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Acetic acid bacteria: physiology and carbon sources oxidation

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Abstract:	<p>Acetic acid bacteria (AAB) are obligately aerobic bacteria within the family Acetobacteriaceae, widespread in sugary, acidic and alcoholic niches. They are known for their ability to partially oxidise a variety of carbohydrates and to release the corresponding metabolites (aldehydes, ketones and organic acids) into the media. Since a long time they are used to perform specific oxidation reactions through processes called "oxidative fermentations", especially in vinegar production. In the last decades physiology of AAB have been widely studied because of their role in food production, where they act as beneficial or spoiling organisms, and in biotechnological industry, where their oxidation machinery is exploited to produce a number of compounds such as L-ascorbic acid, dihydroxyacetone (DHA), gluconic acid and cellulose. The present review aims to provide an overview of AAB physiology focusing carbon sources oxidation and main products of their metabolism.</p>
Response to Reviewers:	<p>Answers to Reviewers</p> <p>Thank you very much for all your comments and suggestions, strongly contributed to improve the manuscript. Below a detailed response for each point raised out is provided.</p> <p>Reviewer #1: This review describes acetic acid bacteria focusing on their metabolism. It is a concise overview of the acetic acid bacteria involved in food fermentation, particularly vinegar production. A few things that should be considered to improve the review:</p> <ul style="list-style-type: none"> - Emphasis on recent taxonomy (a lot of research has been done on AAB taxonomy in the last 5-10 years) - Figure would be good (eg reaction of ethanol to acetic acid with the various enzymes) - Biotechnology needs to be expanded more - VBNC needs to be brought out more in the cultivation section - English expression and language should be checked <p>We have fully considered the criticisms raised out by the reviewer and accordingly the</p>

manuscript was revised.

Recent taxonomy has been added to the paragraph "General characteristics" that in this new version of the manuscript has been renamed as follow:

"General phenotypic characteristics and taxonomic aspects of acetic acid bacteria"
As suggested, a figure (Fig.1) showing ethanol oxidation by ADH and ALDH and location within the cell has been added.

Biotechnological applications of AAB has been expanded more (p. 2 lines 1-23).

As suggested, the paragraph "Isolation and cultivability" was modified and VBNC concept added (p.5, lines 11-15)

The whole manuscript has been revised and a thoroughly English revision has been done.

Specific comments:

Abstract

- use biotechnology rather than biotech

The term was corrected

P3, L7 - assigned rather than allocated

Allocated has been replaced by "assigned" (p.3, line 13), as suggested.

P3, L12 & 14 - write ethanol (EtOH) or acetic acid (AcOH) and then use abbreviation

The correction has been done.

P3, L23 - surface layer

Superficial has been replaced by "surface" (p. 1, line 14)

P4, L1 - acetic acid production is not a fermentation

The sentence was corrected:

AcOH fermentation was replaced by " AcOH production"

P4, L5 - AAB are involved in these other food productions, not only implicated

Implicated was replaced by involved, as suggested.

Page 5 - what is the significance of 1996 in regards to these various foods and the bacteria isolations?

The whole sentence was removed.

Reference is needed for 'Early researchers....'

The reference was added [35] : Dupuy P (1952) Recherche d'une technique d'isolement des Acetobacter du vin. Ann Technol Agric 1:107-112 (p.5. L.19)

Page 6 L 10-15 - this section a few more sentences to explain these statements better.

Lines 14-15: why the difference? Explain the significance between the two versions.

To clarify, we removed the second part of the sentence: "Whereas previous edition of Bergey's Manual [25] recommended the use of a simple medium containing 0.5% yeast extract, 1.5% EtOH, and 2.5% agar."

In the new version of the manuscript, the sentence reports only the medium of the last version of bergey's manual (p. 8, L: 5-9) "The last edition of Bergey's Manual of Systematic Bacteriology [1] proposed standard medium for enrichment and isolation of AAB, with the exception of Ga. europaeus (which strains are AcOH-dependent), containing: D-glucose, 0.05%; EtOH, 1.5%; yeast extract, 0.5%; peptone, 0.3%; cycloheximide, 0.01%; and agar, 0.12%."

Page 7 L 6-11 - this whole section needs a bit of rearranging. These lines should be at the start of the section

The paragraph "Isolation and cultivation" was rearranged. The sentence was simplified and put at the start of paragraph "Isolation and cultivation of AAB strains, especially from fermented beverages, have been described to be problematic, giving rise to an underestimation of species richness when culture dependent methods are applied [31]".

Lines 6-14 - this needs to be expanded to explain these observations and statements. It is suggesting that now all bacteria are growing up/being cultivated. Is this viable but not culturable (VBNC). Or poor growth medium? Highlight the difficulties in cultivating AAB and all the AAB present.

The section was rearranged as follows:

p. 5, lines 11-17 " Low recovery of strains due to the fraction of population that could reach a viable but non-culturable (VBNC) state has been stated. For instance in wine it was shown that VBNC status of spoiling AAB is induced by O₂ deprivation [32]. Whereas in vinegars some studies developed by no culture approaches, such as PCR/DGGE, revealed higher species diversity respect to that detected by culturing methods [33, 34, 3].

Limitations of culturing were in part overcome with the formulation of appropriate media, which allowed the cultivation of no growing strains or slow growing strains."

Page 8 L 1-8 : it would be a good idea to have a figure here for the reaction and enzymes, their location It is always beneficial in a review to have a figure.

As suggested, a figure (Fig.1) showing ethanol conversion and location of enzymes within the cell has been added. The figure represents the ethanol oxidation to acetic acid by the membrane-bound ADH and ALDH and cytoplasmic respiration of ethanol.

P9 L 15 - ... were present in the strain. The genes were present, but was it shown that the pathways were functioning. Genomics show that genes are present but do not confirm that reactions are occurring.

The sentence was removed.

P 10 L 7 - High in what sense? Concentration? Structure?

P 10 L 9 - wide range of applications. This does not make sense or belong here.

To avoid repetition the sentence (p.10, L.6-9) was removed.

The sentence reported in the new version of manuscript is at page 9, lines: 14-18 "The most characteristic reaction is the direct oxidation of glucose into Glucono- δ -lactone, which is oxidized into gluconic acid. This reaction is particularly active in *Gluconobacter* growing at high concentrations of sugars. D-gluconate can be further oxidized to 2-ketogluconate and 2,5-diketogluconate by the gluconate dehydrogenase and 2-ketogluconate dehydrogenase [52]."

P 10 L 14 - last sentence Is this important for high production of vitamin C?

The sentence was removed.

P 11 L 1-5 - another sentence or two to expand this concept would be beneficial for the reader.

To clarify the sentence was rewritten as follows (p. 10, Lines 14-17):

"In *G. oxydans* it was reported that oxidation of glycerol is catalyzed by the membrane-bound glycerol/sorbitol dehydrogenase. This quinoprotein is considered the main polyol-dehydrogenase of *G. oxydans* that exhibits a broad substrate specificity. It catalyzes the oxidation of D-sorbitol, gluconate, and glycerol to L-sorbose, 5 ketogluconate and DHA, respectively [54]."

P 13 L 6-7: this sentence needs to be reworded and explained better. What has been reached?

P 13 L 11 - sugared is not a good word here. Rewrite using high sugar content.

In the new Conclusion section, the sentences were rewritten as follow:

“AAB are food-associated microorganisms that have a long history in oxidative fermentation processes, where they are spoiling or beneficial organisms. The physiological uniqueness of AAB is due to their ability to partially oxidize carbon sources and quantitatively excrete the corresponding compounds in the surrounding media. This feature, besides vinegar, is exploited for the industrial production of a number of compounds from alcohols, sugar and sugar alcohols oxidation. Moreover they are considered promising for the production of the pure form of cellulose. Although the valuable potential of AAB in biotechnological applications, their industrial exploitation is not full developed.

Perspectives to enhance their role as biocatalysts include the availability of genetic stable strains and further advance on their metabolic potential, in order to obtain high carbon substrates conversion efficiency.”

Table 1.

In the legend write AAB in full. At the top of the table, it would be much better to have at least abbreviations of the genera rather than numbers. It would make it much easier for the reader.

AAB was replaced by acetic acid bacteria, as suggested.

Number were replaced by abbreviations.

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1 **Acetic acid bacteria: physiology and carbon sources oxidation**

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1 **Abstract**

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3 2 Acetic acid bacteria (AAB) are obligately aerobic bacteria within the family
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5 3 *Acetobacteriaceae*, widespread in sugary, acidic and alcoholic niches. They are known for
6
7 4 their ability to partially oxidise a variety of carbohydrates and to release the corresponding
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9 5 metabolites (aldehydes, ketones and organic acids) into the media. Since a long time they
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11 6 are used to perform specific oxidation reactions through processes called “oxidative
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13 7 fermentations”, especially in vinegar production.
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18 8 In the last decades physiology of AAB have been widely studied because of their role in
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20 9 food production, where they act as beneficial or spoiling organisms, and in
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22 10 biotechnological industry, where their oxidation machinery is exploited to produce a
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24 11 number of compounds such as L-ascorbic acid, dihydroxyacetone (DHA), gluconic acid
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26 12 and cellulose. The present review aims to provide an overview of AAB physiology
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28 13 focusing carbon sources oxidation and main products of their metabolism.
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35 15 **Keywords:** acetic acid bacteria, oxidative fermentation, cellulose, *Acetobacter*,
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37 16 *Gluconacetobacter*, *Gluconobacter oxydans*
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1 Introduction

2 Acetic acid bacteria (AAB) are strictly aerobic bacteria occurring in sugary, alcoholic and
3 acidic niches such as fruits, flowers and particularly fermented beverages [1, 2, 3, 4].

4 Although foods are the most known sources of AAB, they play role as plant-associated
5 bacteria (N_2 fixing), symbionts of insects and human pathogens [5, 6].

6 The metabolic potential of AAB in these environments is expressed by the partial
7 oxidation of carbohydrates releasing the corresponding products (aldehydes, ketones and
8 organic acids) into the surrounding media. Through processes called “oxidative
9 fermentations”, AAB perform specific oxidation reactions and channel the released
10 electrons to molecular oxygen. Due to these ability they are known since a long time
11 especially for their role in vinegar production [7].

12 Vinegar, an aqueous solution of acetic acid (AcOH) that is produced by AAB from a dilute
13 ethanol (EtOH) solution [8], was the first investigated environment concerning the
14 biological formation of AcOH. Early researches [9] allowed to recognize that the surface
15 layer during vinegar formation, commonly known as “mother of vinegar”, was a mass of
16 living microorganisms causing AcOH production.

17 AAB are also involved in the production of other foods, like palm wine [10], cocoa powder
18 [11], *nata de coco* (a fermented food from coconut), *pulque* (a beverage from agave) and
19 *kombucha*, a slightly acid and sparkling beverage obtained from tea fermentation by a
20 symbiotic culture of AAB and yeasts [8].

21 They can spoil fermented beverages such as wine, cider and beer, where the production of
22 AcOH is undesired [12], whereas in others foods, such as sourdough for bread production
23 [13], AAB can occasionally occur contributing to the acidification of dough.

1 Besides fermented foods, some AAB are used as biocatalysts for the industrial production
2 of a range of compounds, making them important biocatalysts for the development of eco-
3 friendly fermentation processes as an alternative to the chemical synthesis. Strains of *G.*
4 *oxydans* produce enzymes involved in amino acids synthesis e.g. glutamic and aspartic
5 acids thanks to the incomplete set of tricarboxylic acid (TCA) enzymes which could
6 function primarily for glutamate, aspartate and succinate biosynthesis [14].

7 One of the most important biotechnological application of *G. oxydans* is the production of
8 L-ascorbic acid (vitamin C) precursors such as L-sorbose from D-sorbitol and 2-keto-L-
9 gluconic acid from 2,5-Diketo-D-gluconic acid or L-Sorbosone [15].

10 Strains of *G. oxydans* are also exploited for the microbial production of DHA that is used
11 in the pharmaceutical industry as a cosmetic tanning agent and also as an intermediate for
12 the synthesis of various organic chemicals and surfactants [16].

13 Among organic acids, gluconic acid, used as a bulk chemical in the food, textile, medical
14 and construction industries can be produced by *G. oxydans* which oxidize glucose to
15 gluconate by the membrane bound glucose dehydrogenase [17].

16 Other applications of *G. oxydans* are the production of miglitol's precursors, used as a
17 therapeutic drug for the treatment of non-insulin-dependent diabetes mellitus; D-tagatose,
18 used as a bulking agent in food and a non-calorific sweetener; and shikimate, which is a
19 key intermediate for a number of antibiotics [16].

20 Species of *Acetobacter* and *Gluconacetobacter* genera, besides vinegar, are of interest in
21 agricultural field, where especially strains of *Ga. diazotrophicus* have been proved useful
22 for their role as N₂ fixing bacteria [5]. Finally *Ga. xylinus* is well known for the ability to
23 produce high amount of pure cellulose [18].

1 On the basis of the increasing prospective on AAB for food, beverages and other
2 biotechnological applications, this work aims to review physiology of AAB including
3 carbon sources oxidation and metabolites production.

4 **General phenotypic characteristics and taxonomic aspects of acetic acid bacteria**

5 AAB are gram negative or gram-variable, non-spore forming, ellipsoidal to rod-shaped
6 cells that can occur in single, pairs or in short chains. They could be motile due to the
7 presence of peritrichous or polar flagella. Catalase positive and oxidase negative, AAB
8 have an obligate aerobic metabolism, with oxygen as the terminal electron acceptor. The
9 optimum pH for the growth is 5-6.5, while they can grow at lower values (3-4) [1]. Their
10 optimum temperature vary between +28 and +30°C although some species are recognized
11 as thermotolerant [19, 20]. They can produce pigments and also different kinds of
12 exopolysaccharides [21]. Main distinctive traits of AAB are reported in Table 1.

13 AAB are assigned to the family *Acetobacteriaceae* that belongs to the order
14 *Rhodospirillales* as part of the *Alphaproteobacteria*, within the family *Acetobacteraceae*.
15 At present they are represented by the following genera: *Acetobacter*, *Acidomonas*,
16 *Ameyamaea*, *Asaia*, *Gluconacetobacter*, *Gluconobacter*, *Granulibacter*, *Kozakia*,
17 *Neoasaia*, *Neokomagataea*, *Saccharibacter*, *Swaminathania* and *Tanticharoenia*.

18 The most updated data on valid published species of each genus are reported by the List of
19 Prokaryotic names with Standing in Nomenclature [22].

20 Since their first description as “vinegar bacteria”, about 150 years ago [9], classification of
21 AAB has undergone robust changes, with scission, renaming, restoration and emendation
22 of genera and species [23].

23 During the last decades studies on AAB identity and their phylogenetic relationships have
24 been achieved by polyphasic approaches combining phenotypic, chemotaxonomic and

1 genotypic data of strains. Both the use in a polyphasic strategy of 16S rRNA gene as
2 molecular marker, and the formulation of more suitable isolation media, reflect the
3 increased number of new described genera and species.

4 However, identification at the species level is often difficult due to the low resolution
5 power of phenotypic characterization and the high sequence similarity ($\geq 99,5\%$) of 16S
6 rDNA of phylogenetically closely related species [24].

7 Weaknesses of phenotypic characterization are generated by a difficult standardisation of
8 tests, in some cases by low number of discriminant characters and by instability of
9 physiological traits of preserved strains [25].

10 For instance physiological deficiencies caused by inactivation of enzymes, such as the
11 membrane-bound alcohol dehydrogenase (ADH) and cellulose synthase, deriving from
12 genetic instability, can affect reliability of phenotypic assays. The main source of genetic
13 instability has been attributed to mobile genetic elements, mainly transposons, widely
14 distributed in the genome. Insertion sequences (IS) responsible for EtOH oxidation
15 deficiency, like those of the family IS 12528, were found in the chromosome of *A.*
16 *pasteurianus* NCIB 7214 (5 copies) [26], *A. aceti* 1023 (1 copy) and *G. oxydans* IFO
17 12528 (10 copies). Likewise 100 copies of IS 1380 occur in *A. aceti* 1023 and 74 copies in
18 *A. pasteurianus* NBRC 3283 [1, 27]. Also spontaneous cellulose deficient mutants due to
19 the IS 1031 were detected in *Ga. xylinus* ATCC 23769 [28].

20 Advances in taxonomy of AAB derive from the availability of full genome sequences
21 especially of type strains, that allowed the application of new genomic approaches.

22 To solve ambiguities of phylogenetically closely related species, recently the use of
23 different genes as phylogenetic markers, such as housekeeping genes (*dnaK*, *groEL* and
24 *rpoB*) has been proved useful for AAB species differentiation [29]; whereas protein-coding

1 genes, such as those involved in the metabolism of AcOH, have been applied to investigate
2 phylogenetic relationships among *Acetobacter*, *Gluconacetobacter* and *Gluconobacter*
3 genera [30]. Since multigene analysis can resolve ambiguities in phylogenetic
4 reconstructions when a single gene is not enough, it is expected that the availability of
5 more complete genome sequences will increase the application of these approaches.

6 7 **Isolation and cultivability**

8 Isolation and cultivation of AAB strains, especially from fermented beverages, have been
9 described to be problematic, giving rise to an underestimation of species richness when
10 culture dependent methods are applied [31].

11 Low recovery of strains due to the fraction of population that could reach a viable but non-
12 culturable (VBNC) state has been stated. For instance in wine it was shown that VBNC
13 status of spoiling AAB is induced by O₂ deprivation [32]. Whereas in vinegars some
14 studies developed by no culture approaches, such as PCR/DGGE, revealed higher species
15 diversity respect to that detected by culturing methods [33, 34, 3].

16 Limitations of culturing were in part overcome with the formulation of appropriate media,
17 which allowed the cultivation of no growing strains or slow growing strains.

18 A number of conventional culture media to isolate AAB from different sources are
19 reported in literature, which carbon source are mainly glucose, mannitol and EtOH. Early
20 researches proposed a variety of media containing yeast-glucose agar (pH 5.5-6.0),
21 peptone-glucose agar fortified with yeast extract and tomato juice, and media containing
22 EtOH (1.5%) as single carbon source, yeast extract (0.5%) and agar (2.5%) [35]. To
23 control the growth of other bacteria and yeasts, these media can be acidified and/or
24 supplemented with antibiotics and cycloheximide [36].

1 A medium that allows successful isolation from different niches is glucose yeast extract
2 carbonate (GYC) composed by D-glucose, 10%; yeast extract, 0.5%; peptone 0.3%;
3 CaCO₃, 0.12%; and agar, 0.12% [37]. After incubation, colonies of AAB are recognized by
4 the surrounding zones of CaCO₃ clearing. CaCO₃ neutralizes AcOH generated by AAB,
5 preventing physiological stress and cell death [25]. The last edition of Bergey's Manual of
6 Systematic Bacteriology [1] proposed standard medium for enrichment and isolation of
7 AAB, with the exception of *Ga. europaeus* (which strains are AcOH-dependent),
8 containing: D-glucose, 0.05%; EtOH, 1.5%; yeast extract, 0.5%; peptone, 0.3%;
9 cycloheximide, 0.01%; and agar, 0.12%.

10 Important advances in recovering AAB strains from industrial vinegar have been reached
11 introducing a double agar layer (0.5% in the lower layer and 1% in the upper layer) and
12 media containing EtOH and AcOH, in an attempt to simulate the environment of the
13 acetification tanks. Using this approach the new species *Ga. europaeus* isolated from
14 industrial wine vinegar was described [38].

15 It was also stated that isolates from cider or wine vinegar grew readily in Reinforced AE-
16 Medium (RAE), containing D-glucose, 4.0%; peptone, 1.0%; yeast extract, 1%; citric
17 acid, 0.15%; disodium hydrogen phosphate (Na₂HPO₄), 0.38%; glacial AcOH, 1 ml;
18 absolute EtOH, 1 ml; agar, 0.5% upper layer; 1% bottom layer); pH 3.8. The simpler AE
19 medium (glucose, 0.5%; yeast extract, 0.3%; peptone, 0.4%; agar, 0.9%; absolute EtOH,
20 3 ml; glacial AcOH, 3 ml) has been proved suitable for the isolation of strains from spirit
21 vinegars [39, 40].

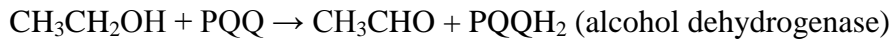
22 Actually, some of the most widely used isolation media are: GYC, AE; YPM
23 medium (yeast extract, 0.5%; peptone, 0.3%; mannitol, 2.5%; agar, 1.2%) and MYA
24 medium (malt extract, 1.5%; yeast extract, 0.5%; agar, 1.5%; and EtOH, 60 ml) [41].

1 **Carbon sources**

2 *Ethanol oxidation*

3 AAB partially oxidize EtOH by two successive catalytic reactions of the ADH and a
4 membrane-bound aldehyde dehydrogenase (ALDH) that are bound to the periplasmic side
5 of the cytoplasmic membrane. The complete oxidation of EtOH occurs at cytoplasmic
6 level by a NAD-ADH and NAD-ALDH. The AcOH produced can be further utilized by
7 acetyl CoA synthase and via TCA cycle (Fig. 1).

8 The membrane-bound ADH and ALDH complexes are tightly linked to the
9 respiratory chain, which transfers electrons via ubiquinone (UQ) and a terminal ubiquinol
10 oxidase to oxygen as final electron acceptor [42]. ADH oxidizes EtOH to acetaldehyde,
11 which is further oxidized to AcOH by ALDH as follows:



14 ADH of many AAB is composed by three subunits. Subunit I is the largest (72-78 kDa)
15 and it is encoded in the *adhA* gene. It possesses a heme *c* and a pyrroloquinoline quinone
16 (PQQ) as cofactors and requires Ca^{2+} to be active, according to the catalytic mechanism
17 given by Anthony (1996) [43] and Goodwin and Anthony (1998) [44]. The subunit II
18 which molecular size range from 44-45 kDa contains three heme *c* moieties and it is
19 encoded in the *adhB* gene. The third and smallest subunit (20 kDa), encoded in the *adhS*,
20 helps the two functional subunits with their association to the membrane protecting the
21 catalytic subunit from proteolysis and it contribute to the correct conformation of the ADH
22 complex for electron transport on the periplasmic surface [26, 45]. Oxidation of EtOH
23 occurs at PQQ site that acts as two-electron redox mediator; electrons are initially
24 transferred to UQ, which will be re-oxidized by a membrane-associated oxidase.

1 Eventually, oxygen is the final electron acceptor, resulting in formation of H₂O and a
2 proton motive force necessary for energy production through a membrane-bound ATPase.

3 AAB possesses also an inactive form of ADH with the same subunit composition of the
4 active form, but having 10 times lower Q-1 reductase activity. However, it exhibits an
5 ubiquinol:ferricyanide oxidoreductase activity, an ethanol:Q-1 and ethanol: ferricyanide
6 oxidoreductase activities. The ubiquinol:ferricyanide oxidoreductase activity is higher in
7 the inactive ADH than in the active enzyme suggesting that it play a role for the regulation
8 of redox levels of UQ/ubiquinol in the cytoplasmic membrane contributing to the
9 functionality of AAB under acidic and high aeration conditions [45].

10 The ALDH complex of AAB is composed of two or three subunits of different molecular
11 masse organized as an operon. It has been reported that the ALDH enzymes from *A. aceti*
12 and *Ga. europaeus* contain three subunits, whereas two subunits were detected in *G.*
13 *suboxydans*, *A. rances*, and *A. polyoxogenes* [1]. Its optimum pH is between 4 and 5,
14 although it can catalyses the oxidation of acetaldehyde to acetate at lower pH values [46].
15 ALDH is sensitive to oxygen concentrations, and when these are low its activity decreases,
16 accumulating acetaldehyde into the media. It is also more sensitive to the presence of
17 EtOH than ADH [47].

18 *Sugars oxidation*

19 AAB are known to have a high oxidative ability against sugars, mainly glucose but also
20 arabinose, fructose, galactose, mannitol, mannose, ribose, sorbose and xylose (Table 1)
21 [48]. They can catabolize sugars through the cytoplasmic hexose monophosphate pathway
22 (Warburg-Dickens pathway) [36]. The Entner-Doudoroff pathway occurs only in cellulose-
23 synthesizing *Acetobacter* and *Gluconacetobacter* strains, where it appears to be more
24 active than the hexose monophosphate cycle [49].

1 The oxidative pentose-phosphate pathway was reported to be the most important route for
2 phosphorylative breakdown of sugars and polyols to CO₂ in *G. oxydans*. Therefore it was
3 predicted that *G. oxydans* has the capability to take up and to channel many polyols, sugars
4 and sugar derivatives into the oxidative pentose phosphate pathway: polyols are first
5 oxidized by soluble dehydrogenases; these products and other ketoses and aldoses are
6 further modified by isomerases and epimerases. Finally, the compounds are
7 phosphorylated by specific or unspecific kinases forming intermediates of the oxidative
8 pentose phosphate pathway [50].

9 *Acetobacter* species can use sugars through the hexose monophosphate pathway and also
10 through the Embden-Meyerhof-Parnas and Entner-Doudoroff pathways [51]. Sugars are
11 further metabolised to CO₂ and H₂O via the TCA pathway, which is not functional in
12 *Gluconobacter*. Sugar is more preferred as carbon source by *Gluconobacter* than by
13 *Acetobacter* because the species of this genus can obtain energy more efficiently by the
14 metabolisation of the sugars via pentose phosphate pathway [48]. The most characteristic
15 reaction is the direct oxidation of glucose into Glucono- δ -lactone, which is oxidized into
16 gluconic acid. This reaction is particularly active in *Gluconobacter* growing at high
17 concentrations of sugars. D-gluconate can be further oxidized to 2-ketogluconate and 2,5-
18 diketogluconate by the gluconate dehydrogenase and 2-ketogluconate dehydrogenase [52].

19 At industrial scale, massive gluconic acid production by *G. oxydans* requires high glucose
20 concentrations, low pH and high aeration rate. The further oxidation to ketogluconic acids
21 is potentially undesirable reaction when using *Gluconobacter* strains for gluconic acid
22 production. Suppression of ketogluconates formation has been achieved performing
23 processes at low pH values [53].

1 *Sugar alcohols oxidation*

2
3 2 AAB can oxidize several sugar alcohols like glycerol to dihydroxyacetone (DHA), D-
4
5 3 mannitol to D-fructose, D- sorbitol to L-sorbose, D-arabitol to xylulose, D and meso-
6
7 4 erythritol to L-erythrulose [1, 7]. Particularly in winemaking, AAB can use glycerol as
8
9 5 carbon source which is converted into DHA by the glycerol dehydrogenase; an enzyme
10
11 6 bound with the cellular membrane inducing a high accumulation of DHA in the media. In
12
13 7 wine, DHA can react with proline producing a "crust-like" aroma and it can bind SO₂
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15 8 reducing its anti-microbial activity [36].
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20 9 In *G. oxydans* it was reported that oxidization of glycerol is catalized by the
21
22 10 membrane-bound glycerol/sorbitol dehydrogenase. This quinoprotein is considered the
23
24 11 main polyol-dehydrogenase of *G. oxydans* that exhibits a broad substrate specificity. It
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26 12 catalyzes the oxidation of D-sorbitol, gluconate, and glycerol to L-sorbose, 5 ketogluconate
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28 13 and DHA, respectively [54].
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32 14 In *G. oxydans* can oxidize glycerol by the enzyme glycerol/sorbitol dehydrogenase
33
34 15 that is considered the major polyol-dehydrogenase with a broad substrate specificity. It
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36 16 catalyzes the oxidation of D-sorbitol, gluconate, and glycerol, producing L-sorbose, 5
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38 17 ketogluconate and DHA, respectively [54].
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42 18 The metabolic pathway of glycerol in *A. pasteurianus*, predicted from *Ga. xylinus*, showed
43
44 19 that glycerol utilization is accompanied by the formation of DHA, cellulose, CO₂, and
45
46 20 small amounts of acetate. In this species, DHA phosphate from glycerol can be produced
47
48 21 by two pathways, one is via DHA catalyzed by glycerol dehydrogenase and the other one
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50 22 is via glycerol 3-phosphate by glycerol kinase. The DHA phosphate may be converted to
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52 23 D-glyceraldehyde 3-phosphate by triosephosphate isomerase and thus enter into the
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54 24 gluconeogenesis pathway [27].
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1 *Organic acid oxidation*

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3 2 The ability of *Acetobacter* and *Gluconacetobacter* strains to oxidize AcOH (Fig 1)
4
5 3 generating the so-called acetate overoxidation occurs via TCA cycle [42]. Other acids such
6
7 4 as lactic, pyruvic, malic, succinic, citric, and fumaric are similarly metabolized. In contrast,
8
9 5 strains of *Gluconobacter* do not have a functional TCA cycle because of deficiencies in the
10
11 6 two key enzymes, *alpha*-ketoglutarate dehydrogenase and succinate dehydrogenase.
12
13 7 Consequently, they are unable to metabolize AcOH and other organic acids. Although the
14
15 8 optimum pH for the oxidation of organic acids by AAB is near 6.0, there is evidence that it
16
17 9 occur at lower values (3.5 to 4.0). In vinegar, for instance, *Acetobacter* species exhibits a
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19 10 biphasic growth curve, where the first corresponds to an EtOH oxidation with AcOH
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21 11 production, and the second to an overoxidation [42].
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28 12 Several strains of *Acetobacter* and *Gluconobacter*, particularly strains of *A. pasteurianus*,
29
30 13 can oxidize lactate to acetoin. Acetoin has a characteristic "butter-like" aroma and flavor
31
32 14 occurring in spoiled wine [36].
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38 **Production of exopolysaccharides**

39
40 17 Dextrans, levans and cellulose are the main exopolysaccharides produced by AAB glucose
41
42 18 metabolism [1]. *Ga. xylinus* species have been regarded as model system for the study of
43
44 19 biochemistry and genetics of cellulose biogenesis. The rate of cellulose production in *Ga.*
45
46 20 *xylinus* is proportional to the rate of cell growth, and the yield is dependent on the carbon
47
48 21 sources. Activators for bacterial cellulose production are compounds like caffeine and
49
50 22 related xanthenes [1]. *Ga. xylinus* synthesizes a cellulose mat that covers the surface of the
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52 23 growth medium in static cultures, whereas round balls of cellulose are formed in shaking
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54 24 cultures. The key enzyme in cellulose synthesis by *Ga. xylinus* is the membrane bound
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1 cellulose synthase which uses UDP-glucose as substrate. The pathway from glucose to
2
3 cellulose consists of the following four enzymatic steps:

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5
6 3 Glucose → glucose-6-phosphate → glucose-1-phosphate → UDP-glucose → cellulose
7

8
9 4 Cells of cellulose-producing AAB are entrapped in the polymer matrix, supporting the
10
11 5 population at the liquid-air interface. This facilitates oxygen and nutrient supply, since the
12
13 6 concentration of nutrients in the cellulose matrix is enhanced by its absorptive properties,
14
15 7 in contrast to the surrounding aqueous environment. Aeration of cultures gives rise to the
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17 8 formation of spontaneous non-cellulose-producing mutants. Most *Ga. xylinus* and *Ga.*
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19 9 *intermedius* strains produce besides the water-insoluble cellulose also a water-soluble
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21 10 polysaccharide called “acetan,” a heteropolymer containing glucose, mannose, glucuronic
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23 11 acid and rhamnose in a molar ratio of 4: 1: 1: 1. Acetan formation seems to influence the
24
25 12 degree of polymerization and crystallinity of the cellulose fibrils [55].
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30 13 Production of exopolysaccharides especially cellulose from AAB seems to be a promising
31
32 14 area of application because of the increasing need of pure cellulose in medical and
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34 15 engineering fields [18].
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40 17 **Conclusion**

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42 18 AAB are food-associated microorganisms that have a long history in oxidative
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44 19 fermentation processes, where they are spoiling or beneficial organisms. The physiological
45
46 20 uniqueness of AAB is due to their ability to partially oxidize carbon sources and
47
48 21 quantitatively excrete the corresponding compounds in the surrounding media. This
49
50 22 feature, besides vinegar, is exploited for the industrial production of a number of
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52 23 compounds from alcohols, sugar and sugar alcohols oxidation. Moreover they are
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54 24 considered promising for the production of the pure form of cellulose. Although the
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1 valuable potential of AAB in biotechnological applications, their industrial exploitation is
2 not full developed.

3 Perspectives to enhance their role as biocatalysts include the availability of genetic stable
4 strains and further advance on their metabolic potential, in order to obtain high carbon
5 substrates conversion efficiency.

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1 **Table 1** Distinctive characteristics of acetic acid bacteria genera

Characteristic	<i>G.</i>	<i>A.</i>	<i>Ga.</i>	<i>Ac.</i>	<i>As.</i>	<i>K.</i>	<i>Sw.</i>	<i>Sa.</i>	<i>N.</i>	<i>Gr.</i>	<i>Am.</i>	<i>T.</i>	<i>Ne.</i>
Production of AcOH	+	+	+	+	-	+	+	v (w/-)	+	v (w/-)	+	-	-
Oxidation of:													
Acetate to CO ₂ and H ₂ O	-	+	+	+	w	w	w	-	-	w	+	-	-
Lactate to CO ₂ and H ₂ O	-	+	+	v (-/w)	w	w	w		-	+	w	-	-
Growth on 0.35% AcOH (pH 3.5)	+	+	+	+	-	+	+	-	+	nd	+	+	-
Growth in the presence of 1% KNO ₃	-	-	-	+	-	-	+	nd	-	nd	-	-	-
Production of keto-d-gluconic acid from d-glucose:													
2,5-diketo-d-gluconic acid	v	-	v	-	-	-	nd	nd	nd	nd	-	+	nd
5-keto-d-gluconic acid	+	v	v	-	+	+	nd	+	+	nd	+	+	nd
2-keto-d-gluconic acid	+	v	v	-	+	+	nd	+	+	nd	+	+	nd
Production of DHA from glycerol	+	v	v	-	v	+	+	-	w	-	w	+	-
Growth on methanol as carbon source	-	v	-	+	-	-	-	-	-	+	vw	-	nd
Production of water soluble brown pigment(s)	v	-	v	-	-	-	+	-	-	nd	-	+	-
Production of γ -pyrones from:													
d-glucose	v	-	v	nd	-	-	nd	nd	nd	nd	nd	nd	nd
d-fructose	+	-	-	nd	v (+/w)	v	nd	nd	nd	nd	nd	nd	nd
Acid production from:													
l-arabinose	+	v	v	+	+	+	+	+	+	nd	nd	nd	nd
d-arabinose	+	-	-	v	+	v	nd	-	w	nd	nd	nd	nd
d-xylose	+	v	v	+	+	+	v	+	+	-	nd	nd	nd
l-rhamnose	-	-	-	-	v	-	-	-	w	nd	nd	nd	nd
d-glucose	+	v	+	+	+	+	+	+	+	w	nd	nd	nd
d-galactose	+	v	+	+	+	+	+	+	+	nd	nd	nd	nd
d-mannose	+	v	v	+	+	+	+	+	+	nd	nd	nd	nd
d-fructose	+	-	+	-	+	-	v	v	+	nd	nd	nd	nd
l-sorbose	+	-	v	nd	+	-	nd	-	-	nd	nd	nd	nd
Melibiose	+	-	-	v	+	+	nd	+	+	nd	nd	nd	nd
Sucrose	+	-	-	-	+	v	nd	+	+	-	nd	nd	nd
Raffinose	-	-	-	-	-	+	nd	-	+	nd	-	w	-
d-mannitol	+	-	v	-	v	-	-	+	w	-	-	-	-
d-sorbitol	+	-	-	-	v	-	+	-	+	-	-	-	-

Dulcitol	-	-	-	-	v	-	v	-	w	-	-	-	-
Glycerol	+	-	+	+	+	+	+	-	+	v (w/-)	w	+	-
EtOH	+	+	+	+	-	+	+	-	+	+	+	+	-
Production of cellulose	-	-	v	-	-	-	nd	-	nd	nd	nd	nd	nd
Production of levan-like mucous substance(s) from sucrose	-	v	-	-	-	+	nd	-	-	nd	-	-	-
Growth in the presence of 30% d-glucose	-	v	v	+	+	-	nd	+	+	nd	-	+	+
Motility and flagellation	N-m (mostly) or po	N-m or pe	N-m or pe	N-m or pe	N-m or pe	N-m	pe	N-m	N-m	N-m	pe	N	N
Major ubiquinone	Q10	Q9	Q10	Q10	Q10	Q10	Q10	Q10	Q10	nd	Q10	Q10	Q10
G + C content (mol%)	54-64	52-64	56-67	62-63	59-61	56-57	57-60	52-53	63.1	59	66.0	65.6	56.8

- 1 Adapted from [24, 56, 57]. *G.*: *Gluconobacter*; *A.*: *Acetobacter*; *Ga.*: *Gluconacetobacter*; *Ac.*: *Acidomonas*; *As.*: *Asaia*; *K.*: *Kozakia*; *Sw.*: *Swaminathania*; *Sa.*:
2 *Saccharibacter*; *N.*: *Neoasaia*; *Gr.*: *Granulibacter*; *Am.*: *Ameyamaea*; *T.*: *Tanticharoenia*; *N.*: *Neokomagataea*. +: positive, -: negative, w: weak, v: variable, nd: not
3 determined, N-m: non-motile, po: polar, pe: peritrichous.

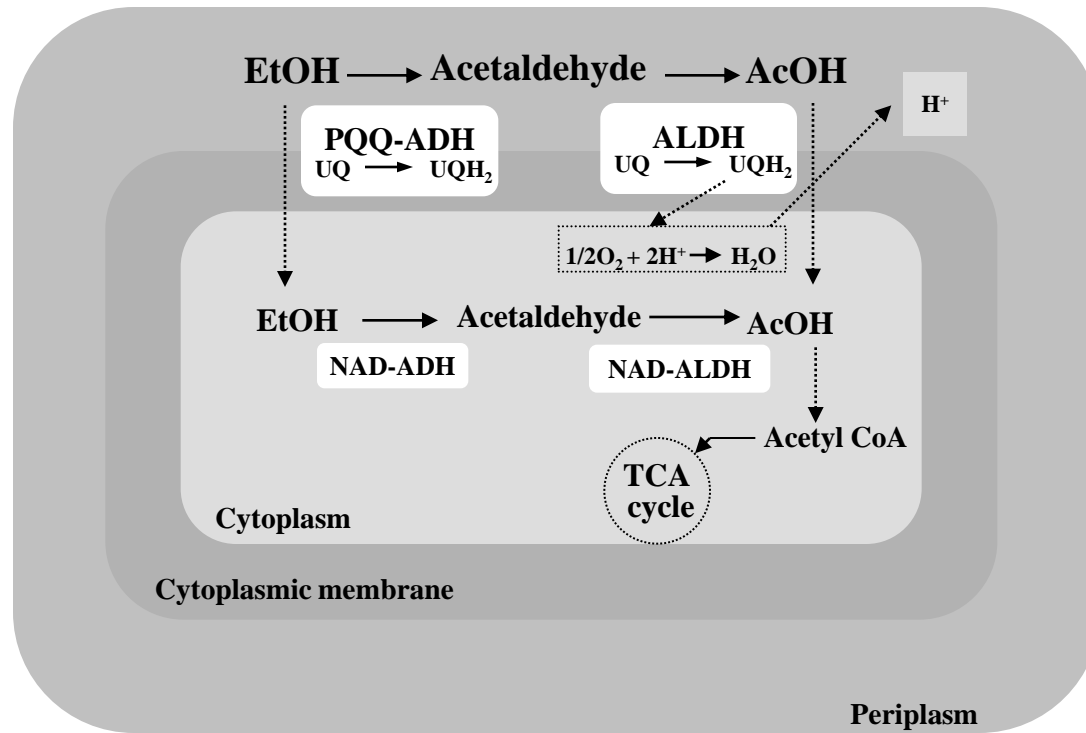


Fig.1 Ethanol oxidation by PQQ-ADH and ALDH at the outer surface of cytoplasmic membrane and by NAD-ADH and NAD-ALDH in the cytoplasm

Answers to Reviewers

Thank you very much for all your comments and suggestions, strongly contributed to improve the manuscript. Below a detailed response for each point raised out is provided.

Reviewer #1:

This review describes acetic acid bacteria focusing on their metabolism. It is a concise overview of the acetic acid bacteria involved in food fermentation, particularly vinegar production.

A few things that should be considered to improve the review:

- Emphasis on recent taxonomy (a lot of research has been done on AAB taxonomy in the last 5-10 years)
- Figure would be good (eg reaction of ethanol to acetic acid with the various enzymes)
- Biotechnology needs to be expanded more
- VBNC needs to be brought out more in the cultivation section
- English expression and language should be checked

We have fully considered the criticisms raised out by the reviewer and accordingly the manuscript was revised.

Recent taxonomy has been added to the paragraph "General characteristics" that in this new version of the manuscript has been renamed as follow:

"General phenotypic characteristics and taxonomic aspects of acetic acid bacteria"

As suggested, a figure (Fig.1) showing ethanol oxidation by ADH and ALDH and location within the cell has been added.

Biotechnological applications of AAB has been expanded more (p. 2 lines 1-23).

As suggested, the paragraph "Isolation and cultivability" was modified and VBNC concept added (p.5, lines 11-15)

The whole manuscript has been revised and a thoroughly English revision has been done.

Specific comments:

Abstract

- use biotechnology rather than biotech

The term was corrected

P3, L7 - assigned rather than allocated

Allocated has been replaced by "assigned" (p.3, line 13), as suggested.

P3, L12 & 14 - write ethanol (EtOH) or acetic acid (AcOH) and then use abbreviation

The correction has been done.

P3, L23 - surface layer

Superficial has been replaced by "surface" (p. 1, line 14)

P4, L1 - acetic acid production is not a fermentation

The sentence was corrected:

AcOH fermentation was replaced by “ AcOH production”

P4, L5 - AAB are involved in these other food productions, not only implicated

Implicated was replaced by involved, as suggested.

Page 5 - what is the significance of 1996 in regards to these various foods and the bacteria isolations?

The whole sentence was removed.

Reference is needed for 'Early researchers....'

The reference was added [35] : Dupuy P (1952) Recherche d'une technique d'isolement des Acetobacter du vin. Ann Technol Agric 1:107-112 (p.5. L.19)

Page 6 L 10-15 - this section a few more sentences to explain these statements better.

Lines 14-15: why the difference? Explain the significance between the two versions.

To clarify, we removed the second part of the sentence: “Whereas previous edition of Bergey's Manual [25] recommended the use of a simple medium containing 0.5% yeast extract, 1.5% EtOH, and 2.5% agar.”

In the new version of the manuscript, the sentence reports only the medium of the last version of bergey’s manual (p. 8, L: 5-9) “The last edition of Bergey's Manual of Systematic Bacteriology [1] proposed standard medium for enrichment and isolation of AAB, with the exception of *Ga. europaeus* (which strains are AcOH-dependent), containing: D-glucose, 0.05%; EtOH, 1.5%; yeast extract, 0.5%; peptone, 0.3%; cycloheximide, 0.01%; and agar, 0.12%.”

Page 7 L 6-11 - this whole section needs a bit of rearranging. These lines should be at the start of the section

The paragraph “Isolation and cultivation” was rearranged. The sentence was simplified and put at the start of paragraph “Isolation and cultivation of AAB strains, especially from fermented beverages, have been described to be problematic, giving rise to an underestimation of species richness when culture dependent methods are applied [31]”.

Lines 6-14 - this needs to be expanded to explain these observations and statements. It is suggesting that now all bacteria are growing up/being cultivated. Is this viable but not culturable (VBNC). Or poor growth medium? Highlight the difficulties in cultivating AAB and all the AAB present.

The section was rearranged as follow:

p. 5, lines 11-17 “ Low recovery of strains due to the fraction of population that could reach a viable but non-culturable (VBNC) state has been stated. For instance in wine it was shown that VBNC status of spoiling AAB is induced by O₂ deprivation [32]. Whereas in vinegars some studies developed by no culture approaches, such as PCR/DGGE, revealed higher species diversity respect to that detected by culturing methods [33, 34, 3].

Limitations of culturing were in part overcome with the formulation of appropriate media, which allowed the cultivation of no growing strains or slow growing strains.”

Page 8 L 1-8 : it would be a good idea to have a figure here for the reaction and enzymes, their location It is always beneficial in a review to have a figure.

As suggested, a figure (Fig.1) showing ethanol conversion and location of enzymes within the cell has been added. The figure represents the ethanol oxidation to acetic acid by the membrane-bound ADH and ALDH and cytoplasmic respiration of ethanol.

P9 L 15 - ... were present in the strain. The genes were present, but was it shown that the pathways were functioning. Genomics show that genes are present but do not confirm that reactions are occurring.

The sentence was removed.

P 10 L 7 - High in what sense? Concentration? Structure?

P 10 L 9 - wide range of applications. This does not make sense or belong here.

To avoid repetition the sentence (p.10, L.6-9) was removed.

The sentence reported in the new version of manuscript is at page 9, lines: 14-18

“The most characteristic reaction is the direct oxidation of glucose into Glucono- δ -lactone, which is oxidized into gluconic acid. This reaction is particularly active in *Gluconobacter* growing at high concentrations of sugars. D-gluconate can be further oxidized to 2-ketogluconate and 2,5-diketogluconate by the gluconate dehydrogenase and 2-ketogluconate dehydrogenase [52].”

P 10 L 14 - last sentence Is this important for high production of vitamin C?

The sentence was removed.

P 11 L 1-5 - another sentence or two to expand this concept would be beneficial for the reader.

To clarify the sentence was rewritten as follow (p. 10, Lines 14-17):

“In *G. oxydans* it was reported that oxidization of glycerol is catalized by the membrane-bound glycerol/sorbitol dehydrogenase. This quinoprotein is considered the main polyol-dehydrogenase of *G. oxydans* that exhibits a broad substrate specificity. It catalyzes the oxidation of D-sorbitol, gluconate, and glycerol to L-sorbose, 5 ketogluconate and DHA, respectively [54]. “

P 13 L 6-7: this sentence needs to be reworded and explained better. What has been reached?

P 13 L 11 - sugared is not a good word here. Rewrite using high sugar content.

In the new Conclusion section, the sentences were rewritten as follow:

“AAB are food-associated microorganisms that have a long history in oxidative fermentation processes, where they are spoiling or beneficial organisms. The physiological uniqueness of AAB is due to their ability to partially oxidize carbon sources and quantitatively excrete the corresponding compounds in the surrounding media. This feature, besides vinegar, is exploited for the industrial production of a number of compounds from alcohols, sugar and sugar alcohols oxidation. Moreover they are considered promising for the production of the pure form of cellulose. Although the valuable potential of AAB in biotechnological applications, their industrial exploitation is not full developed.

Perspectives to enhance their role as biocatalysts include the availability of genetic stable strains and further advance on their metabolic potential, in order to obtain high carbon substrates conversion efficiency.”

Table 1.

In the legend write AAB in full. At the top of the table, it would be much better to have at least abbreviations of the genera rather than numbers. It would make it much easier for the reader.

AAB was replaced by acetic acid bacteria, as suggested.

Number were replaced by abbreviations.

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Title of the article

ACETIC ACID BACTERIA: PHYSIOLOGY AND CARBON SOURCES
UTILIZATION

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