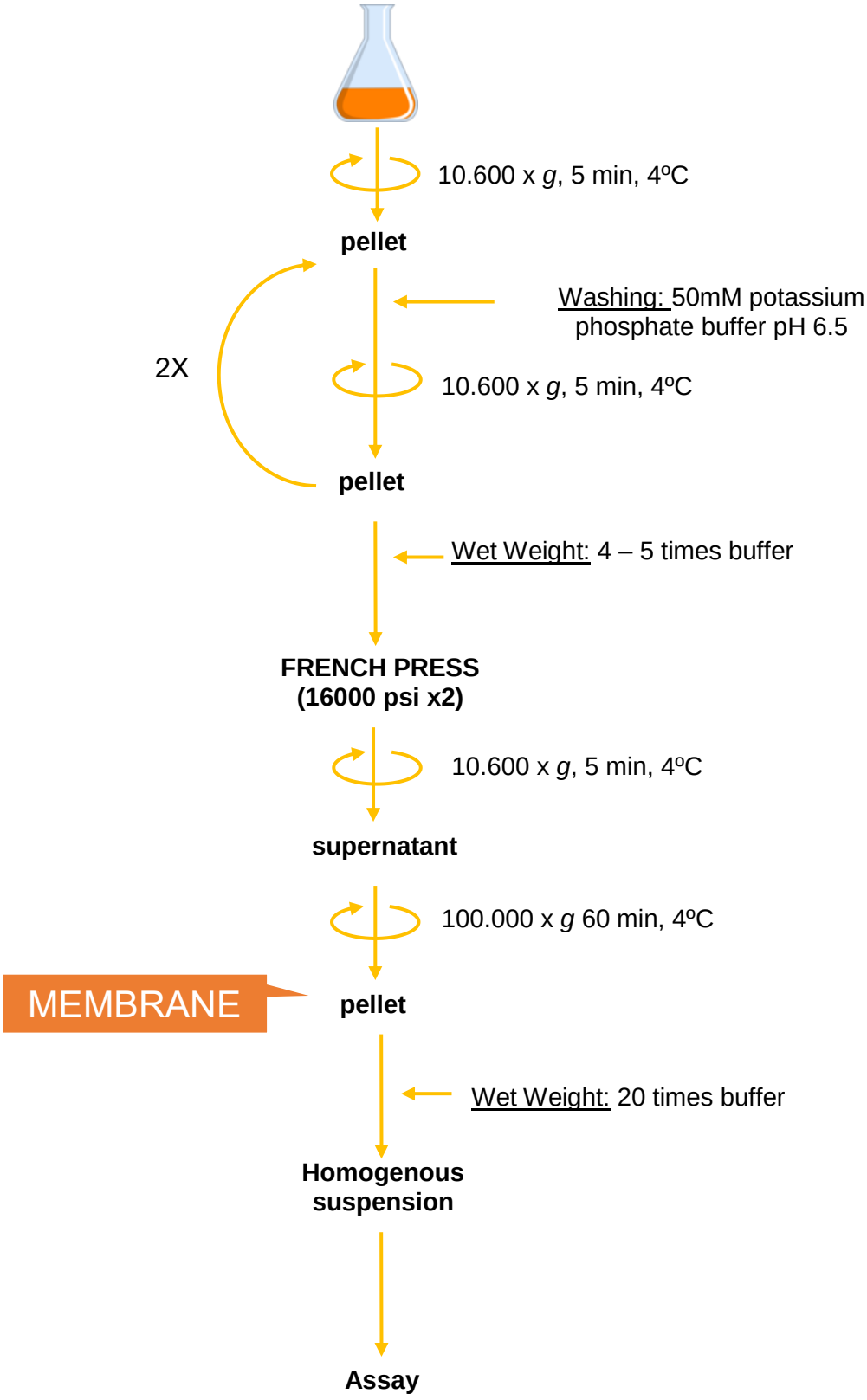
The amount of NADPH formed in this reaction is stoichiometric to the amount of D-glucose. NADPH is measured by the increase of its light absorbance at 334, 340 or 365 nm. On completion of reaction (3), F-6-P is converted to G-6-P by phosphoglucose isomerase (PGI) (4).



G-6-P reacts in turn with NADP forming D-gluconate-6-phosphate and NADPH. The amount of NADPH obtained in this reaction is stoichiometric to the amount of D-fructose. The increase in NADPH is measured by means of its light absorbance.

5. MEMBRANE-BOUND DEHYDROGENASE ACTIVITY

5.1. Preparation Membrane Fraction



5.2. Protein determination

The protein concentration was determined by a modified Lowry method (Dulley and Grieve, 1975) using a bovine serum albumin as the standard.

Solution A

Sodium carbonate	20 g/L
Sodium hydroxide	4 g/L
Sodium dodecyl sulfate	5 g/L

Solution B

Copper (II) sulfate pentahydrate	2g/L
Potassium sodium tartrate	10 g/L

Solution C

Folin-Ciocalteu reagent (Wako Pure Chem.).

In test tubes:

1. Prepare a standard curve by diluting the BSA stock with distilled water (total volume 400 µL);
2. Prepare the dilution of the samples (total volume 400 µL).
3. In all the test tube prepared before:
4. Add 2 mL of Solution A + Solution B (50:1) and mix with vortex. Stand for 10 min at 25ºC
5. Add 200 µL of Solution C and mix quickly with vortex. Incubate for 20 min at 25ºC.
6. Measure the absorbance at 750 nm

5.3. Enzyme Assay

The enzyme activity of PQQ and FAD- dependent dehydrogenases could be analyzed using artificial electron acceptors like potassium ferricyanide or phenazine methosulfate (PMS). In the case of membrane-bound GADH and 2KGDH enzymes as contain heme *c* component in their molecule or membrane fraction, their activity is easily determined with potassium ferricyanide (Adachi et al., 2007). In this case, the reduction rate of ferricyanide to ferrocyanide is quantitative to the amount of substrate oxidized. However, enzyme activity of mGDH and GLDH should be measured with DCIP-PMS, because

these enzymes do not contain heme *c* component. In the reaction, DCIP acts as an electron acceptor and PMS as a mediator, the reduction of DCIP is measured at 600 nm.

6. DNA EXTRACTION OF AAB

Total DNA was extracted using the modified CTAB method (cetyl trimethyl ammonium bromide), as described by Ausubel et al. (1992).

Cells are harvested from culture medium, wine or vinegar and centrifuged for 5 min in at 14000 rpm.

1. Resuspend the pellet in 520 µL of TE Buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8).
2. Add 30 µl of SDS 20% and 6 µl of proteinase K (20 mg/mL). Mix using the vortex
3. Incubate the mixture during 1h at 37ºC
4. Add 150 µl of NaCl 5M and 140 µl of 10% CTAB in 0,7M NaCl
5. Incubate the suspension during 10 min at 65ºC
6. Incubate on ice for 10-15 min
7. Add one volume of chloroform:isoamyl alcohol (24:1) and mix manually until homogenize
8. Incubate the mixture on ice for 5 min
9. Centrifuge at 4ºC and 10000 rpm for 10 min (wash again with chloroform:isoamyl alcohol until not observe the inter-phase)
10. Transfer the aqueous phase to an Eppendorf tube with 380 µl of isopropanol
11. Mix until observe the precipitation of the DNA
12. Incubate at -20ºC for 5 min
13. Centrifuge at 4ºC at 10000 rpm for 10 min
14. Eliminate the supernatant

7. AAB IDENTIFICATION

7.1. Amplification of the 16S-23S rRNA gene ITS region (Ruiz et al., 2000)

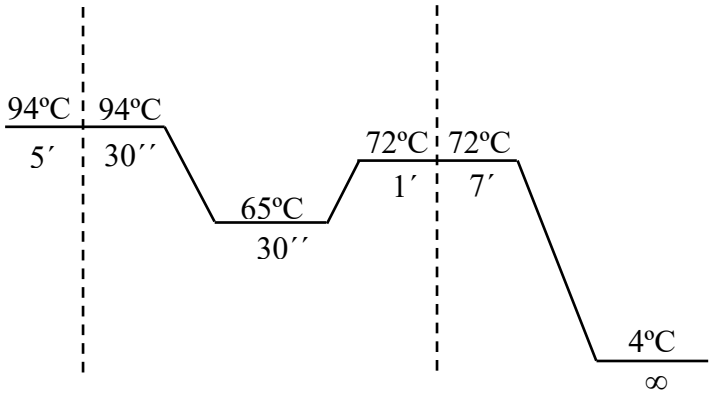
Primers used to amplify the ITS 16S-23S rDNA are:

ITS1, 5'-ACCTGCGGCTGGATCACCTCC-3'

ITS2, 5'-CCGAATGCCCTTATCGCGCTC-3'

Amplification mix	µL for final volume = 50 µL
Primer ITS1 (10 ρM)	1.5 µL
Primer ITS2 (10 ρM)	1.5 µL
dNTPs (each dNTP 10 mM)	1.0 µL
MgCl2 (100 mM)	3.0 µL
Buffer Taq 10x, without Mg. (Biotaq)	1.0 µL
Taq DNA polymerasa (Biotaq)	0.5 µL
H2O milli-Q	40.5 µL
DNA	1.0 µL

PCR conditions:



Five microliters of the amplified DNA are mixed with 2 µL of bromophenol blue and detected by electrophoresis on a 1% (w/v) agarose gel (Boehringer Mannheim). The length of the amplification product is determined by comparison with a 100 bp DNA ladder (Roche Diagnostics, Mannheim, Germany).

7.2. Band purification with QIAquick Gel Extraction Kit (Quiagen, Netherlands)

With QIAquick Gel Extraction kit, 80% of DNA ranging from 70 bp to 10 kb could be recovered. First, the band is excised from the gel and the agarose is dissolved in a buffer with a pH indicator to determine if the pH of the solution is optimal for DNA binding. then, the mixture is added to the spin column. The silica membrane of the column retains the nucleic acids in high salt-conditions, impurities are washed and after, pure DNA is eluted with low salt buffer or water. DNA fragments purified with the kit system are ready for direct use (sequencing, restriction digestion, etc…).

7.3. Cloning ITS products into pGEM- T Easy vector

pGEM-T Easy Vector is suitable for the cloning of PCR products. The vector has a 3´ terminal thymidine in both ends, that improves the efficiency of ligation of a PCR product into the plasmid.

For the preparation of the cloning reaction, the mix contains:

2X Rapid Ligation Buffer	5 µL
pGEM-T Easy Vector	1 µL
PCR product	2 µL
T4 DNA Ligase	1 µL
Deionized water	1 µL

Mix by pipetting and incubate for 1 hour at room temperature.

7.3.1. Transformation of JM109 High Efficiency Competent Cells

1. Prepare LB/ampicillin/ IPTG/ X-Gal plates.
2. Centrifuge the ligation reactions and add 2 µl to a sterile Eppendorf on ice.
3. Add 50 µl of JM109 High Efficiency Competent Cells to 2 µl of the ligation reactions and incubate on ice for 20 minutes.
4. Heat shock for 45-50 seconds at exactly 42ºC. Incubate on ice for 2 minutes.
5. Add S.O.C medium (Invitrogen - ThermoFisher Scientific, USA) and incubate for 1.5 hours at 37ºC with shaking.

6. Plate. Select white colonies and growth in LB liquid media with ampicillin.

7.4. Plasmid extraction with NucleoSpin® Plasmid QuickPure

With the NucleoSpin Plasmid QuickPure the pelleted bacteria are resuspended (Buffer A1) and plasmid DNA is liberated from the *E.coli* host cells by SDS/alkaline lysis (Buffer A2). Buffer A3 neutralizes the resulting lysate and creates appropriate conditions for binding of plasmid DNA to the silica membrane of the column. Precipitated protein, genomic DNA, and cell debris are then pelleted by a centrifugation step. The supernatant is loaded onto a column. Contaminations like salts, metabolites, and soluble macromolecular cellular components are removed by a simple washing with ethanolic Buffer A4. Pure plasmid DNA is finally eluted under low ionic strength conditions with alkaline Buffer AE (5mm Tris/HCl, Ph 8.5).

7.4.1. Digestion

Cloned plasmid	5 µL
Buffer H	2 µL
Enzyme *Eco* RI	1 µL
Distilled water	12 µL

Incubate at 37ºC for 1 hour. Visualize the fragments in agarose gel 1.5%

REFERENCES

Adachi, O., Ano, Y., Toyama, H., Matsushita, K., 2007. Biooxidation with PQQ- and FAD-Dependent Dehydrogenases. In: Schmid, R.D., Urlacher, V.B. (Eds.), Modern Biooxidation Enzymes, Reactions and Applications, Weinheim: Wiley-vch Verlag GmbH & Co KGaA.

Ausubel, F. M., Brent, R., Kingston, R., Moore, D. D., Seidman, J. G., Smith, J. A., Struh, K., 1992. Short Protocols In Molecular Biology: Wiley, John.

Dulley, J.R., Grieve, P.A., 1975. A simple technique for eliminating interference by detergents in the Lowry method of protein determination. Anal. Biochem. 64, 136–141.

Riou, C., Nicaud, J.M., Barre, P., Gaillardin, C., 1997. Stationary-phase gene expression in Saccharomyces cerevisiae during wine fermentation. Yeast 13, 903–915.

Ruiz, A., Poblet, M., Mas, A., Guillamón, J. M., 2000. Identification of acetic acid bacteria by RFLP of PCR-amplified 16S rDNA and 16S-23S rDNA intergenic spacer. Int. J. Syst. Evol. Microbiol. 50, 1981-1987.

Sambrook, J., Russell, D. W., 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

UNIVERSITAT ROVIRA I VIRGILI
SELECTION AND OPTIMIZATION OF ACETIC ACID BACTERIA FOR D-GLUCONIC ACID PRODUCTION
Florencia Sainz Perez

APPENDIX 2

Impact of gluconic fermentation of strawberry using acetic acid bacteria on amino acids and biogenic amines profile

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Food Chemistry 178, 221-228 (2015)

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ABSTRACT

This paper studies the amino acid profile of beverages obtained through the fermentation of strawberry purée by surface culture using three strains belonging to different acetic acid bacteria species (one of *Gluconobacter japonicus*, one of *Gluconobacter oxydans* and one of *Acetobacter malorum*) isolated from strawberry. An HPLC-UV method involving Diethyl ethoxymethylenemalonate (DEEMM) was adapted and validated. From among an entire set of 21 amino acids, multiple linear regression showed that glutamine, alanine, arginine, tryptophan, GABA and proline were significantly related to the fermentation process. Furthermore, linear discriminant analysis classified 100% of the samples correctly in accordance with the microorganism involved. *G. japonicus* consumed glucose most quickly and achieved the greatest decrease in amino acid concentration. None of the 8 biogenic amines were detected in the final products, which could serve as a safety guarantee for these strawberry gluconic fermentation beverages, in this regard.

Keywords: HPLC; DEEMM; nitrogen compounds; beverages; strawberry products; acetic acid bacteria

APPENDIX 2

1. INTRODUCTION

Strawberry (*Fragaria x ananassa* Duch.) is one of the most economically important fresh and processed fruits (Hancock, Sjulin & Lobos, 2008) and a source of bioactives (Álvarez-Fernández, Hornedo-Ortega, Cerezo, Troncoso & García-Parrilla, 2014a; Cerezo, Cuevas, Winterhalter, Garcia-Parrilla & Troncoso, 2010; Stürtz, Cerezo, Cantos-Villar & Garcia-Parrilla, 2011). Hence, there is a wide variety of processed strawberry products, such as purée, jams, juices, beverages, fruit preparations, etc. (Fügel, Carle & Schieber, 2005; Hui, Barta, Canor, Gusek, Sidhu & Sinha, 2006). Recently, strawberry fermented products, such as wines and vinegars, have been produced as a good solution for using strawberry surpluses and as an alternative method for conserving this perishable fruit (Hidalgo, Torija, Mas & Mateo, 2013; Ubeda, et al., 2013).

Gluconic acid is abundantly available in grains, fruits and other foodstuffs, such as rice, meat, dairy products, honey and fermented products like wine and vinegar. It is a mild organic acid, which has applications in the food industry (Deppenmeier, Hoffmeister & Prust, 2002; Ramachandran, Fontanille, Pandey & Larroche, 2006; Singh & Kumar 2007). It is produced from glucose by different microorganisms, which include bacteria, yeast and some ectomycorrhizal fungus. Among them, some genera of the family *Acetobacteraceae,* such as *Gluconobacter,* are used industrially to produce gluconic acid (Deppenmeier & Ehrenreich, 2009; Ramachandran et al., 2006). There are several works that have studied gluconic acid fermentations. However, most of them are focused on biotechnology and its applications (Deppenmeier et al., 2002; Gupta, Singh, Qazi, & Kumar, 2001; Ramanchandran et al., 2006; Singh & Kumar, 2007) and reports focusing on the gluconic fermentation of fruits are scarce.

Acetic acid bacteria (AAB) can utilize a wide range of compounds as sources of nitrogen, from simple inorganic compounds to complex compounds, including amino acids (Merrick & Edwards, 1995). *Gluconobacter* strains are able to grow using ammonium ion as their sole source of nitrogen (Deppenmeier & Ehrenreich, 2009; Gupta et al., 2001). However, AAB have been shown to consume amino acids in the conversion of ethanol

into acetic acid (Callejón, Troncoso & Morales, 2010). Hence, free amino acids present in the medium could also be a good source of nitrogen for these bacteria, in addition to ammonium ion. The amino acid content of fruits and fruit derived products is studied since they contribute to the final aroma and taste, among other properties (Mandrioli, Mercolini & Raggi, 2013).

Furthermore, some biogenic amines can be directly formed from amino acids by decarboxylation. These compounds can be formed and degraded during the normal metabolism of living organisms, although they have been quantified especially in fermented food and beverages such as cheeses, dry fermented sausages or wine (Ancín-Azpilicueta, González-Marco & Jiménez-Moreno, 2008; ten Brink, Damink, Joosten, & Huis in't Veld, 1990; Kirschbaum, Rebscher, & Brückner, 1999). High concentrations of biogenic amines in final products could be due to employ poor quality raw materials, contamination and food processing and storage under unsuitable conditions (Önal, 2007; ten Brink at al., 1990). Biogenic amines, in particular histamine and tyramine, can cause health problems when are present in food in a high concentration (ten Brink at al., 1990). These compounds could cause wide effects on consumer such as headache, inflammations, irritation, hypertension and hypotension (Ancín-Azpilicueta et al., 2008; ten Brink at al., 1990). The European legislation does not have a biogenic amines threshold, but European Food Safety Authority (EFSA) has elaborated a scientific opinion on the risk associated with their formation in fermented products (European Food Seafty Authority (EFSA) Panel on Biological Hazards (BIOHAZ), 2011).

Several techniques have been developed for analyzing amino acids and biogenic amines in foods (Callejón et al., 2010; Hernández-Orte, Ibarz, Cacho & Ferreira, 2003; Önal, 2007; Peña-Gallego, Hernández-Orte, Cacho & Ferreira, 2012). Nevertheless, the analytical technique most frequently employed for the determination of amino acids and biogenic amines is HPLC with C18 reverse-phase columns (Peña-Gallego et al., 2012). This method is less time-consuming than other techniques and the instrumentation used

is simple (Hernández-Orte et al., 2003). The direct detection of amino acids by HPLC yields matrix interferences (Callejón, Tesfaye, Torija, Mas, Troncoso & Morales, 2008) and biogenic amines do not have good absorption properties in the visible, ultraviolet or fluorescence wavelength ranges (Peña-Gallego, Hernández-Orte, Cacho & Ferreira, 2009). For these reasons, the determination of these compounds requires a chemical derivatization to improve detection limits and to avoid matrix interference (Callejón et al., 2010; Gómez-Alonso, Hermosín-Gutiérrez & García-Romero, 2007; Peña-Gallego et al., 2012). The reagents most widely used are 2,2-dihydroxy-1,3-indanedione (Ninhydrin), dansyl chloride (DnsCl), dabsyl Chloride (DbsCl), phenylisothiocyanate (PITC), o-phthaldialdehyde (OPA), 6-aminoquinolyl N-hydroxysuccinimidyl carbamate (AQC) and diethyl ethoxymethylenemalonate (DEEMM), among others (Callejón et al., 2010; Peña-Gallego et al., 2012). Some of these techniques have been able to determine amino acids and biogenic amines simultaneously, such as the method proposed by Gómez-Alonso et al. (2007), which used DEEMM as the derivatization agent to increase the specific absorbance of the analytes, followed by reversed phase HPLC and UV-vis photodiode array detection. This method has been proposed for wines and beers.

The aims of this study were: (a) to adapt an analytical method to determine the profile of amino acids, biogenic amines and ammonium ion in different gluconic acid fermented products and in the starting substrate (strawberry purée) by HPLC using DEEMM as the derivatization agent; (b) to study the differences in amino acid consumption by the different AAB strains employed; (c) to verify whether the fermented products can be discriminated or grouped according to the strain that performed the fermentation, taking the amino acid profiles as variables, and (d) to check whether these fermented beverages are safe for human consumption by determining the concentrations of biogenic amines.

2. MATERIALS AND METHODS

2.1. Reagents and standards

Most of the amino acid standards were purchased from Fluka (Buchs, Switzerland). The aspartic acid, glutamic acid, histidine, alanine, lysine, γ-aminobutiric acid (GABA), biogenic amines, ammonium sulphate, diethyl ethoxymethylenemalonate, acetic acid glacial, boric acid, 2-aminoadipic acid (internal standard) and sodium azide were supplied by Sigma-Aldrich (Steinheim, Germany). The glycine, ornithine, methanol (HPLC grade) and acetonitrile (HPLC grade) were acquired from Merck (Darmstadt, Germany). The sodium acetate and sodium hydroxide were obtained from Panreac (Castellar del Vallès, Barcelona). The ultrapure water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

The stock standard solutions were prepared individually by dissolving the pure compounds in HCl 0.1 N. The calibration solutions were prepared by diluting the stock standard solutions with water.

2.2. Samples

We analyzed samples of the fermentation of strawberry purée using a surface cultures of acetic acid bacteria (AAB), supplied by HUDISA, S.A. (Lepe, Spain). These gluconic fermentations were conducted in the laboratories of the Biochemistry and Biotechnology Department (Facultat d'Enologia, Universitat Rovira i Virgili. Tarragona, Spain). These fermentations were carried out with different AAB strains: one of *Acetobacter malorum* (3 samples), one of *Gluconobacter oxydans* (3 samples) and one of *Gluconobacter japonicus* (2 samples). The initial substrate used for these processes was also studied. AAB were grown in a GY medium (1% yeast extract and 5% glucose) and incubated at 28 °C with stirring. The fermentation substrate consisted of mixing 90% strawberry purée with 10% rectified concentrated must (Concentrados Pallejà, Riudoms, Spain). For each fermentation, 500 mL of the substrate was inoculated with $2x10^6$ cell/mL of the AAB strains in a 1 L Erlenmeyer flask. Fermentations were performed at 28 ºC with a stirring

speed of 128 rpm. In all the cases the fermentations were considered finished after 10 days of fermentation, when more than 90% of the initial glucose had been consumed. Only *G. japonicus* strain practically exhausted glucose after the 10 days. All samples were frozen immediately after sampling. Table 1 displays the sample codes and their concentrations in glucose, fructose and gluconic acid.

Table 1. Samples codes and glucose, fructose and gluconic acid concentration in strawberry purée and gluconic fermented.

Samples		Codex	Glucose (g/L)	Fructose (g/L)	Gluconic acid (g/L)
Strawberry purée		*SP*	62	62	-
Strawberry gluconic acid fermentation beverages (SGFB)	*Acetobacter malorum*	*SGFAM1*	4,74	51,72	49,91
		SGFAM2	6,68	54,69	45,73
		SGFAM3	2,25	51,33	47,78
	Gluconobacter oxydans	*SGFGO1*	4,74	56,96	51,95
		SGFGO2	4,12	50,47	46,16
		SGFGO3	2,57	54,22	49,48
	Gluconobacter japonicus	*SGFGJ1*	0,93	50,94	52,38
		SGFGJ2	0,78	47,49	47,95

2.3. Sample preparation

First, 2 mL of sample were centrifuged at 6000 rpm for 15 min (Eppendorf centrifuge 5415R, Hamburg, Germany). The derivatization of amino acids and biogenic amines was performed by diethyl ethoxymethylenemalonate (DEEMM). For this, 700 µL of borate buffer 1 M (pH = 9), 300 µL of methanol, 400 µL of standard or sample, 10 µL of internal standard (L-2-aminoadipic acid, 1 g/L) and 12 µL of DEEMM were mixed in a covered vial, which was introduced into an ultrasound bath for 30 min. Later, the sample was heated at 70 °C for 2 hours to allow the complete degradation of excess DEEMM and reagent byproducts (Gómez-Alonso et al. 2007). All samples were filtered through a membrane filter with a mean pore size of 0.45 µm (Millipore) prior to use.

2.4. Equipment

HPLC analysis was carried out in Waters HPLC system consisting of a Waters 717 autosampler injector and a Waters 1525 Binary HPLC pump system controller connected to a Waters 996 photodiode array detector. Data treatment was performed in a Waters Millennium data station. The column consisted of a LiChroCART® 250-4 LiChrospher® 100 RP-18 (5 µm, 250 x 4.6 mm) from Merck (Darmstadt, Germany) and a 4.0 x 3.0 mm guard column from Analytical Phenomenex (Torrance, CA, USA). The column was thermostatized at 45 °C in a column header module controlled by Waters TCM HPLC Temperature Controller. The gradient program employed is shown in Table 2; it was similar to the one used by Gómez-Alonso et al. (2007). The injection volume was 10 µL and the separation was obtained at a flow rate of 0.9 mL/min. Mobile phase A consisted of a 25 mM acetate buffer (pH 5.8) with 0.02% sodium azide, and mobile phase B was an 80:20 mixture of acetonitrile and methanol. A photodiode array detector monitored at 280 and 269 nm was used for detection. All mobile phases were filtered through a membrane filter with a mean pore size of 0.45 µm (Millipore) prior to use.

Table 2. Eluent gradient for HPLC method.

Time (min)	A (%)	B (%)
0	90	10
20	90	10
26	87	13
32	83	17
34	83	17
43	75	25
48	75	25
53	70	30
58	70	30
65	60	40
72	28	72
75	25	75
77	20	80
79	20	80
85	0	100

2.5. Statistical analysis

All statistical analyses were performed by means of Statistica software (StatSoft, 2004). One-way ANOVA was performed to evaluate significant differences between types of samples (significance levels $p<0.05$). Multiple linear regression (MLR) was performed to evaluate relationships between amino acid concentrations and glucose consumption.

In addition, Principal Component Analysis (PCA) followed by Lineal Discriminant Analysis (LDA) were employed to evaluate whether the profiles of amino acids and biogenic amines were different enough to distinguish between the gluconic acid fermentations analyzed.

3. RESULTS AND DISCUSSION

3.1. Method validation

The analytical method used to perform this work was originally developed by Gómez-Alonso et al. (2007) to determine amino acids in wines and beers. Slight modifications in gradient and temperature were performed to obtain better peak resolution in the strawberries and their fermented products. Thus, the modified method was validated in terms of linearity, sensitivity (detection and quantification limits) and precision (repeatability and intermediate precision) response according to AOAC criteria (AOAC, 1993). Table 3 displays these validation parameters. Linear range was obtained using a value close to the limit of quantification (LOQ) as the lowest concentration. Thus, the lowest point of the linear range was lower than that described by Gómez-Alonso et al. (2007) in most cases. In all the analytes, R^2 were above 0.99, showing a linear relationship between standard concentration and detector response.

Room temperature in our laboratory oscillates widely. Therefore, we set the temperature at 45 ºC to achieve repeatability and intermediate precision within AOAC limits (AOAC, 1993).

Due to the high concentration of asparagine and its proximity to serine, peak overlapping could not be prevented, so the two were quantified together (asparagine-serine) as previously described (Hermosín, Chicón & Dolores Cabezudo, 2003).

3.2. Sample analysis

The proposed method was applied to determine amino acids and biogenic amines in strawberry purées and beverages obtained after gluconic fermentation.

Table 4 displays amino acid concentration in samples obtained by surface culture fermentation with different AAB strains. A total of 31 compounds were determined, including 22 amino acids, 8 biogenic amines and ammonium ion.

Asparagine-serine, alanine and glutamine were the major amino acids in the substrate as shown Fig. 1, which is in accordance to the strawberry amino acids profile (Moing, Renaud, Gaudillère, Raymond, Roudeillac & Denoyes-Rothan, 2001; Perez, Rios, Sanz & Olias, 1992). On the other hand, the amino acid profiles changed after gluconic fermentation (Fig. 2). In general, most amino acids followed a similar trend, and theirconcentration change during fermentations with different AAB strains. In order to search for a relationship between amino acids and glucose consumption, we performed a correlation matrix analysis, which revealed the most significant correlations. Hence, we selected glutamine, alanine, arginine, GABA, proline and tryptophan for subsequent multiple linear regression analysis. We tested 3 methods: standard, forward stepwise and backward stepwise inclusion of variables, and obtained an R^2 of 0.9874 for standard and forward stepwise analysis and an R^2 of 0.9807 for backward stepwise analysis. The following variables were included in the forward stepwise analysis (in order): alanine, arginine, glutamine, tryptophan and GABA. However, only glutamine and arginine were included in the backward stepwise analysis model.

Table 3. Results of regression analysis of calibration curves, LOD, LOQ, repeatability and intermediate precision.

Compounds	λ (nm)	RT (min)	Linear range (µg/L)	Ecuation y= ax + b	R^2	LOD (µg/L)	Repeatability (n=5)		Intermediate precision (n=5)	
							Mean (mg/L)	RSD (%)	Mean (mg/L)	RSD (%)
Aspartic acid	280	3.8	0.5-100	y = 0.055x + 0.1715	0.9929	0.15	41.94	1.27	41.84	2.47
Glutamic acid	280	4.4	0.5-150	y = 0.0542x + 0.1309	0.997	0.10	35.98	0.73	35.79	2.58
Asparagine	280	7.4	0.5-800	y = 0.0382x + 0.1171	0.9964	0.15	62.34	2.14	62.14	1.18
Serine	280	7.4	0.5-100	y = 0.073x + 0.0107	0.9997	0.15	55.78	0.49	55.83	0.11
Glutamine	280	8.3	5-800	y = 0.0484x + 0.5199	0.9985	0.10	26.70	1.74	25.43	2.65
Histidine	280	10.4	0.5-100	y = 0.0529x - 0.0306	0.9977	0.15	43.14	0.29	43.37	0.44
Glycine	280	11.9	0.5-100	y = 0.1127x + 0.0299	0.9999	0.05	36.84	1.02	36.55	2.30
Threonine	280	12.2	1-100	y = 0.0611x - 0.0267	0.9987	0.05	35.68	2.06	35.36	1.51
Alanine	280	19.8	1-150	y = 0.0689x + 0.0793	0.9987	0.05	53.60	1.95	52.66	1.32
Arginine	280	21.2	5-100	y = 0.0383x + 0.0045	0.9962	0.05	39.86	1.57	40.03	3.98
GABA	280	22	0.5-150	y = 0.0678x - 0.0427	0.9996	0.05	45.29	1.01	44.03	4.68
Proline	280	24.8	may-00	y = 0.025x - 0.3434	0.9973	0.3	66.97	1.27	58.01	13.90
Tyrosine	280	35.6	1-100	y = 0.021x + 0.7793	0.99	0.05	61.31	2.81	66.06	2.17
Valine	280	43.4	1-100	y = 0.0624x + 0.169	0.9988	0.05	41.45	1.51	40.73	1.25
Methionine	280	44	0.5-100	y = 0.0339x - 0.0147	0.9975	0.05	60.75	0.60	60.17	0.47
Cysteine	280	46.7	0.5-100	y = 0.0267x + 0.0521	0.997	0.05	43.82	6.06	48.49	7.92
Isoleucine	280	49.7	0.5-100	y = 0.0467x + 0.0901	0.9957	0.05	15.94	7.89	14.51	3.21
Tryptophan	280	57.1	0.5-100	y = 0.0818x - 0.0572	0.9974	0.05	45.76	1.14	46.14	4.07
Leucine	280	50.9	0.5-100	y = 0.0633x + 0.2051	0.9993	0.05	44.18	2.27	46.53	9.34
Phenylalanine	280	51.5	1-100	y = 0.0383x + 0.0011	0.9994	0.05	97.25	1.54	95.31	2.72
Ornithine	280	56.9	0.5-100	y = 0.0256x + 0.037	0.9976	0.05	52.79	1.55	51.00	3.32
Lysine	280	60.3	0.5-100	y = 0.077x + 0.0198	0.9997	0.05	40.04	0.76	39.92	0.75
Histamine	280	34.5	0.5-100	y = 0.0286x + 0.0246	0.9936	0.05	87.14	4.89	78.50	4.84
Agmatine	280	38.7	0.5-100	y = 0.025x + 0.1354	0.9921	0.05	23.08	7.62	22.63	4.47
Spermidine	280	60.7	0.5-100	y = 1.0326x - 0.0169	0.9991	0.10	1.97	0.33	1.96	0.98
Tyramine	280	71.5	0.5-100	y = 0.0627x - 0.0644	0.995	0.05	42.83	1.44	41.60	2.45
Putrescine	280	74.9	0.5-100	y = 0.0582x + 0.151	0.9958	0.05	52.82	1.61	51.94	1.35
Tryptamine	280	75.1	0.5-100	y = 0.055x + 0.1047	0.9975	0.05	25.49	1.04	25.15	2.55
Cadaverine	280	75.9	0.5-100	y = 0.0561x + 0.1219	0.9943	0.05	43.75	1.45	42.98	1.15
Phenylethylamine	280	75.9	0.5-100	y = 0.0461x + 0.0044	0.9998	0.05	44.77	0.54	43.36	3.19
Ammonium	269	37.8	1-100	y = 0.0714x + 0.0631	0.9996	0.05	20.25	3.84	20.41	5.27

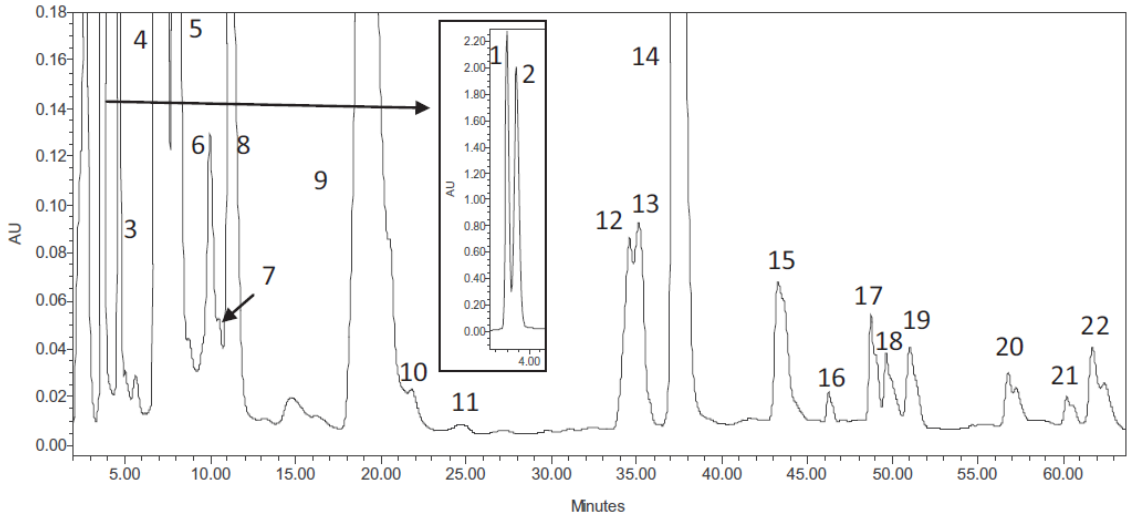

Figure 1. Strawberry purée (substrate) HPLC Chromatogram at 280 nm. 1, aspartic acid; 2, glutamic acid; 3, internal standard; 4, asparagine-serine; 5, glutamine; 6, histidine; 7, glycine; 8, threonine; 9, alanine; 10, arginine; 11, γ-aminobutiric acid; 12, proline; 13, tyrosine; 14, ammonium ion; 15, valine; 16, methionine; 17, isoleucine; 18, tryptophan; 19, leucine; 20, phenylalanine; 21, ornithine; 22, lysine.

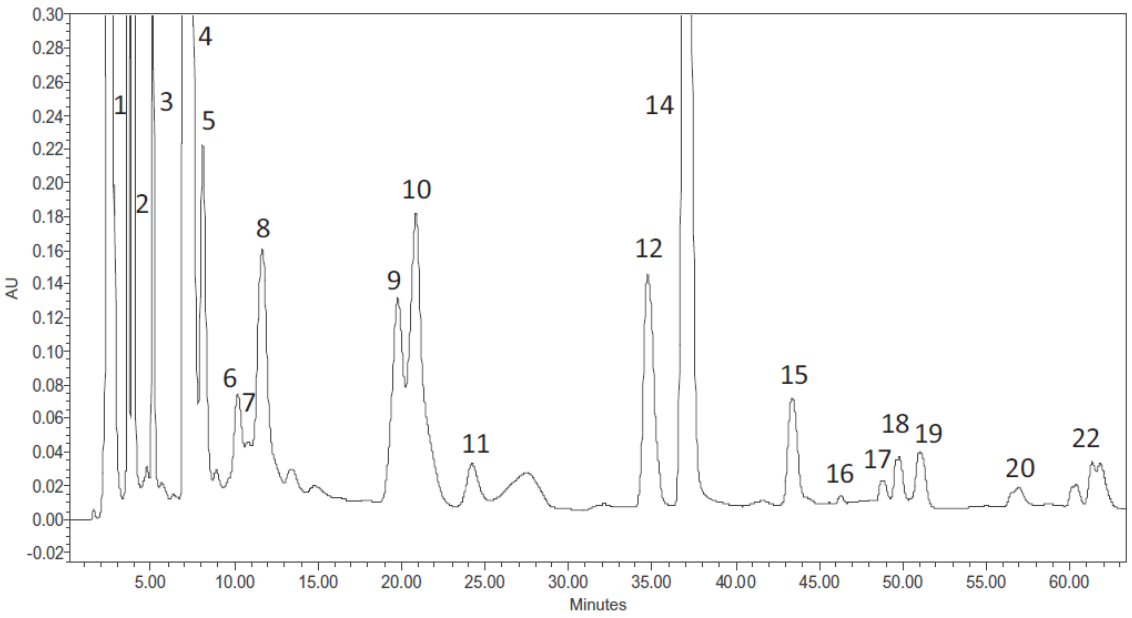

Figure 2. *Gluconobacter japonicus* strain HPLC Chromatogram at 280 nm. 1, aspartic acid; 2, glutamic acid; 3, internal standard; 4, asparagine-serine; 5, glutamine; 6, histidine; 7, glycine; 8, threonine; 9, alanine; 10, arginine; 11, γ-aminobutiric acid; 12, proline; 14, ammonium ion; 15, valine; 16, methionine; 17, isoleucine; 18, tryptophan; 19, leucine; 20, phenylalanine; 22, lysine.

Glutamine, alanine and tryptophan showed a significant decrease. These amino acids were mostly consumed by AAB. They have already been shown to be a good nitrogen source for microorganisms such as AAB and yeast (Arias-Gil, Garde-Cerdán & Ancín-Azpilicueta, 2007; Joubert, Bayens & De Ley, 1961; Sánchez & Demain, 2002).

Conversely, other amino acids, such as arginine, GABA and proline, increased significantly during fermentation. Among them, the increase of arginine was the most pronounced. A probable cause of the arginine increase in the final beverages is that this amino acid is known to be stored in microorganism vacuole under nitrogen-available conditions, and it is left in the media after autolysis (Carrasco & Pérez-Ortín, 2003; Leigh & Dodsworth, 2007). increases in GABA have been previously reported following the fermentation of blackberry with *Lactobacillus brevis* (Kim, Lee, Ji, Lee & Hwang, 2009). On the other hand, proline is not usually consumed in a rich nitrogen medium (Callejón et al., 2008). Besides, this amino acid could increase its concentration during cider fermentation by AAB (Valero, Berlanga, Roldan, Jimenez, García & Mauricio, 2005).

In all the samples fermented with gluconic acid, asparagine-serine was the most abundant amino acid followed by arginine, glutamic acid, proline and ammonium ion. Gluconic fermentation did not modify the most essential amino acid from strawberry purée substrate. Generally, tryptophan was the only amino acid that was significantly consumed for all AAB strains tested significantly. Conversely, arginine increased wide its concentration in all fermentations (Table 4). This is important because it is an essential amino acid to fetus, infant and disease adults with disease such as endothelial dysfunction, cystic fibrosis or sickle cell disease vasculopathy (Wu, 2009; Wu et al., 2009).

Additionally, gluconic fermentation preserves polyphenols, which exert a bioactive effect and play an important role in the sensory properties (Álvarez-Fernández, Hornedo-Ortega, Cerezo, Troncoso & García-Parrilla, 2014b).

Biogenic amines were not detected in the substrates or beverages. This was expected since according to Cipolla, Havouis and Moulinoux (2010) strawberries were included in the group of food containing less than 100 nmol/g/ml of polyamines. Moreover, biogenic amines of endogenous origin are found in low concentrations (Önal, 2007).

Table 4. Concentration of amino acids, biogenic amines and ammonium ion in strawberry purée and gluconic fermented.

Compounds	Mean concentrations (mg/L) ±			
	SP	SGFAM	SGFGO	SGFGJ
Aspartic acid	45.98[b,c] ± 0.79	34.10[a] ± 1.53	31.20[a,d] ± 1.29	38.77[c] ± 4.14
Glutamic acid	58.53[d] ± 0.20	58.39[d] ± 1.98	56.38[d] ± 2.45	48.43[a,b,c] ± 3.50
Asparagine-serine	717.17[d] ± 12.09	711.73 ± 21.89	654.92 ± 35.04	594.00[a] ± 18.56
Glutamine	102.96[b,c,d] ± 0.88	30.49[a,d] ± 0.64	25.11[a,d] ± 1.37	18.35[a,b,c] ± 2.18
Histidine	22.34[b,d] ± 0.42	17.74[a,d] ± 1.89	18.34[d] ± 2.31	15.21[a,b,c] ± 0.87
Glycine	1.48[b] ± 0.32	2.28[a] ± 0.64	2.00 ± 1.17	1.82 ± 1.21
Threonine	37.86 ± 2.73	36.52 ± 1.58	33.75 ± 2.22	34.24 ± 1.57
Alanine	113.93[b,c,d] ± 3.13	28.26[a,d] ± 0.46	17.56[a] ± 2.15	19.97[a,b] ± 2.52
Arginine	6.43[b,c,d] ± 0.91	59.35[a] ± 1.69	63.16[a] ± 3.01	63.73[a] ± 0.72
GABA	1.89[b,c,d] ± 0.89	5.12[a,c] ± 0.74	6.73[a,b] ± 1.31	5.99[a] ± 0.87
Proline	13.74[b,c,d] ± 1.34	36.55[a] ± 2.78	41.46[a]± 2.17	38.96[a] ± 2.35
Tyrosine	1.58[c,d] ± 1.20	1.18 ± 0.51	ND	ND
Valine	6.16[d] ± 0.82	4.10[d] ± 0.58	3.50[d] ± 0.74	1.99[a,b,c] ± 0.19
Methionine	10.12 ± 1.11	11.47 ± 1.37	12.47 ± 2.22	12.44 ± 1.44
Cysteine	ND	ND	ND	ND
Isoleucine	4.69[d] ± 0.53	3.34[d] ± 1.12	4.25[d] ± 1.20	0.34[a,b,c] ± 1.13
Tryptophan	2.11[b,c,d] ± 0.09	0.70[a] ± 0.12	0.90[a] ± 0.44	0.70[a] ± 0.15
Leucine	0.64 ± 0.55	0.83 ± 1.47	0.62 ± 1.04	0.65 ± 0.80
Phenylalanine	7.97[d] ± 0.78	9.65 ± 1.95	9.18 ± 1.82	9.87[a] ± 0.70
Ornithine	4.08[d] ± 0.13	2.09 ± 1.50	1.48 ± 0.50	ND
Lysine	ND	1.83 ± 0.59	2.59 ± 1.06	1.69 ± 0.71
Histamine	ND	ND	ND	ND
Agmatine	ND	ND	ND	ND
Spermidine	ND	ND	ND	ND
Tyramine	ND	ND	ND	ND
Putrescine	ND	ND	ND	ND
Tryptamine	ND	ND	ND	ND
Cadaverine	ND	ND	ND	ND
Phenylethylamine	ND	ND	ND	ND
Ammonium	39.51[c,d] ± 0.17	49.04[d] ± 5.19	57.58[a,d] ± 3.63	64.64[a,b,c] ± 2.26

ND: non detected.
RSD: Relative standard deviation.
Codes of samples are explained in Table 1.
[a]Shows significant differences between substrat and other samples according to ANOVA test (p<0.05).
[b]Shows significant differences between *A.malorum* and other samples according to ANOVA test (p<0.05).
[c]Shows significant differences between *G.oxydans* and other samples according to ANOVA test (p<0.05).
[d]Shows significant differences between *G.japonicus* and other samples according to ANOVA test (p<0.05).

During fermentative processes, especially by lactic acid bacteria, the concentration of biogenic amines increases significantly (Landete, Ferrer & Pardo, 2007). However, the non-presence of biogenic amines in the gluconic fermentations may be due to the fact that AAB are not able to produce these nitrogen compounds (Landete et al., 2007).

Although amino acids and ammonium showed a similar trend during all gluconic fermentations, we observed some differences, depending on the bacteria involved in the process. Thus, *G. japonicus* strain was the one that showed the highest consumption of amino acids. In fact, significant differences in amino acid profile were observed between

G. japonicus strain and the other two strains. Glutamine, histidine, valine, ornithine, glutamic acid and isoleucine were consumed by *G. japonicus* strain in a higher proportion than by the rest of the strains during fermentation. According to these results, we could conclude that *G. japonicus* strain showed the greatest activity during fermentation. This strain demonstrated the fastest glucose consumption, as no glucose was present in the medium after 10 days of fermentation.

In order to explore differences between the strains, multivariate statistical analysis was applied. First, we performed a principal component analysis (PCA). We selected the most significant variables to gather different samples using the variable contribution obtained in PCA. Hence, Factor 1 includes glutamine, alanine, arginine and proline, and Factor 2 includes glutamic acid, asparagine-serine, isoleucine, leucine and phenylalanine. Afterwards, a linear discriminant analysis (LDA) was performed to evaluate whether the profile of amino acids and ammonium ion were different enough to distinguish the samples analyzed in this study based on substrate and AAB strain. For this purpose, we selected the variables mentioned above in Factor 1 and 2. Both standard and forward stepwise analyses were performed in LDA and the AAB strain was the grouping variable. The classification matrix was 100% for the standard and the forward stepwise analysis. However, while the standard analysis considered all these variables, the forward stepwise analysis did not select glutamic acid or phenylalanine. The scatterplot of canonical scores of the standard and forward stepwise analyses are shown in Fig. 3. As it can be observed, the selected variables were able to clearly separate the substrate from the gluconic acid products. However, although the final products showed a similar amino acid profile, the LDA was able to group the samples from gluconic acid fermented beverages according to the strain involved in their fermentation process.

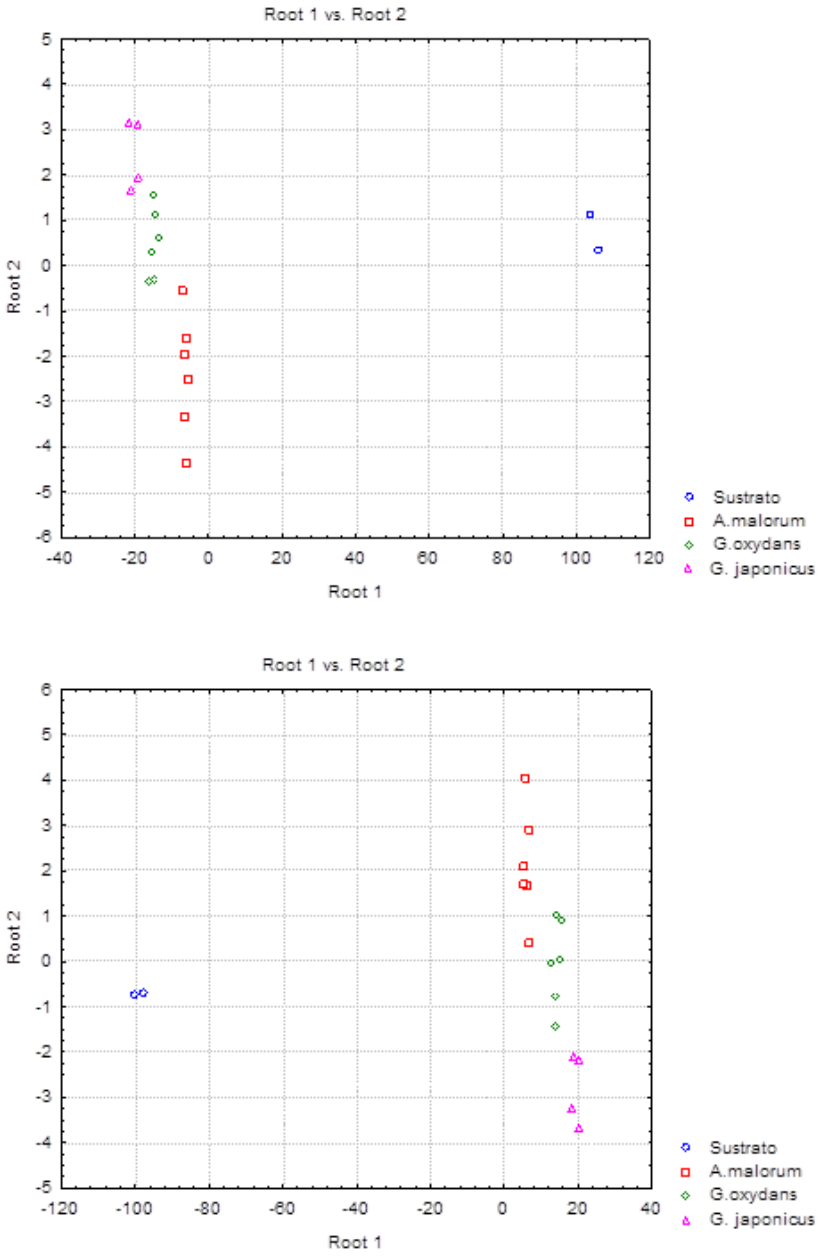

Figure 3. The scatterplot of canonical scores of standard (A) and forward stepwise (B) analysis.

4. CONCLUSIONS

A method for the determination of amino acid and biogenic amines in gluconic acid fermentation was successfully adapted, obtaining adequate values and demonstrating good linearity and precision, as well as low detection and quantification limits. Its utility for the routine analysis of amino acids and biogenic amines in this type of product has been shown.

APPENDIX 2

The fermented products did not contain biogenic amines. The amino acid profile, specifically the concentrations of glutamine, alanine, arginine, proline, glutamic acid, asparagine-serine, isoleucine, leucine and phenylalanine allows the discrimination of the beverages according to the AAB strain responsible for the fermentation. Fermentation with *G. japonicus* resulted in major amino acid concentration changes.

ACKNOWLEDGMENTS

We wish to thank the Ministry of Science and Innovation of the Spanish government for its financial support through projects AGL2010-22152-C03-01 and AGL2010-22152-C03-02.

REFERENCES

Álvarez-Fernández, M. A., Hornedo-Ortega, R., Cerezo, A. B., Troncoso, A. M., & García-Parrilla, M. C. (2014a). Effects of the strawberry (*Fragaria ananassa*) purée elaboration process on non-anthocyanin phenolic composition and antioxidant activity. *Food Chemistry, 164*, 104-112.

Álvarez-Fernández, M. A., Hornedo-Ortega, R., Cerezo, A. B., Troncoso, A. M., & García-Parrilla, M. C. (2014b). Non-anthocyanin phenolic compounds and antioxidant activity of beverages obtained by gluconic fermentation of strawberry. *Innovative Food Science & Emerging Technologies,* in press.

Ancín-Azpilicueta, C., González-Marco, A., & Jiménez-Moreno, N. (2008). Current knowledge about the presence of amines in wine. *Critical Reviews in Food Science and Nutrition, 48*, 257–275.

Peer Verified Methods Program, Manual on Policies and Procedures. (1993). Arlington, VA: AOAC.

Arias-Gil, M., Garde-Cerdán, T., & Ancín-Azpilicueta, C. (2007). Influence of addition of ammonium and different amino acid concentrations on nitrogen metabolism in spontaneous must fermentation. *Food Chemistry, 103*(4), 1312-1318.

Callejón, R., Tesfaye, W., Torija, M., Mas, A., Troncoso, A., & Morales, M. (2008). HPLC determination of amino acids with AQC derivatization in vinegars along submerged and surface acetifications and its relation to the microbiota. *European Food Research and Technology, 227*(1), 93-102.

Callejón, R., Troncoso, A., & Morales, M. (2010). Determination of amino acids in grape-derived products: A review. *Talanta, 81*(4), 1143-1152.

Carrasco, P., & Pérez-Ortín, J. E. (2003). Arginase activity is a useful marker of nitrogen limitation during alcoholic fermentations. *Systematic and Applied Microbiology, 26*(3), 471-479.

Cerezo, A. B., Cuevas, E., Winterhalter, P., Garcia-Parrilla, M. C., & Troncoso, A. M. (2010). Isolation, identification, and antioxidant activity of anthocyanin compounds in camarosa strawberry. *Food Chemistry, 123*(3), 574-582.

Cipolla, B. G., Havouis, R., & Moulinoux, J. (2010). Polyamine reduced diet (PRD) nutrition therapy in hormone refractory prostate cancer patients. *Biomedicine & Pharmacotherapy, 64*(5), 363-368.

Deppenmeier, U., Hoffmeister, M., & Prust, C. (2002). Biochemistry and biotechnological applications of *Gluconobacter* strains*. Applied Microbiology and Biotechnology, 60*(3), 233-242.

Deppenmeier, U., & Ehrenreich, A. (2009). Physiology of acetic acid bacteria in light of the genome sequence of *Gluconobacter oxydans*. *Journal of Molecular Microbiology and Biotechnology, 16*(1-2), 69-80.

European Food Safety Authority (EFSA) Panel on Biological Hazards (BIOHAZ). (2011). Scientific Opinion on risk based control of biogenic amine formation in fermented foods. *EFSA Journal*, *9* (10), 2393.

Fügel, R., Carle, R., & Schieber, A. (2005). Quality and authenticity control of fruit purées, fruit preparations and jams—A review. *Trends in Food Science & Technology, 16*(10), 433-441.

Gómez-Alonso, S., Hermosín-Gutiérrez, I., & García-Romero, E. (2007). Simultaneous HPLC analysis of biogenic amines, amino acids, and ammonium ion as aminoenone derivatives in wine and beer samples. *Journal of Agricultural and Food Chemistry, 55*(3), 608-613.

Gupta, A., Singh, V. K., Qazi, G., & Kumar, A. (2001). *Gluconobacter oxydans*: Its biotechnological applications. *Journal of Agricultural and Food Chemistry, 3*(3), 445-456.

Hancock, J., Sjulin, T., & Lobos, G. (2008). Strawberries. *Temperate fruit crop breeding* (pp. 393-437) Springer.

Hermosín, I., Chicón, R. M., & Dolores Cabezudo, M. (2003). Free amino acid composition and botanical origin of honey. *Food Chemistry, 83*(2), 263-268.

Hernández-Orte, P., Ibarz, M., Cacho, J., & Ferreira, V. (2003). Amino acid determination in grape juices and wines by HPLC using a modification of the 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) method. *Chromatographia, 58*(1-2), 29-35.

Hidalgo, C., Torija, M., Mas, A., & Mateo, E. (2013). Effect of inoculation on strawberry fermentation and acetification processes using native strains of yeast and acetic acid bacteria*. Food Microbiology, 34*(1), 88-94.

Hui, Y., Barta, J., Canor, M., Gusek, T., Sidhu, J., & Sinha, N. (2006). *Handbook of fruits and fruit processing*. Blackwell Publishing, Iowa.

APPENDIX 2

Joubert, J., Bayens, W., & De Ley, J. (1961). The catabolism of amino acids by acetic acid bacteria. *Antonie Van Leeuwenhoek, 27*(1), 151-160.

Kim, J. Y., Lee, M. Y., Ji, G. E., Lee, Y. S., & Hwang, K. T. (2009). Production of γ-aminobutyric acid in black raspberry juice during fermentation by *Lactobacillus brevis* GABA100*. International Journal of Food Microbiology, 130*(1), 12-16.

Kirschbaum, J., Rebscher, K., & Brückner, H. (1999). Determination of Biogenic Amines in Fermented Beverages and Vinegars by Pre-column Derivatization with para-Nitrobenzyloxycarbonyl Chloride (PNZ-Cl) and Reversed-Phase LC. *Chromatographia, 49*, 117–124.

Landete, J.M., Ferrer, S., & Pardo, I. (2007). Biogenic amine production by lactic acid bacteria, acetic bacteria and yeast isolated from wine. *Food Control, 18*(12), 1569-1574.

Leigh, J. A., & Dodsworth, J. A. (2007). Nitrogen regulation in bacteria and archaea. *The Annual Review of Microbiology, 61*, 349-377.

Mandrioli, R., Mercolini, L., & Raggi, M. A. (2013). Recent trends in the analysis of amino acids in fruits and derived foodstuffs. *Analytical and Bioanalytical Chemistry, 405*(25), 7941-7956.

Merrick, M. J., & Edwards, R. A. (1995). Nitrogen control in bacteria. *Microbiological Reviews, 59*(4), 604-622.

Moing, A., Renaud, C., Gaudillère, M., Raymond, P., Roudeillac, P., & Denoyes-Rothan, B. (2001). Biochemical changes during fruit development of four strawberry cultivars. *Journal of the American Society for Horticultural Science, 126*(4), 394-403.

Önal, A., 2007. A review: Current analytical methods for the determination of biogenic amines in foods*. Food Chemistry, 103*(4), 1475-1486.

Peña-Gallego, A., Hernández-Orte, P., Cacho, J., & Ferreira, V. (2009). Biogenic amine determination in wines using solid-phase extraction: A comparative study. *Journal of Chromatography A, 1216*(15), 3398-3401.

Peña-Gallego, A., Hernández-Orte, P., Cacho, J., & Ferreira, V. (2012). High-performance liquid chromatography analysis of amines in must and wine: A review. *Food Reviews International, 28*(1), 71-96.

Perez, A. G., Rios, J. J., Sanz, C., & Olias, J. M. (1992). Aroma components and free amino acids in strawberry variety chandler during ripening. *Journal of Agricultural and Food Chemistry, 40*(11), 2232-2235.

Ramachandran, S., Fontanille, P., Pandey, A., & Larroche, C. (2006). Gluconic acid: Properties, applications and microbial production. *Food Technology and Biotechnology, 44*(2), 185-195.

Sánchez, S., & Demain, A. L. (2002). Metabolic regulation of fermentation processes. *Enzyme and Microbial Technology, 31*(7), 895-906.

Singh, O. V., & Kumar, R. (2007). Biotechnological production of gluconic acid: Future implications*. Applied Microbiology and Biotechnology, 75*(4), 713-722.

StatSoft, Inc., 2004. STATISTICA (data analysis software system), version 7. www.statsoft.com

Stürtz, M., Cerezo, A. B., Cantos-Villar, E., & Garcia-Parrilla, M. C. (2011). Determination of the melatonin content of different varieties of tomatoes (*Lycopersicon esculentum*) and strawberries (*Fragaria ananassa*). *Food Chemistry, 127*(3), 1329-1334.

ten Brink, B., Damink, C., Joosten, H., & Huis In't Veld, J. (1990). Occurrence and formation of biologically active amines in foods. *International Journal of Food Microbiology, 11*(1), 73-84.

Ubeda, C., Callejón, R., Hidalgo, C., Torija, M., Troncoso, A., & Morales, M. (2013). Employment of different processes for the production of strawberry vinegars: Effects on antioxidant activity, total phenols and monomeric anthocyanins. *LWT-Food Science and Technology, 52*(2), 139-145.

Valero, E., Berlanga, T. M., Roldan, P. M., Jimenez, C., García, I., & Mauricio, J. C. (2005). Free amino acids and volatile compounds in vinegars obtained from different types of substrate. *Journal of the Science of Food and Agriculture, 85*(4), 603-608.

Wu, G. (2009). Amino acids: Metabolism, functions, and nutrition. *Amino Acids, 37*(1), 1-17.

Wu, G., Bazer, F. W., Davis, T. A., Kim, S. W., Li, P., Rhoads, J. M., Satterfield, M. C., Smith, S. B., Spencer, T. E., Yin, Y. (2009). Arginine metabolism and nutrition in growth, health and disease. *Amino Acids, 37*(1), 153-168.