Oxidative fermentation of glucose and ethanol in designed media and cooked grape must by acetic acid bacteria

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ABSTRACT

In this study, acetic acid bacteria strains were investigated for their ability to oxidize different carbon sources producing the corresponding oxidative products. Bacterial strains were cultivated in seven designed media and their acetification ability was assessed. The most performing strains were further tested to evaluate gluconic acid production in cooked grape must. Organic acids, sugars, and ethanol concentrations were assayed by high-pressure liquid chromatography. Overall, the findings showed high variability amongst strains of the same species, especially amongst *Gluconobacter oxydans* strains. However, strains ATCC 621H and DSM 3503[T] resulted to be the highest gluconic acid producers in all tested conditions. This study shows that grape must can be further valorized by selective fermentations for the production of gluconic based products.

1. Introduction

The acetic acid bacteria (AAB) metabolize carbohydrates via periplasmic oxidations producing the corresponding oxidative products, which are released into the growing media. These conversions are generally known as oxidative fermentations. Furthermore, AAB group comprises highly versatile members having a variety of metabolic activities including exopolysaccharides production, such as levan and bacterial cellulose [1,2].

Due to the variety of metabolized substrates and, even more, their adaptive capacity to grow in different environments, like high acidity and high sugar niches, AAB are extremely feasible microorganisms for food, and biotechnological applications.

Considering the oxidative metabolism of AAB, among genera, members of *Acetobacter* are well-known in food-industry for their ability to convert ethanol (EtOH) into acetic acid (AcOH); whereas members of *Gluconobacter* genus are known to incompletely oxidize a variety of sugars, such as D-glucose, into D-gluconic acid (GlcA) and its derivatives [3]. On the other hand, *Novacetimonas* genus (recently established as a dissection of the *Komagataeibacter* genus) include species with a great variety of metabolic abilities, such as production of AcOH, GlcA, and bacterial cellulose [4]. For these peculiar traits, AAB are involved in the production of several fermented foods and beverages, like vinegar and kombucha tea, characterized by the presence of AcOH and/or GlcA [5–9]. In more recent times, they are exploited for the production of

pure bacterial cellulose, as a versatile biopolymer useful for food and biomedical applications [10].

GlcA is a mild organic acid; it is non-corrosive, non-toxic, and non-volatile, and is a natural constituent of some plants, wine, and honey [11]. It is used as an additive in pharmaceutical, textile, building and, especially, in the food industry for its ability to lower pH and for providing a refreshing sour taste to beverages.

The synthesis of GlcA in *Gluconobacter* occurs by the direct oxidation of glucose into glucono-δ-lactone catalyzed by the membrane-bound pyrrolo-quinolinequinone-dependent gluconate dehydrogenase (PQQ-GDH). Glucono-δ-lactone is stable in acid conditions, but it can spontaneously hydrolyse to GlcA under neutral and alkaline conditions or can be converted to GlcA by a membrane-bound gluconolactonase. D-gluconate can be further oxidized to keto-acids. In *G. oxydans*, at periplasmic site, GlcA is oxidized to 2-keto-D-gluconate by the flavoprotein gluconate dehydrogenase (GADH); 5-keto-D-gluconate is produced by a PPQ-dependent protein bound to the cytoplasmic membrane. Finally, 2,5-keto-D-gluconate can be formed by the flavoprotein 2-keto-D-gluconate dehydrogenase (2KGADH) [12].

The global production of GlcA in 2022 was estimated at 800 thousand tonnes [13], while the Global Industry Analysts estimated the global GlcA market at US$ 725.6 millions and forecasted it to reach $1.3 billions by 2030, growing at a CAGR of 7.6 % over that period [14]. The growing demand for GlcA, in association with the current effort towards the real implementation of a circular economy, encourages the research

in integrating agro-food by-products as a revalorization strategy for microbial GlcA production, decreasing the production by the chemical synthesis [15]. Indeed, in the last few years, several studies focused on optimizing GlcA production from biomass such as corn cob [16], sugar cane bagasse [17], grape bagasse mixed with potato powder [18], corn stover [19], and potato pulp hydrolysate [20]. Most of the studies utilized *G. oxydans* strains and tested different methods to enhance GlcA production. High conversion rates ranging from 71.5 to 96.3 % were reported, highlighting the suitability of AAB for GlcA production starting from food biomass or agri-food byproducts.

In Italy, viticulture has been part of traditional agricultural practices since ancient times [21]. In 2022, the agricultural area reserved for vineyards was 698 thousand hectares (47 and 651 thousand hectares respectively for table grapes and wine grape), reaching a total grape harvest of 8.121 thousand tonnes (1.015 and 7.106 thousand tonnes, respectively, for table grapes and wine grapes) [22]. Sometimes atmospheric conditions interfere with the optimal ripening of the grapes and, therefore, the resulting musts have a low sugar content and cannot be used to produce wine (Regulation (EU) 2019/33). Thus, grape must can be concentrated through cooking technique to increase the sugars concentration [23]. However, the surplus grape musts can be valorized by microbial conversions [24]. Indeed, grape must contains fermentable sugars (e.g glucose and fructose) [12,25], whose content increases once cooked. Cooked grape must (CGM) is obtained by direct heating grape must until a reduction of volume variable from 10 to 70 % is reached [26]. CGM has already been tested as a potential substrate for GlcA production. However, most of the studies involved fungi like *Aspergillus niger* [27–29]. To the best of our knowledge, no works on the valorization of CGM by AAB fermentations are available. Here, we evaluate AAB for converting sugared substrates, like CGM, into valuable new products, and accordingly the possibility to reduce surplus and/or CGM not optimal for conventional uses, proposing a new way of biotransformation. Our hypothesis is that CGM could be a suitable feedstock for AAB fermentation, leading to the production of Glca products. This could be exploited for the design of new products in the context of sustainable productions obtained through selected microbial conversions. Therefore, this study, aimed at determining whether AAB strains could have a role in the valorization of agri-food products like CGM, enabling the production of a GlcA beverage. First, we evaluated the ability of different AAB strains to produce GlcA in CGM through static and shaking cultivation systems. AAB strains were screened based on their ability to grow in designed culture media containing different carbon sources, then, they were tested in CGM. The strains ATCC 621H and DSM 3503^T produced the highest GlcA amount and were further tested in diluted and undiluted CGM, confirming a high GlcA production yield. Our findings emphasise the fundamental role that AAB could have for the valorization of agri-food raw materials products, including wastes products.

2. Materials and methods

2.1. Bacterial strains and culture conditions

In this study, 10 AAB strains obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) and UMCC (Unimore Microbial Culture Collection, Reggio Emilia, Italy) were used (Table 1). Strains were rehydrated from −80 °C storage conditions in GY broth (glucose 50.0 g/L, and yeast extract 10.0 g/L) and used at a final concentration of 1×10^8 cells mL^{-1}. Standard cultivation and preservation of AAB strains were conducted in GY at 28 °C for 3 days. Before use, all the media were sterilized by autoclaving at 121 °C for 15 min.

2.2. Designed media at different carbon sources content

The first screening was performed by cultivating AAB strains in 100

Table 1
Bacterial strains used in this study.

Bacterial strain	Species	Isolation source
DSM 7148	*G. cerinus*	Flower of *Rheum undullatum* [30]
DSM 9533^T	*G. cerinus*	Cherry (*Prunus* sp.) [30]
DSM 7146^T	*G. frateurii*	Strawberry [30]
ATCC 621H	*G. oxydans*	Descendent of ATCC 621 [30]
DSM 2003	*G. oxydans*	–
DSM 3503^T	*G. oxydans*	Beer [30]
DSM 3508^T	*A. aceti*	Alcohol turned to vinegar [30]
DSM 3509^T	*A. pasteurianus*	Beer [30]
UMCC 1754 = AB0220	*A. pasteurianus*	Vinegar [31]
DSM 5602^T	*N. hansenii*	Vinegar brewery [30]

mL Erlenmeyer flasks containing 50 mL of seven different media namely G, E, A, GA, GE, EA, GEA, whose composition is reported Table 2. Media were formulated based on literature review [31–33] to test bacterial strains' response to different environmental conditions. Bacterial cultures were incubated at 28 °C in static conditions for 12 days. Media and materials were sterilized by autoclaving at 121 °C for 15 min. Absolute EtOH was filtered (0.45 μm, Cellulose Acetate Filter, VWR) and added to media after sterilization.

2.3. Cultivation in cooked grape must

Fresh red grape must (variety Sangiovese) was obtained from a local winery situated in the northern Emilia Romagna Region (Italy). Bacterial cultures were inoculated in 100 mL Erlenmeyer flasks containing a mixture of GY and cooked grape must (50 % v/v of CGM and 50 % v/v GY). The inoculum was performed at a final concentration of 1×10^8 cells mL^{-1} and bacterial cultures were incubated at 28 °C for 15 days. The final working volume was set at 50 mL. GlcA production was checked at 0, 3, 6, 9, 12, and 15 days.

Best performing strains were further tested on undiluted CGM (CGM180) and CGM diluted with distilled water to reach a sugars concentration of 80 (CGM80) and 60 (CGM60) g/L. The cultivation was conducted in static and shaking (120 rpm) conditions in 100 mL Erlenmeyer flasks with a working volume of 50 mL and an inoculum at final concentration of 1×10^8 cells mL^{-1}. Incubation was performed at 28 °C in static condition for 12 days.

2.4. pH and titratable acidity determination

The pH and the titratable acidity of samples was determined using a pH meter (Titroline easy, Schott, Mainz, Germany).

Titratable acidity was determined by titration with a NaOH 0.05 M solution. Samples (1 mL) were diluted with distilled water (9 mL) and NaOH solution was carefully added until a pH of 7.2 was reached. Results were expressed as g/100 mL of AcOH though the following expression:

Titratable acidity (g/100 mL AcOH): VNaOH * MNaOH * MWAcOH/ (Vvinegar + VH$_2$O)

2.5. Analytical determination by high-pressure liquid chromatography

Qualitative and quantitative analysis of AcOH, GlcA, glucose, fructose, and EtOH was carried out by injecting 20 μm of sample into a Jasco LC-Net II/ADC apparatus equipped with a Jasco pump PU-2080 Plus, a

Table 2
Composition of the seven media formulated for a first bacterial screening.

	G	E	A	GA	GE	EA	GEA
Glucose (g/L)	50.0	/	/	50.0	50.0	/	1.0
Yeast extract (g/L)	5.0	5.0	5.0	5.0	5.0	5.0	5.0
AcOH (mL/L)	/	/	30.0	30.0	/	30.0	30.0
EtOH (mL/L)	/	30.0	/	/	30.0	30.0	20.0

UV detector set at 210 nm for organic acids determination (Jasco UV-2070 Plus), and a RI detector for glucose and EtOH determination (JASCO RI-2031 plus). Samples were diluted with bi-distilled water and filtered through 0.45 µm PTFE membranes (VWR international). Diluted samples were then transferred into glass vials for injection. Molecules separation occurred using an isocratic method by flowing samples into a system composed of a precolumn (BIO-RAD micro-guard cation H+ cartridges 30x4.6 mL) and two Aminex HPX-87H column (300 × 7.8 mm). Chromatographic conditions were set based on a previous work [34]. Briefly, the mobile phase was composed of 0.005 N sulfuric acid and 5 % of acetonitrile using a flow of 0.6 mL/min. The column was heated at 60 °C with an Eldex CH-150 oven. HPLC standards were purchased from Sigma-Aldrich (Milan, Italy), and five solutions with increasing analyte concentration were prepared for each. Quantitative analysis was performed using calibration curves. For each standard, the regression coefficient was over 0.996. Peak identifications were conducted using the functions provided by ChromNAV software.

2.6. Statistical analysis

Experimental data were analyzed using R v 4.2.3 [35] at a significance level of $p = 0.05$ and reported as the average of the triplicate ± standard deviation. A one-way ANOVA was used to determine statistical significance, whereas Tukey post-hoc test was used to determine statistical differences among samples.

3. Results and discussion

3.1. Variation of titratable acidity in designed media

In this study, 10 AAB strains were cultivated in 7 different media containing carbon sources (glucose, EtOH and AcOH) at various concentrations, and the production of organic acids was evaluated. The chosen AAB strains belong to species of *Acetobacter*, *Gluconobacter* and *Novacetimonas* genera. The variation in titratable acidity at the end of the cultivation time was set as a criterion for assessing bacterial growth and ability to use different carbon sources, and consequently, as a turning point for selecting strains to test in CGM.

The highest increase in titratable acidity was observed in G and GE media for all tested strains, except for DSM 3509^T, which was the less performing strain in most of the media (Fig. 1). Media G and GE contain glucose or glucose and EtOH, respectively, which are the most used carbon sources of AAB [3,36]. EtOH oxidation is typical in vinegar-producing systems leading to AcOH production, whereas glucose oxidation is typical of kombucha-producing systems and other sugared environments leading to the production of GlcA [2,3]. *Acetobacter*, *Komagataeibacter* and *Novacetimonas* species prevail in vinegar-producing systems due to their high ethanol-oxidation capacity, releasing AcOH into the media. On the other hand, *Gluconobacter* species are mainly responsibles for GlcA production in sugared environments.

In this study, although glucose and/or ethanol were present, in media containing AcOH (A, GA, EA and GEA) as carbon source a slight variation or a general reduction in titratable acidity was observed (Fig. 1). Therefore, AcOH presence at 3 % v/v could have severely inhibited bacterial growth of all the tested strains. Indeed, as reported, AcOH could act as growth-inhibitor by penetrating the cells membrane, disintegrating membrane transport processes, and causing an increase of acidity into the cell [2,37]. Even though several studies have reported an increase of AcOH tolerance in AAB while glucose is used in combination with ethanol [38–40], no such behaviour was observed for 8 bacterial strains out of 10, with the only exceptions of DSM 2003 and DSM 3503^T. On the contrary, AcOH and EtOH might have acted synergically, inhibiting bacterial growth on media EA and GEA [41,42]. On the other hand, the reduction of titratable acidity could be linked to the catabolism of AcOH in absence of EtOH by AAB. In vinegar-like systems, strains belonging to *Acetobacter*, *Gluconacetobacter*, and *Novacetimonas* genera exhibit a biphasic growth curve, where the first correspond to EtOH oxidation into AcOH, and the second to the overoxidation of AcOH.
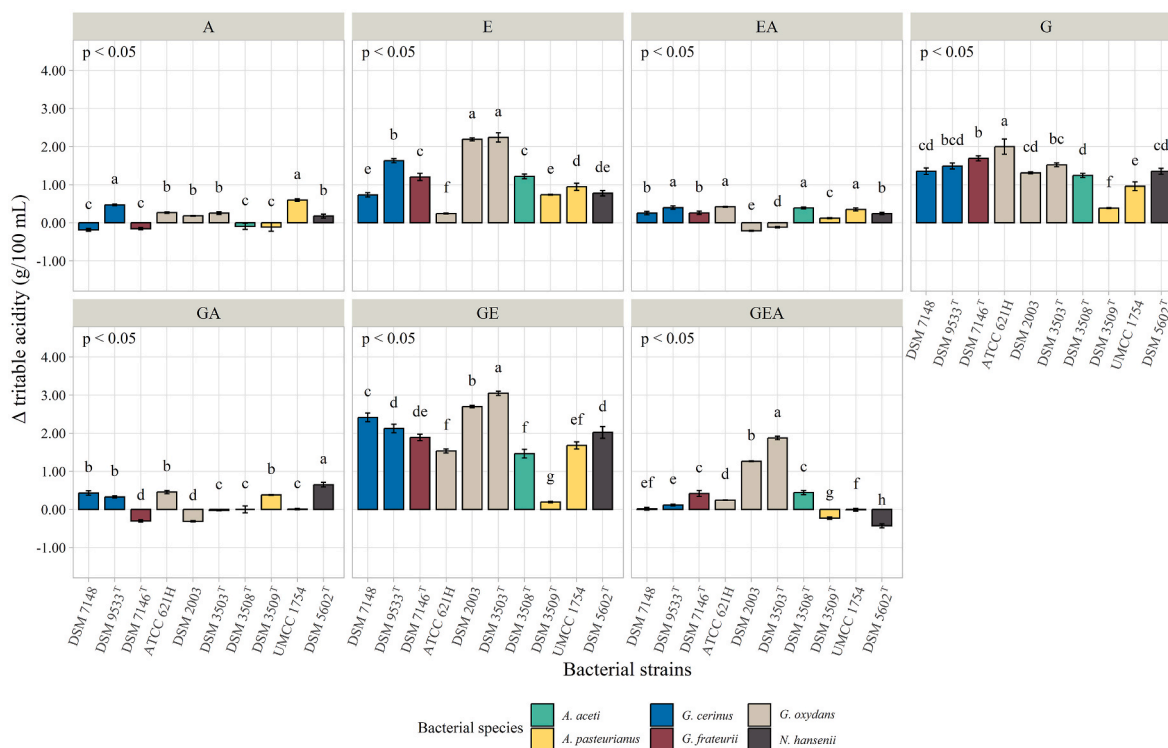

Fig. 1. Variation of titratable acidity (g acetic acid/100 mL) after 12 days of fermentation in 7 different media by 10 acetic acid bacteria strains. Bar plots indicate the average titratable acidity variation by three replicates ± standard deviation. Significant differences among titratable acidity variation are shown by different letters ($p < 0.05$).

Thus, AcOH is oxidized to CO_2 and H_2O through the TCA cycle, as a strategy to survive. leading to a reduction of the acidity of the system [43,44]. The reduction in AcOH concentration was particularly clear when UMCC 1754 = AB0220 was cultivated in A medium (Fig. 2). Previously, the strain has been used at industrial scale to produce vinegar [45], and subculture cultivability and phenotypic stability have been assessed after 9 years of preservation [46]. This strain has also shown high suitability as selected starter culture for both laboratory and prototype scale wine fermentation, showing high AcOH production and fast start-up of the acetification process [47]. In this frame, Gullo and co-workers [47] reported the ability of UMCC 1754 to oxidize AcOH, confirming what was observed in this study. On the contrary, no consumption of AcOH was observed for strains DSM 2003, ATCC 621H, and DSM 3503[T] in none of the media (Fig. 3). Indeed, bacteria belonging to *Gluconobacter* genus deficiency in two key enzymes, alpha-ketoglutarate dehydrogenase [EC 1.2.4.2] and succinate dehydrogenase [EC 1.3.5.1], making them unable to metabolize AcOH [3].

Gluconobacter strains have been reported to partially utilize EtOH [48,49], however, D-glucose is reported as the preferred carbon source. Consistently, for *G. oxydans* strains the highest increases of titratable acidity were recorded in media containing glucose as one of the carbon sources (Fig. 1), apart from medium GA which contain also AcOH. On the contrary, *Acetobacter* strains were reported to prefer alcohol-rich environments, exhibiting high EtOH oxidation and lower oxidation of sugars or sugar alcohols [50]. *A. pasteurianus* UMCC 1754 and *A. aceti* DSM 3508[T] showed the highest increase of titratable acidity in GE medium, thus when both glucose and EtOH were present. However, generally, in GE medium all the strains, apart from DSM 3509[T], showed a high increase in titratable acidity. Both EtOH and glucose act as energy source and as starting compounds to produce AcOH and GlcA, respectively.

Based on media composition, high variability in terms of acetification were observed amongst ATCC 621H and other *G. oxydans* strains. In presence of EtOH, ATCC 621H showed a significantly lower titratable acidity variation compared to DSM 2003 and DSM 3503[T].

In E, EA, GE, and GEA media, EtOH was almost depleted during DSM

3503[T] and DSM 2003 cultivation. In the case of ATCC 621H, by contrast, a remarkable amount was detected in each media (Fig. 3). As a result of EtOH utilization, AcOH production was observed both for DSM 2003 and DSM 3503[T], whereas no increase was recorded for ATCC 621H.

Gluconobacter species have been reported to have a biphasic growth, where they can first accumulate the products of the partial oxidation and later utilize them to promote the growth [51]. As can be observed in Fig. 3d, in G medium, for all the strains a sharp decrease in GlcA concentration was recorded when glucose was depleted. Indeed, in *Gluconobacter* species in absence of glucose, GlcA could be transported and, successively, phosphorylated into 6-phosphogluconate. Through the Entner-Doudoroff pathway or pentose phosphate pathway, 6-phosphogluconate is further metabolized [52]. In addition, GlcA could be also oxidized to 2-keto-D-gluconate and 2,5-diketo-D-gluconate by a GADH [EC 1.1.99.3] and 2KGADH [EC 1.1.99.22], respectively [4,36,53]. Coherently, no reduction in AcOH was recorded since *Gluconobacter* strains lack the glyoxylic acid shunt and the tricarboxylic acid cycle is incomplete [54].

From the results of the first step, the best strains were selected using average titratable acidity increase as the main criterion. However, based on CGM composition higher relevance was given to results obtained in G and GE media. Therefore, six strains were selected, namely *G. oxydans* DSM 2003, ATCC 621H, DSM 3503[T], *G. cerinus* DSM 7148, DSM 9533[T], and *G. frateurii* DSM 7146[T].

3.2. Substrate suitability for gluconic acid production

A physico-chemical characterization of CGM was performed (Table 3). Fructose was found to be the main carbon source, followed by glucose. The pH of CGM was 3.25. Since high sugar concentration and low pH can negatively affect AAB growth [55], in this study, strains were inoculated into a 50:50 mix of CGM and standard GY medium. The growth in CGM-GY was set as a further screening by evaluating GlcA production during 15 days of incubation.

Results showed high variability in GlcA production among tested strains (Fig. 4). This evidence is consistent with previous studies [11,56]
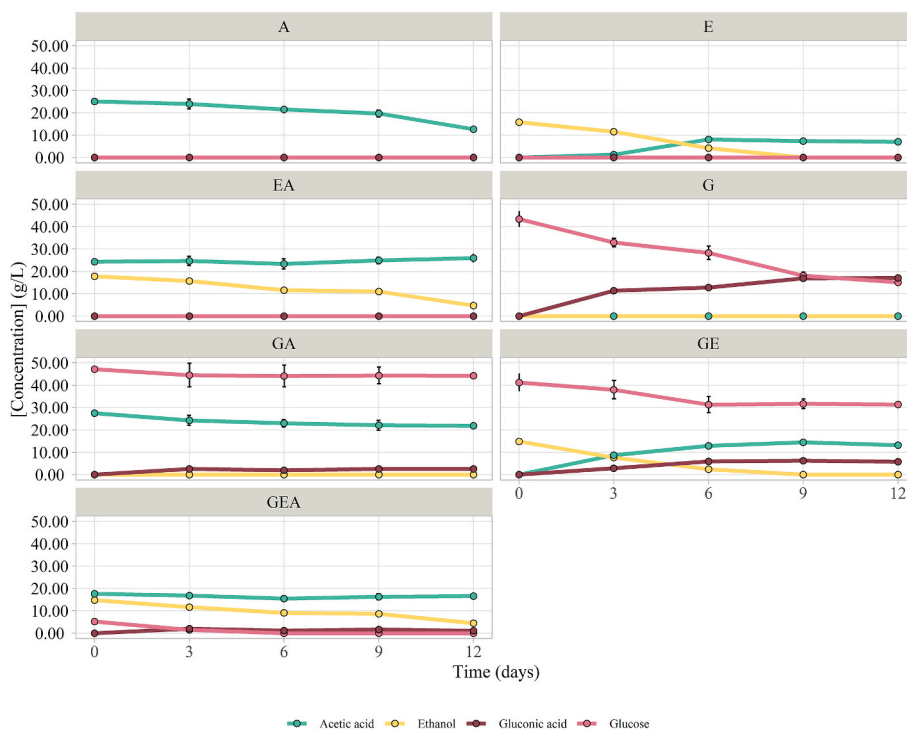

Fig. 2. Variation (g/L) of acetic acid, glucose, ethanol, and gluconic acid concentration during 12 days fermentation. *A. pasteurianus* UMCC 1754 was cultivated in A, E, EA, G, GA, GE, and GEA media. Values are expressed as the mean of triplicate measurements ± standard deviation. *G. oxydans*.
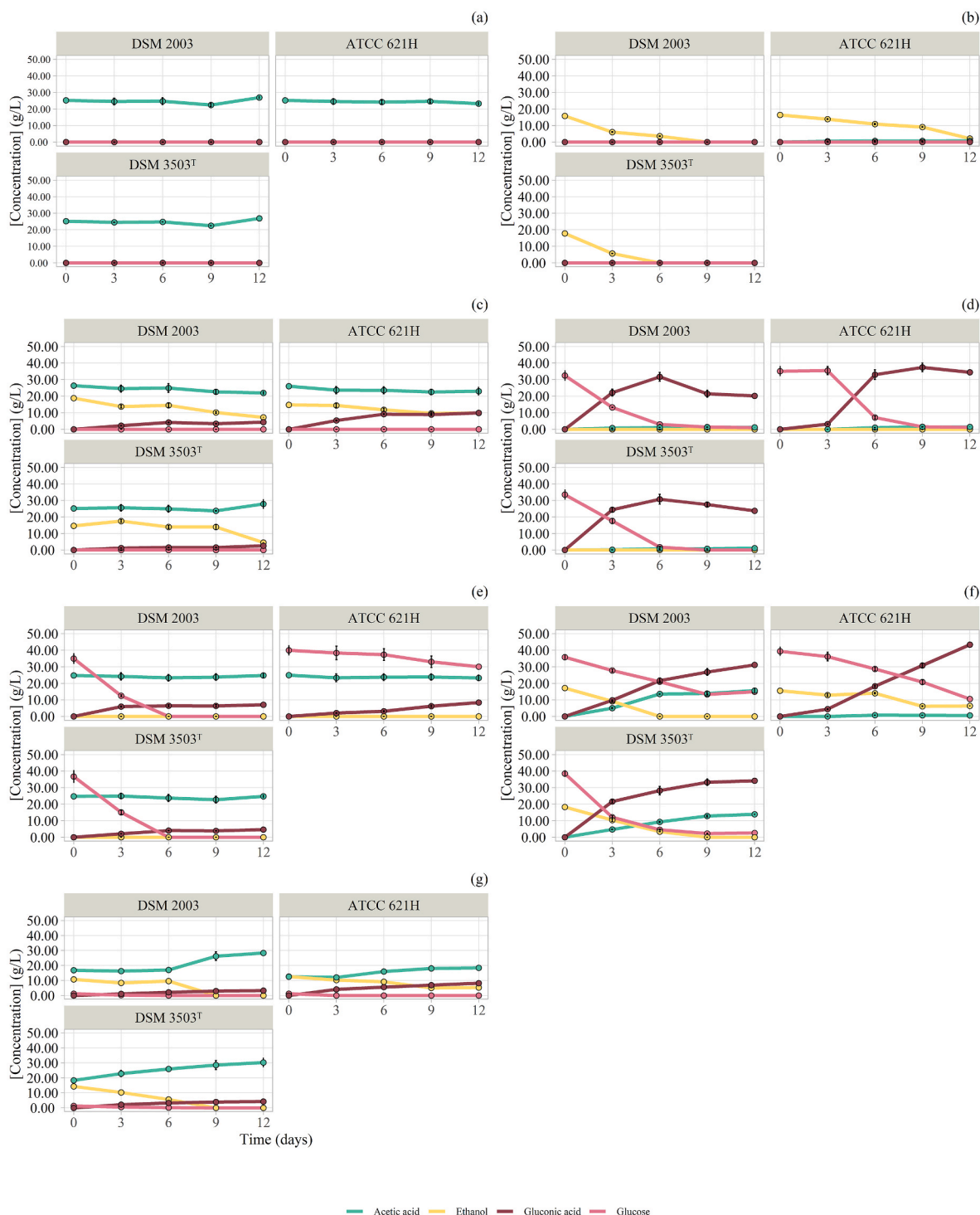
Fig. 3. Variation (g/L) of acetic acid, glucose, ethanol, and gluconic acid concentration during 12 days fermentation. *G. oxydans* DSM 2003, ATCC 621H and DSM 3503[T] were cultivated in (a) A, (b) E, (c) EA, (d) G, (e) GA, (f) GE, and (g) GEA media. Values are expressed as the mean of triplicate measurements ± standard deviation.

reporting GlcA production as a phenotypic trait strictly dependent on the bacterial strain.

Even if at low rate, GlcA production was observed for all the strain after 6 days of cultivation in CGM-GY. *G. frateurii* 7146[T] and *G. oxydans* DSM 2003 had an average production, whereas *G. cerinus* DSM 9533[T] and DSM 7148 showed a considerably lower production of GlcA compared to other strains. A possible explanation could be related to the high sugar concentration present in the medium, which overly stressed

the bacterial cells lowering GlcA production [57]. *G. oxydans* DSM 3503[T] and *G. oxydans* ATCC 621H resulted to have the highest GlcA production, which is in accordance with studies reporting both strains as high GlcA producers [58,59].

A main characteristic of *Gluconobacter* sp. is its ability to rapidly oxidize many organic compounds to the corresponding acids and ketones. Therefore, the low production of some strains or the reduction of GlcA concentration between 12 and 15 days of incubation, such as in

Table 3
Physico-chemical composition of cooked grape must.

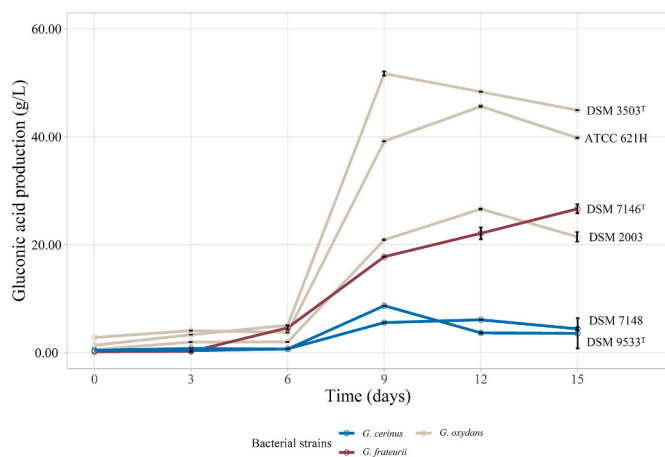|	Concentration
Glucose (g/L)	80.76 ± 2.36
Fructose (g/L)	103.39 ± 5.96
AcOH (g/L)	7.11 ± 0.33
GlcA (g/L)	1.52 ± 0.12
pH	3.25 ± 0.06
Titratable acidity (g AcOH/100 mL)	2.26 ± 0.18
°Brix	26.0 ± 0.2

Fig. 4. Gluconic acid production (g/L) during cultivation in a 50:50 mix of cooked grape must and GY medium. Production values are expressed as the mean of triplicate measurements ± standard deviation.

ATCC 621H (Fig. 4), may be related to the further oxidation to keto-gluconates via pentose phosphate pathway.

3.3. Effect of cooked grape must sugar concentration on the production of GlcA

As observed in Fig. 4, DSM 3503^T and ATCC 621H resulted to be the highest producers of GlcA in CGM-GY, therefore they were tested for GlcA production in CGM180, CGM80, and CGM60. The production of GlcA and the consumption of glucose and fructose were monitored along 12 days of cultivation through HPLC analysis (Fig. 5 a,b,c).

For all the strains, the lowest production of GlcA was observed in CGM180, probably due to the inhibition effect of the high sugar concentration [52]. We observed a slight decrease in glucose and fructose concentration, which were mainly consumed in the first 6 days of fermentation. A similar trend was observed in CGM80, but GlcA production resulted to be higher at the end of fermentation. CGM60 turned out to be the best medium for both DSM 3503^T and ATCC 621H. The latter had a faster consumption of glucose and consequently a faster production of GlcA after the third day of fermentation. However, after 9 days we observed no further increase in GlcA concentration. A putative explanation of this phenomenon could be the lower pH values of the media, independently from the cultivation regime (shaking and static systems) (Table 4), which led to a gradual decrease in cells viability and to a repression of GlcA production [11].

Similar to what Matsushita and co-workers [36] described for alcohol dehydrogenase inactivation in acidic growth conditions, the shutdown in GlcA production could be related to the inactivation of the PQQ-GDH. Indeed, as reported by Sainz and co-workers [11], a large accumulation of GlcA which dropped pH values can lead to a reduction in cells viability and GlcA production. The release of GlcA via porins present in the outer membrane and the consequent accumulation of acids outside the cells [54,60] could have contributed to the formation

of an acidic environment.

It is well-known that *G. oxydans* strains are obligate aerobic micro-organisms, and their metabolism is strictly dependent on the sufficient presence of dissolved oxygen in the growth medium. During growth on glucose-based media, a low amount of oxygen is needed for growth, whereas a major amount is consumed for the membrane-bound oxidation of glucose [58]. Therefore, sufficient oxygen supply is an essential factor in oxidation reaction involving PQQ-GDH for producing GlcA [52, 61]. In this study, the amount of oxygen supplied was not determined, however strains DSM 3503^T and ATCC 621H were also cultivated in shaking condition, and the amount of GlcA obtained compared to that produced in static regime after 12 days of incubation (Fig. 5d). In CGM180 after cultivation in shaking condition, compared to static condition, no increases in GlcA production were observed for DSM 3503^T, whereas for ATCC 621H GlcA production reduced drastically. A slight increase was obtained when ATCC 621H was cultivated in CGM60, reaching 26.02 g/L of GlcA at the end of the fermentation. On the contrary, no significant variations for DSM 3503^T were observed. However, when both strains were cultivated in CGM80 under shaking conditions, the GlcA production increased considerably. In detail, for ATCC 621H an increase of 124 % was obtained, while for DSM 3503^T the increase was equal to 62 %. Data are in agreement with Siberbach and co-workers [58] who reported a positive influence of the shaking system on the activity of the extremely oxygen-dependent PQQ-GDH. Thus, the results confirmed the possibility to partially overcome low GlcA yield or environment stress-related inhibition effects by increasing dissolved oxygen values in the substrate.

4. Conclusions

In this study, AAB strains were screened for their ability to grow on media containing different carbon sources, i.e., glucose, EtOH, and AcOH, and, subsequently, tested for producing GlcA in undiluted and diluted CGM.

Based on our results, tested strains showed an extremely high variability during the growth in formulated media, confirming that strains belonging to *G. oxydans* species strongly differ for the preferential use and order of carbon substrates, as well as for the ability to produce GlcA. In CGM and GY medium DSM 3503^T and ATCC 621H were the highest GlcA producers. High GlcA content contributes to improve the sensorial complexity of foods; in fact GlcA is an appreciated compound in food products where both acidity and mild astringency are required. Moreover, with the premise of contributing to research on the valorization of agri-food by-products implementing more sustainable food productions, in this study we explored the use of CGM as an appropriate substrate to be fermented by the selected AAB strains. Diluted CGM was found to be a suitable substrate to obtain GlcA as the main oxidative compound. Fermentation in shaking condition permitted to obtain high GlcA production in both CGM60 and CGM80. These evidences highlight the possibility to exploit CGM as a fermentable substrate by AAB, also in the new perspective to valorize agro-food wastes. At the same time, the potentiality of ATCC 621H and DSM 3503^T as microbial starters for CGM fermentation to obtain GlcA has been emphasized. The selection of *G. oxydans* strains could be the starting point to produce new fermented beverages containing GlcA.

This work successfully explored CGM as a fermentable substrate by treating it as a resource, fully applying the concept of resource revalorization from agri-food waste through innovative food-biotechnology. Our findings underline the crucial importance of selecting high-performance microorganisms for specific food-waste, even more so if it is possible to both make the production process more sustainable but also increase the content of a specific component such as, in this case, GlcA. Further studies will be conducted for the evaluation of potential functional compounds and for implementing prototypal scale experiments in the light of industrial exploitation.
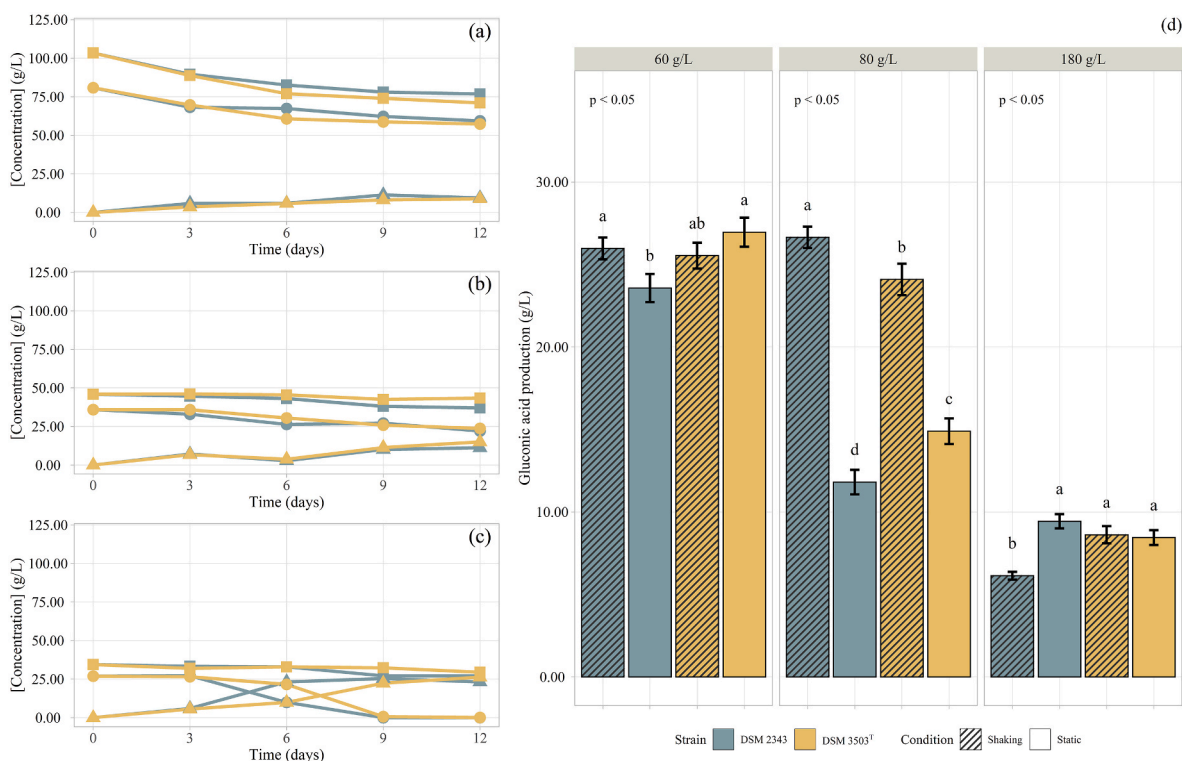
Fig. 5. Glucose utilization (●), fructose utilization (■) and gluconic acid production (▲) by *G. oxydans* DSM 3503T and *G. oxydans* ATCC 621H during static cultivation in cooked grape must at 180 (a), 80 (b), and 60 (c) g/L of total sugars. (d) Gluconic acid production by DSM 3503T and DSM 23243 after 12 days of cultivation in cooked grape must with 180, 80, and 60 g/L of total sugars in static and shaking condition. Bar plots indicate the average gluconic acid production by three replicates ± standard deviation. Significant differences are shown by different letters (p < 0.05).

Table 4

pH values after 12 days of fermentation in cooked grape must at different sugars concentration: 180 (CGM180), 80 (CGM80), and 60 (CGM60) g/L.

	CGM180		CGM80		CGM60	
	Shaking	Static	Shaking	Static	Shaking	Static
DSM 3503[T]	$3.12^b \pm 0.04$	$3.11^a \pm 0.09$	$2.87^a \pm 0.12$	$3.12^a \pm 0.06$	$2.76^a \pm 0.04$	$2.76^a \pm 0.11$
ATCC 621H	$3.26^a \pm 0.06$	$3.25^a \pm 0.12$	$2.76^a \pm 0.04$	$2.96^a \pm 0.08$	$2.75^a \pm 0.08$	$2.84^a \pm 0.08$

Data are expressed as means ± standard deviations. Different letters indicate statistical differences within the same column at p < 0.05.

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CRediT authorship contribution statement

Marcello Brugnoli: Writing – original draft. **Elsa Cantadori:** Writing – original draft, Formal analysis. **Mattia Pia Arena:** Writing – review & editing. **Maria Gullo:** Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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