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Abstract: Acetic acid bacteria (AAB) are a group of microorganisms highly used in the food industry. However, its use can be limited by the insufficient information 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 selected 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.

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Dear Prof. Cocolin

Please find enclosed the revised version of the manuscript FOOD-D-16-00755R1 submitted to International Journal of Food Microbiology.

We have modified the manuscript according to reviewers' comments.

Thank you very much for your attention.

Yours sincerely

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Responses to the reviewers

Reviewer #1: The manuscript has been well improved.
There is just one thing which is in the answers to review wrongly interpreted and has to be corrected. It concerns the fact that the other papers have mentioned before that the growth requirement of AAB are specific for each strain: the paper of Trcek and Teuber (2000) has stated that ….almost each strain of the AAB has a unique growth requirements what induces problems in using an appropriate minimal medium……Sainz et al. are now interpreting that this paper is not dealing with specific AAB requirement for nitrogen, however, nitrogen is one of the defined component of the minimal medium and therefore the mentioned statement concerns generally all growth factors in chemically defined growth medium. So, it has to be added, that the other authors have noticed before the specific growth requirements among strains of AAB and citation(s) has to be added.

The citation has been included in the text in the following sentence and in the list of references (Lines 577-579):
Lines 79-81. "Later, Treck and Teuber (2002) mentioned the difficulty to define an appropriate minimal medium for AAB growth because almost each strain of AAB has unique growth requirements."

Reviewer #3: The manuscript has been thoroughly revised and the current version is acceptable for publications. There are a few additional comments that I would like to make which are as follows;

The following responses were provided;

1. Although we are aware of the relevance of total acidity and pH, all the solutions were buffered and we have performed some studies with larger volumes we have not detected dramatic changes in pH, although it always decline. Furthermore, this is meant for food industry application, where batch processes are habitual. Thus, chemostat studies are not appropriate for our goals.

This explanation has been included in the text.
Lines 340-346. "The study was done in batch conditions with defined media and controlled temperature. Acidity and pH were left to evolve freely along the process. We are aware of the relevance of total acidity and pH, and thus all the solutions were buffered. In previous studies with larger volumes, no dramatic changes in pH were observed, although it always declined. Chemostat conditions would allow us to better control all parameters. However, in food industry batch processes are habitual."

2. Line 153-154: Is gamma amino butyric acid an amino acid? In chemical terms it is an amino acid. However, it is not an alpha amino acid and is not forming proteins. However, in nature gamma amino butyric acid is present in natural musts, for instance, and we are using a medium mimicking these musts.

This explanation has been included in section Materials and Methods, when the media composition is described.

Lines 164-165. “Gaba was included in the study because although this compound is not an alpha amino acid, it is present in natural musts and we were trying to mimic these musts.”

My suggestion to the above responses is that it could be much better if included under appropriate sections in the discussion. This to cater for the wide perspectives that IFJM readers.

As explained above, both comments are now included in the text.

Highlights

The growth of selected strains has been measured as a function of nitrogen source

The nitrogen requirements are heavily strain- and species-dependent.

The combined nitrogen sources yield better growth than single nitrogen sources.

Acetobacter malorum strains performed better in the presence of proline.

Effect of ammonium and amino acids on the growth of selected strains of *Gluconobacter* and *Acetobacter*

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ABSTRACT

Acetic acid bacteria (AAB) are a group of microorganisms highly used in the food industry. However, its use can be limited by the insufficient information 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 selected 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

1. Introduction

Acetic acid bacteria (AAB) are strictly aerobic microorganisms that 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; gluconic acid, 2-keto-D-gluconic acid and 5-keto-D-gluconic acid from 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 from extreme habitats and their high mutability (Mas et al., 2007). Furthermore, low cultivability could also be attributed to Viable But Not Culturable state that have been described for these microorganisms (Millet and Lonvaud-Funel, 2000; Torija et al., 2010). 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/or mannitol as the carbon sources most widely used for the preparation of these enrichment media (Entani, 1985; Sokollek et al., 1998). 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 media is that there is no control over the nitrogen composition, and it is not possible to

study AAB nitrogen requirements because the media are not chemically defined. 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). Later, Treck and Teuber (2002) mentioned the difficulty to define an appropriate minimal medium for AAB growth because almost each strain of AAB has unique growth requirements. Finally, many new genera and species of AAB have been incorporated in the last years (Yamada et al., 2012; Trček and Barja, 2015), but only those growth data required for the description of these new species have been described.

In recent years, studies of nitrogen and AAB have mainly focused on ensuring that nitrogen was sufficiently available 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 wild 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 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 main aim of this work was to determine which nitrogen sources were the best for the growth of the selected strains and what was the minimum concentration needed to promote its growth. We determined the nitrogen requirements of these three

wild strains, and used 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 minimum 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).

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,

143 nitrogen, vitamins) and brought to 1 L with distilled water. The three media tested had
144 an initial sugar concentration of 5% (w/v), composed by equimolar concentrations of D-
145 glucose and D-fructose, and 10 mL/L vitamins (100X) and 1 mL/L oligo elements
146 (1000X) were added to each medium. The concentrated solution of vitamins (100X)
147 was prepared with 2 g/L myo-inositol; 0.15 g/L calcium pantothenate; 0.025 g/L
148 thiamine hydrochloride; 0.2 g/L nicotinic acid; 0.025 g/L pyridoxine; and 3 mL biotin
149 (100 mg/L). The oligo elements solution (1000X) was comprised of 4 g/L manganese
150 sulfate monohydrate; 4 g/L zinc sulfate heptahydrate; 1 g/L copper sulfate pentahydrate;
151 1 g/L potassium iodide; 0.4 g/L cobalt chloride hexahydrate; 1 g/L boric acid; and 1 g/L
152 ammonium heptamolybdate.

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

158 Amino acids solutions have been prepared with distilled water at a concentration of 2.5
159 g N/L and filtered. The amino acids used were: alanine (Ala); arginine (Arg);
160 asparagine (Asn); aspartic acid (Asp); cysteine (Cys); phenylalanine (Phe); γ-
161 Aminobutyric acid (Gaba); glycine (Gly); glutamic acid (Glu); glutamine (Gln);
162 histidine (His); isoleucine (Ile); leucine (Leu); lysine (Lys); methionine (Met); ornithine
163 (Orn); proline (Pro); serine (Ser); threonine (Thr); tryptophan (Trp) and valine (Val).
164 Gaba was included in the study because although this compound is not an alpha amino
165 acid, it is present in natural musts and we were trying to mimic these musts.
166 Ammonium was always used in form of ammonium chloride.

167 All the chemicals were from Sigma Aldrich (Germany).

168 2.2.2. Growth monitoring

169 For all experiments, the initial culture had optical density (OD; 600 nm) of ca. 0.1.
170 Assays were performed using a microplate reader SpectroStar Nano (BMG LABTECH)
171 at 28 ºC in triplicate. The absorbance was measured every 30 minutes for 100 h, that is,
172 200 measurements, with stirring at 500 rpm for 80 seconds prior to each reading. For
173 the representation of the growth the OD readings were normalized by dividing each
174 value by its initial OD, so all graphs began at value 1. To compare the different
175 conditions tested, the maximum OD increase, expressed as the highest factor by which
176 the OD had increased over time, was determined and the maximum growth rate was
177 calculated. The maximum growth rate was the slope obtained in the exponential phase
178 of the OD plotted against the time.

179 2.3 Statistical procedures

180 Data were analysed using a one-way ANOVA, and significant differences were
181 determined using Tukey´s method ($p < 0.05$). The differences in maximum OD increase
182 and maximum growth rate for each strain were compared among the different nitrogen
183 concentrations in the same medium, different media at the same nitrogen concentration,
184 and finally, different nitrogen sources. Values represented with the same letter were not
185 significantly different. All statistical analyses were carried out using SPSS Statistics 23.

186 **3. Results**

187 3.1. Selection of optimal media and nitrogen concentrations

188 First, the six strains were grown in the three media (YNB, M9 and SM) without the
189 addition of amino acids, ammonium, vitamins or oligo elements to assure that they
190 could not grow without a supply of external nitrogen. No strain was able to grow in
191 these three media (data not shown), confirming that no assimilable nitrogen was

192 available in these media and also that nitrogen was an essential growth factor. Then,
193 different nitrogen concentrations in these three media were tested. In Table 2, the results
194 obtained for the six strains grown in YNB, M9 and SM are shown. In general, *G.*
195 *japonicus* strains presented the highest growth in all the media tested and in all the
196 concentrations of nitrogen that were used in this study.

197 In YNB medium, the *G. japonicus* strain CECT 8443 showed better growth than the
198 type strain (LMG 1373) in all the nitrogen concentrations tested; CECT 8443 growth
199 was twelve times higher than the growth of LMG 1373 in nitrogen concentrations of
200 300 mg N/L and 1 g N/L. High concentrations of nitrogen seemed to be unfavourable
201 for LMG 1373 growth in this medium, but not for strain CECT 8443. Therefore, 25 mg
202 N/L can be considered an optimal concentration for the growth of both strains in this
203 medium. Essentially no differences were observed in maximum growth rates either
204 between strains or among nitrogen concentrations. Surprisingly, in the other two media
205 (M9 and SM), the type strain (LMG 1373) presented better growth than CECT 8443,
206 with the only exception being growth in M9 with the addition of 300 mg N/L. In the M9
207 medium, LMG 1373 presented its maximum growth rate with the addition of 100 mg
208 N/L, whereas strain CECT 8443 presented maximum growth at the concentration range
209 between 100 and 300 mg N/L, with very similar values for both the maximum growth
210 and rate. However, higher concentrations (1 g N/L) resulted in a decrease in growth,
211 unlike the pattern that was observed in the YNB medium. Moreover, the maximum
212 growth rates for CECT 8443 were clearly lower than the ones obtained for LMG 1373.
213 Finally, in SM, the maximum growth of LMG 1373 increased as it did the nitrogen
214 concentration, and the growth of this strain showed an opposite trend in YNB. In
215 contrast, CECT 8443 displayed a peak in the maximum OD increase 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 increase 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 increase 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 maximum OD increase 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 increase was observed between 100 and 1000 mg N/L. The best nitrogen concentration was 100 mg N/L, in which the maximum OD increase 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 increase 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

241 grew better than the type strain DSM 14337 (Table 2). In YNB medium, strain DSM
242 14337 performed better with the concentration of 25 mg N/L, presenting a growth
243 similar to that of CECT 7742. On the other hand, CECT 7742 growth was favoured by
244 higher nitrogen concentrations, with 300 mg N/L as the most optimum nitrogen
245 concentration for this strain in YNB medium. In M9 medium, the growth of both *A.*
246 *malorum* strains improved when the nitrogen concentration was higher, showing the
247 best growth in the range between 100 and 1000 mg N/L. Moreover, in this medium, the
248 differences between the strains were lower; in general, the difference between them was
249 less than two-fold. Finally, DSM 14337 did not grow in SM media regardless of the
250 nitrogen concentration, while CECT 7742 presented a similar growth pattern and an
251 identical maximum growth rate between 100 and 1000 mg N/L, reaching the maximum
252 OD increase with the concentration of 300 mg N/L.

253 After this preliminary study, it was necessary to select the best medium and the lowest
254 nitrogen concentration that was favourable for the growth of each strain. Whenever
255 possible, the same medium and nitrogen concentration were selected for strains
256 belonging to the same AAB species to allow a better comparison between them. For *G.*
257 *japonicus* strains, YNB media with a concentration of 25 mg N/L was selected.
258 Although this medium was not ideal for the type strain LMG 1373, its growth was
259 sufficient, and this was the best medium for the wild strain CECT 8843. In the case of
260 the *G. oxydans* strains, 621H grew poorly in all media, but the best growth was
261 observed in YNB with the concentration of 300 mg N/L. Alternatively, Po5 grew well
262 in all media, presenting good growth in YNB with the concentration of both 300 mg
263 N/L and 25 mg N/L. Therefore, both concentrations were chosen for comparison
264 purposes between *G. oxydans* strains, and we extended this comparison to all the
265 *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.

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 supporting 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). In the low nitrogen concentration (Fig. 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. Under high nitrogen concentration (300 mg N/L) (Fig. 2), highly significant (< 0.05) 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 increase 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.

Finally, the growth response to the different nitrogen sources was similar in both *A. malorum* strains (CECT 7742 and DSM 14337) (Fig. 4). Neither the presence of the

316 majority of the amino acids nor the ammonium ion was enough to enhance the growth
317 of these strains. In fact, no growth was observed with these sources, and only five
318 amino acids were able to favour growth of the *A. malorum* strains, Ser, Gln, Glu, Ala
319 and especially Pro. The presence of 100 mg N/L in the medium in the form of Pro
320 increased the growth of both strains to 1.5 times the growth observed in the control
321 medium. For CECT 7742, Ala also enhanced growth.

322 In all the figures, the values were significantly different from the controls, except in
323 621H strain growing with His as sole nitrogen source. Thus, these indications have been
324 avoided for clarity.

325 **4. Discussion**

326 Few studies have dealt with the nutrition of AAB; in the 1950s, some studies about the
327 nutritional requirements of AAB were performed (Foda and Vaughn, 1953;
328 Raghavendra Rao and Stokes, 1953; Rainbow and Minston, 1953). More recently,
329 studies have been mainly focused on the consumption of amino acids and ammonium
330 during the acetification process and the differences among the substrates used for the
331 production of wine vinegar (Álvarez-Cáliz et al., 2012, 2014; Callejón et al., 2008;
332 Maestre et al., 2008; Valero et al., 2003). Therefore, the main aim of this work was to
333 determine which nitrogen sources were the best for the growth of the selected strains
334 and what was the minimal concentration needed to promote its growth. Thus, three
335 different media (YNB, M9 and SM) were used, but the carbon source was the same in
336 all cases to prevent changes in the nitrogen utilization due to the carbon source, as
337 described by Raghavendra Rao and Stokes (1953). In our study, we first worked with a
338 complete solution of amino acids and ammonium in various media that are commonly
339 used to culture bacteria to determine the best medium and the minimum nitrogen
340 concentration that supported the growth of each strain tested. The study was done in

batch conditions with defined media and controlled temperature. Acidity and pH were left to evolve freely along the process. We are aware of the relevance of total acidity and pH, and thus all the solutions were buffered. In previous studies with larger volumes, no dramatic changes in pH were observed, although it always declined. Chemostat conditions would allow us to better control all parameters. However, in food industry batch processes are habitual.

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 wild 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 minimum 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

391 acid consumption during wine acetification have highlighted that Pro is one of the most-
392 consumed amino acids; however, it is normally not fully depleted because its
393 concentration is clearly higher than that of the other amino acids (Álvarez-Cáliz et al.,
394 2012; Callejón et al., 2008; Maestre et al., 2008; Morales et al., 2001). Other good
395 nitrogen sources for the *Acetobacter* strains used in this study were Ala, Glu, Gln and
396 Ser. It is important to note that these strains hardly grew in presence of only
397 ammonium. In fact, there is contradictory information about the use of ammonium
398 sulfate as the sole source of nitrogen by *Acetobacter* strains, but these differences rely
399 on the strain and the carbon source present in the medium (Brown and Rainbow, 1956;
400 Rainbow and Mitson, 1953). Additionally, this statement should be extended to the
401 other AAB genera, as most of the strains used in these previous studies that were
402 considered *Acetobacter* strains actually belong to the *Gluconobacter* genus. We can
403 confirm this statement with our results because the *G. oxydans* strain Po5 presented this
404 opposite behaviour: full recovery of growth when ammonium is added at 25 mg N/L
405 and very low growth with the addition of 300 mg N/L, probably indicating an inhibition
406 of this compound at high concentrations. On the other hand, O'Sullivan. (1974)
407 reported that some amino acids, such as Thr and homoserine, inhibited growth of *A.*
408 *aceti* strains, whereas the presence of Met and Ile could reverse this effect. However, in
409 our case, although the presence of Thr did not improve the growth of both *A. malorum*
410 strains, we cannot assert that there was an inhibition of growth by this amino acid, only
411 that this amino acid, similar to Met and Ile, cannot support the growth of these strains as
412 the sole nitrogen source.

413 In the case of the *G. japonicus* strains, no single amino acid or ammonium ion could
414 replace the complete nitrogen solution because the growth was strongly affected in the
415 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.

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Figure legends

Figure 1. The maximum OD increase 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 (□).

Figure 2. The maximum OD increase 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 (□).

Figure 3. The maximum OD increase 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.

Figure 4. The maximum OD increase 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 1437 (■) and CECT 7742 (□).

618 Table 1. Strains used in this study.

Species	Strain	Origin or isolation source	Reference
G.japonicus	LMG 1373^{T}	Myrica rubra	Malimas et al. (2009)
	CECT 8443	Grape must	Navarro et al. (2013)
G.oxydans	621H	-	(Henneberg, 1987) De Ley. (1961)
	Po5	Vinegar	Vegas et al. (2010)
A.malorum	DSM 14337^{T}	Rotting apple	Cleenwerck et al. (2002)
	CECT 7742[a]	Strawberry vinegar	Hidalgo et al. (2013)

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620 T: type strain

621 [a] This strain has been incorrectly referenced as CECT 7749 in previous studies (Hidalgo et al., 2013 and
622 Sainz et al., 2016)

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Table 2. Comparison of the growth of the selected AAB strains by determination of the maximum OD increase and the maximum rate 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	Maximum OD increase*	Maximum Rate	Maximum OD increase	Maximum Rate	Maximum OD increase	Maximum Rate	Maximum OD increase	Maximum Rate	Maximum OD increase	Maximum Rate
G.japonicus	LMG 1373^{T}	YNB	$15.90 \pm 0.03^{A,a}$	$0.11 \pm 0.01^{A,a}$	$10.73 \pm 0.03^{A,b}$	$0.14 \pm 0.01^{A,b}$	$3.84 \pm 0.06^{A,c}$	$0.10 \pm 0.00^{A,ac}$	$4.62 \pm 0.05^{A,cd}$	$0.11 \pm 0.01^{A,acd}$	$4.51 \pm 0.03^{A,cd}$	$0.12 \pm 0.01^{A,abd}$
		M9	$12.51 \pm 0.02^{B,a}$	$0.13 \pm 0.02^{AB,a}$	$16.87 \pm 0.15^{B,b}$	$0.14 \pm 0.03^{AB,a}$	$20.98 \pm 0.14^{B,b}$	$0.19 \pm 0.07^{B,a}$	$12.84 \pm 0.14^{B,ac}$	$0.18 \pm 0.03^{B,a}$	$11.39 \pm 0.13^{B,ac}$	$0.18 \pm 0.03^{B,a}$
		SM	$21.69 \pm 0.01^{B,a}$	$0.10 \pm 0.02^{AB,a}$	$20.48 \pm 0.01^{C,a}$	$0.13 \pm 0.00^{AB,a}$	$24.95 \pm 0.01^{C,ab}$	$0.09 \pm 0.00^{A,b}$	$29.24 \pm 0.01^{C,bc}$	$0.12 \pm 0.01^{A,b}$	$32.09 \pm 0.03^{C,c}$	$0.13 \pm 0.02^{A,b}$
	CECT 8443	YNB	$40.88 \pm 0.11^{A,a}$	$0.11 \pm 0.01^{A,a}$	$36.18 \pm 0.05^{A,ab}$	$0.12 \pm 0.00^{A,a}$	$35.29 \pm 0.08^{A,b}$	$0.11\pm 0.00^{A,a}$	$57.87 \pm 0.08^{A,c}$	$0.14\pm 0.00^{A,b}$	$55.57 \pm 0.04^{A,c}$	$0.12 \pm 0.00^{A,a}$
		M9	$7.14 \pm 0.03^{B,a}$	$0.09 \pm 0.00^{AB,a}$	$9.78 \pm 0.12^{B,b}$	$0.10 \pm 0.01^{AB,a}$	$13.97 \pm 0.21^{B,c}$	$0.09 \pm 0.00^{AB,a}$	$14.56 \pm 0.20^{B,c}$	$0.09 \pm 0.01^{B,a}$	$9.73 \pm 0.02^{B,b}$	$0.09 \pm 0.01^{B,a}$
		SM	$11.82 \pm 0.21^{C,a}$	$0.10 \pm 0.01^{AB,a}$	$16.65 \pm 0.03^{C,b}$	$0.10 \pm 0.03^{AB,a}$	$18.23 \pm 0.05^{C,b}$	$0.11 \pm 0.03^{AB,a}$	$16.92 \pm 0.14^{B,b}$	$0.11 \pm 0.03^{B,a}$	$13.91 \pm 0.19^{C,b}$	$0.10 \pm 0.02^{B,a}$
G.oxydans	621H	YNB	$2.84 \pm 0.02^{A,a}$	$0.07 \pm 0.02^{A,a}$	$2.80 \pm 0.02^{A,a}$	$0.05 \pm 0.00^{A,a}$	$4.01 \pm 0.02^{A,a}$	$0.05 \pm 0.01^{A,a}$	$8.41 \pm 0.02^{A,b}$	$0.07 \pm 0.00^{A,a}$	$0.99 \pm 0.01^{A,c}$	$0.05 \pm 0.01^{A,a}$
		M9	$3.16 \pm 0.02^{A,a}$	$0.05 \pm 0.00^{AB,a}$	$3.20 \pm 0.02^{AB,a}$	$0.05 \pm 0.00^{A,a}$	$3.42 \pm 0.04^{A,a}$	$0.05 \pm 0.00^{A,a}$	$2.99 \pm 0.02^{B,a}$	$0.06 \pm 0.00^{AB,a}$	$0.93 \pm 0.01^{A,b}$	$0.03 \pm 0.00^{A,a}$
		SM	$1.92 \pm 0.01^{B,a}$	$0.05 \pm 0.01^{AB,a}$	$4.15 \pm 0.01^{B,b}$	$0.09 \pm 0.01^{B,a}$	$7.55 \pm 0.02^{B,c}$	$0.09 \pm 0.02^{B,a}$	$6.46 \pm 0.01^{C,c}$	$0.05 \pm 0.01^{B,a}$	$6.43 \pm 0.02^{B,c}$	$0.07 \pm 0.00^{B,a}$
	Po5	YNB	$16.07 \pm 0.07^{A,a}$	$0.17 \pm 0.02^{A,a}$	$16.76 \pm 0.03^{A,a}$	$0.17 \pm 0.01^{A,a}$	$16.88 \pm 0.17^{A,a}$	$0.13 \pm 0.02^{A,ab}$	$22.92 \pm 0.07^{A,b}$	$0.07 \pm 0.00^{A,b}$	$7.51 \pm 0.01^{A,c}$	$0.12 \pm 0.01^{A,ab}$
		M9	$12.65 \pm 0.00^{B,a}$	$0.09 \pm 0.02^{B,a}$	$20.53 \pm 0.07^{B,b}$	$0.08 \pm 0.01^{B,a}$	$28.22 \pm 0.03^{B,c}$	$0.11 \pm 0.02^{A,a}$	$27.97 \pm 0.07^{C,c}$	$0.15 \pm 0.03^{B,b}$	$23.30 \pm 0.26^{B,b}$	$0.14 \pm 0.03^{AB,b}$
		SM	$12.84 \pm 0.22^{B,a}$	$0.20 \pm 0.02^{A,a}$	$17.09 \pm 0.03^{A,ab}$	$0.21 \pm 0.02^{A,a}$	$22.16 \pm 0.05^{A,bc}$	$0.20 \pm 0.02^{B,a}$	$20.01 \pm 0.05^{B,bcd}$	$0.17 \pm 0.02^{B,ab}$	$21.20 \pm 0.13^{B,bcd}$	$0.09 \pm 0.02^{A,b}$
A.malorum	DSM 14337^{T}	YNB	$3.45 \pm 0.01^{A,a}$	$0.04 \pm 0.00^{A,a}$	$1.05 \pm 0.01^{A,b}$	$0.04 \pm 0.00^{A,ab}$	$2.02 \pm 0.01^{A,c}$	$0.05 \pm 0.01^{A,abc}$	$1.64 \pm 0.04^{A,c}$	$0.06 \pm 0.00^{A,c}$	$2.46 \pm 0.03^{A,c}$	$0.04 \pm 0.00^{A,abc}$
		M9	$2.89 \pm 0.01^{A,a}$	$0.06 \pm 0.00^{AB,a}$	$5.76 \pm 0.02^{B,b}$	$0.06 \pm 0.01^{B,a}$	$8.88 \pm 0.01^{B,c}$	$0.07 \pm 0.01^{AB,a}$	$10.65 \pm 0.01^{B,d}$	$0.07 \pm 0.01^{A,a}$	$8.74 \pm 0.02^{B,c}$	$0.06 \pm 0.01^{B,a}$
		SM	$0.84 \pm 0.02^{B,a}$	$0.05 \pm 0.00^{AB,a}$	$1.18 \pm 0.01^{A,b}$	$0.05 \pm 0.00^{AB,a}$	$1.44 \pm 0.00^{C,c}$	$0.05 \pm 0.00^{AB,a}$	$1.24 \pm 0.00^{A,bd}$	$0.05 \pm 0.00^{AB,a}$	$1.29 \pm 0.00^{C,bcd}$	$0.05 \pm 0.00^{AB,a}$
	CECT 7742	YNB	$3.10 \pm 0.02^{A,a}$	$0.05 \pm 0.01^{A,a}$	$4.90 \pm 0.02^{A,b}$	$0.08 \pm 0.02^{A,b}$	$6.80 \pm 0.03^{A,bc}$	$0.06 \pm 0.01^{A,abc}$	$10.1 \pm 0.00^{A,d}$	$0.08 \pm 0.00^{A,bc}$	$6.24 \pm 0.03^{A,bc}$	$0.14 \pm 0.01^{A,d}$
		M9	$7.80 \pm 0.01^{B,a}$	$0.08 \pm 0.01^{B,a}$	$9.40 \pm 0.05^{B,ab}$	$0.08 \pm 0.01^{A,a}$	$12.8 \pm 0.04^{B,bc}$	$0.10 \pm 0.01^{B,a}$	$13.0 \pm 0.07^{B,cd}$	$0.08 \pm 0.00^{AB,a}$	$14.1 \pm 0.00^{B,d}$	$0.09 \pm 0.01^{B,a}$
		SM	$5.70 \pm 0.01^{C,a}$	$0.06 \pm 0.01^{A,a}$	$6.18 \pm 0.01^{A,a}$	$0.05 \pm 0.01^{B,a}$	$11.65 \pm 0.01^{B,b}$	$0.08 \pm 0.01^{AB,b}$	$13.62 \pm 0.01^{B,bc}$	$0.08 \pm 0.00^{AB,bc}$	$12.24 \pm 0.00^{C,bc}$	$0.08 \pm 0.01^{B,bc}$

T: type strain

*Maximum OD increase represents the highest factor by which the OD had increased over time

Values represented with different letter were significantly different ($p<0.05$). In maximum OD increase, 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 maximum rate, the same comparisons were done and differences were marked with italics